

EDITORIAL: What's New for 1996

The 1996 **Instructions to Authors for *Bioconjugate Chemistry*** has been updated to inform authors about practical aspects of electronic manuscript submission and the preparation of illustrations. On an experimental basis, graphics files such as ChemDraw structures may be submitted on disk for highest quality reproduction of some illustrations.

Authors are requested to provide a copy of all illustrations reduced to single column width (8.25 cm, 3.5 in.) to check legibility. Lettering and numbering should be no smaller than 2 mm in height upon reduction. Multiple part figures should be combined on a single page.

We will continue to offer authors of accepted **Review** articles the opportunity to submit an illustration for possible use on the cover of the issue where their article appears.

Thanks to Our Reviewers

It is a pleasure to acknowledge the contributions of the reviewers of manuscripts submitted for publication in Volume 6 of *Bioconjugate Chemistry*. Their unselfish service has been essential in the shaping of the sixth volume of the journal. The scientific community is fortunate that its members are willing to share their time and specialized knowledge in this way. The list of reviewers is on the following page.

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COMMUNICATIONS

Block Polycationic Oligonucleotide Derivative: Synthesis and Inhibition of Herpes Virus Reproduction

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The block polycationic oligonucleotide (oligo) consisting of a phosphodiester 12-mer linked to the polycation chain at the 3'-end and cholesteryl group at the 5'-end was synthesized. The polycation chain was grown on the solid support using the monomer, H-phosphonate of 1-O-(4,4'-dimethoxytrityl)-1,3-butanediol. Amino groups were introduced in the polymer backbone using 1,4-diaminobutane, and then the oligo chain was formed at the free end of the polymer. The last stage of the synthesis was the attachment of the cholesteryl group to the 5'-end of the oligo prior to cleavage and deprotection of the copolymer. The nucleotide sequence of this copolymer, **CGTTCCTCCTGC**, was complementary to the splicing site of immediate early (IE) mRNA 4 and 5 of herpes simplex virus type 1 (HSV-1). The stability of the duplexes formed between the copolymer and the complementary 12-mer was similar to that of unmodified oligo. The stability of the block polycationic oligo against phosphodiesterase digestion was significantly increased compared to that of the unmodified oligo. The block polycationic oligo inhibited the reproduction of HSV-1 in Vero cells; however, the effect was significantly less than the effect of 12-mer oligo modified with cholesterol at the 5'-end. The decreased antiviral activity of the copolymer is explained by the polycation-induced stimulation of the virus infection.

Antisense oligonucleotides (oligos) are selective inhibitors of gene expression and viral reproduction (1-3). Two significant obstacles currently limiting the therapeutical application of oligos are poor uptake into cells and rapid degradation by nucleases. To resolve these problems, a variety of structural modifications to oligo have recently been advanced. Specifically, we and other investigators have demonstrated that the addition of hydrophobic substituents to the oligo chain can significantly increase their antisense activity (4-6). Reduction of a net negative charge of oligo backbone also enhances oligo uptake and stability, as has been shown for methyl phosphonates (7) and other derivatives (2, 3), including novel cationic backbone substituents, aminoethyl phosphonates (8). One way to reduce the net negative charge of the DNA molecule is to link it with a polycation. Leonetti et al., (9) have described oligo conjugated with poly(L-lysine) that exhibited greater specific activity on cells compared to unmodified oligos. Other authors are designing various conjugated structures incorporating cationic peptide and oligo blocks (10). The electrostatic binding of polycations to negatively charged polynucleotide molecules has also been shown to increase the (1) stability against nuclease digestion, (2) uptake into the cells, and (3) biological activity of these polynucleotides (cell transfection and antisense inhibition of gene expression) (11).

It has been suggested (12, 13) that a polycation molecule is able to electrostatically bind with both the negatively charged deoxyribosephosphate backbone of the oligo and the acidic phospholipids of the plasma membrane, thus increasing the stability of the nucleic acid and its uptake into target cells. This paper describes the synthesis, stability, and specific activity of a block polycationic derivative of oligo, consisting of a 12-mer phosphodiester oligo linked to a cholesteryl group at the 5'-end and a polycationic chain at the 3'-end. The oligo sequence, **CGTTCCTCCTGC**, was complementary to the splicing site of immediate early (IE) mRNA 4 and 5 of herpes simplex virus type 1 (HSV-1). This target site was first identified by Kulka et al. (14, 15). The sequence-specific inhibition of HSV-1 reproduction with the hydrophobically modified oligo **CGTTCCTCCTGC** has previously been reported (16, 17). This paper evaluates the effects of the polycationic derivative of this oligo on reproduction of HSV-1 in Vero cells.

The block polycationic oligo (Figure 1) was produced by automated DNA synthesis. During the first stage of this synthesis (Scheme 1), the polycation chains were grown on a solid support modified with 1-O-DMT-1,3-butanediol¹ using a 380B-02 DNA synthesizer (Applied Biosystems, U.S.A.) at a 10 μ mol scale. The monomer **2**, H-phosphonate of 1-O-DMT-1,3-butanediol, was prepared as previously reported (18). Briefly, 3.6 g (40 mmol) of butane-1,3-diol (Merck) dissolved in 50 mL of anhydrous pyridine (Aldrich) was reacted with 6.8 g (20

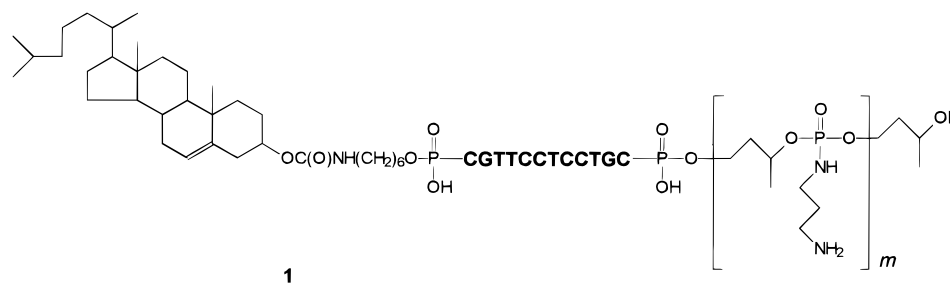
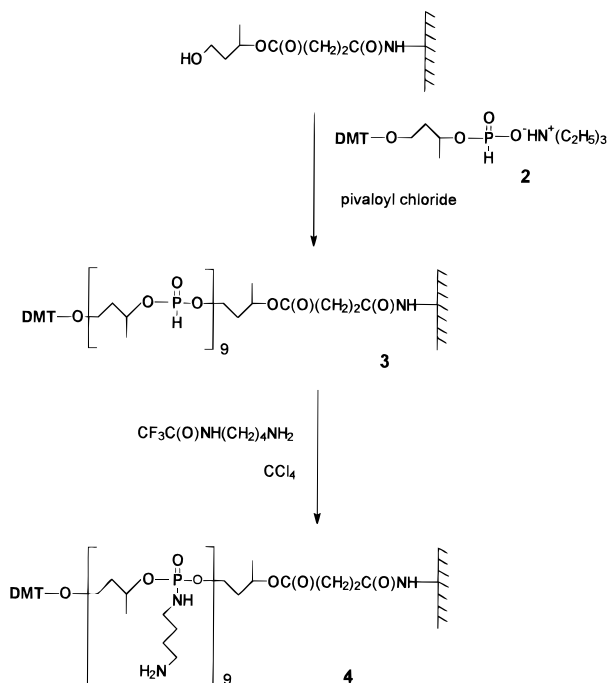
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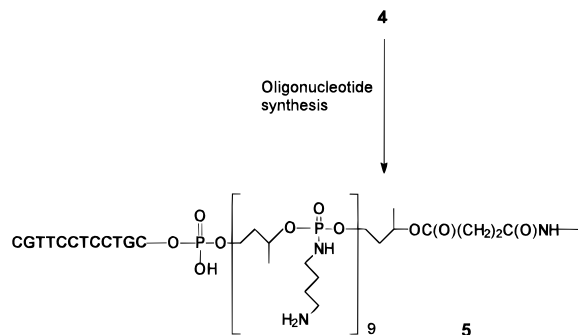
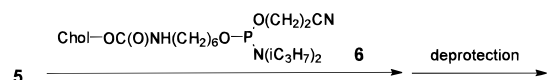
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¹ Abbreviations: Chol, cholesteryl group; DMT, 4,4'-dimethoxytrityl group; EDTA, ethylenediaminetetraacetic acid; PAGE, polyacrylamide gel electrophoresis; PFU, patch-forming unit; 2,4,6-TNBS, 2,4,6-trinitrobenzenesulfonic acid.

**Figure 1.** Block polycationic oligo structure.**Scheme 1. Growth of the Polycation Chain**

mmol) of 4,4'-dimethoxytrityl chloride (Sigma) for 1.5 h at 20 °C. The completion of the reaction was determined using thin layer chromatography on the silica gel plates developed with chloroform/methanol (95:5). The R_f of the product was 0.6. The reaction mixture was added to 200 mL of an 8% aqueous solution of the sodium bicarbonate, the product was extracted with chloroform, and chloroform was evaporated. The resulting oily intermediate (4.7 g, 12 mmol) was dissolved in 30 mL of anhydrous 1,4-dioxane containing 2.32 g (18 mmol) of diisopropylethylamine (Aldrich), and the reaction system was then supplemented with 3.64 g (18 mmol) of salicyl chlorophosphate (Aldrich) which was added using a syringe in an argon atmosphere. The reaction mixture was incubated for 1 h at 20 °C, and formation of the product was monitored using thin layer chromatography as described above (R_f of the product was 0.05). After the completion of the reaction, 10 mL of water was added to the reaction mixture for 30 min, and then the solvents evaporated. The product was dissolved in 100 mL of chloroform, and the solution obtained was extracted with 100 mL of 8% aqueous solution of the sodium bicarbonate, then 100 mL of 0.2 M triethylammonium acetate solution, and finally 100 mL of water. The chloroform was evaporated, and the oily residue containing the monomer **2** was purified on a silica gel chromatographic column, using a stepwise gradient of (1) chloroform, (2) 3% methanol in chloroform, and (3) 6% methanol in chloroform. The yield of the **2** was 4.1 g (7.3 mmol, 63%). During the automated synthesis, a 6-fold excess of **2** (with respect to the 1,3-butanediol linked to the support) was used as a building

Scheme 2. Propagation of the Oligo Chain**Scheme 3. Introduction of the Cholesteryl Group**

block at each condensation cycle, and a 3-fold excess of pivaloyl chloride in pyridine/acetonitrile (1:1) was used as an activating agent. The yield of the polymer **3** after nine condensation cycles was about 96%, as determined by DMT cation release test (498 nm; extinction coefficient, 14 300) (19). Polymer **3** was then oxidized using the Atterton-Todd reaction (19) for 30 min at room temperature by 10-fold excess (with respect to the total concentration of phosphonate groups) of *N*-(trifluoroacetyl)-1,4-diaminobutane in chloroform/pyridine (1:1). During the second stage of the synthesis, standard phosphoramidite chemistry was used to form the oligo chain at the free end of the polymer attached to the solid support (Scheme 2). At the third stage of the synthesis (Scheme 3), the cholesteryl group was introduced at the 5'-end of the oligo using the *N*-[6-[(cholesteryloxy)carbonyl]amino]hexyl]-*O*-(2-cyanoethyl)-*N,N'*-diisopropylphosphoramidite **6** as previously described (16). The copolymeric product was cleaved from the support and deprotected by treatment with concentrated aqueous ammonia for 17 h at 50 °C. The copolymer was purified by reverse phase high-performance liquid chromatography (HPLC) on a Silasorb C₁₆ column (9 × 240 mm, 10 μm, NPO "Chromatographia", Moscow, Russia) using a linear gradient of acetonitrile (5 to 40%) in 20 mM triethylammonium acetate buffer (pH 7.5). The presence of free amino groups in the copolymer was determined using a ninhydrine test. The net yield of the desired copolymer was 27%; the remaining amount accounted for shorter oligomers and oligomers that did not contain amino groups. The copolymer obtained revealed one major band in PAGE (20% gel, 7 M urea, tris-borate buffer, pH 8.3) and migrated substantially more slowly than a 12-mer oligo, modified at the 5'-end with cholesteryl groups as previously described (16). Titration with 2,4,6-TNBS (20) indicated that the concentration of the free amino groups was 11.2 μmol per μmol of the oligo. In this experiment, the oligo concentration was determined spectrophotometrically (18), and it is probable that

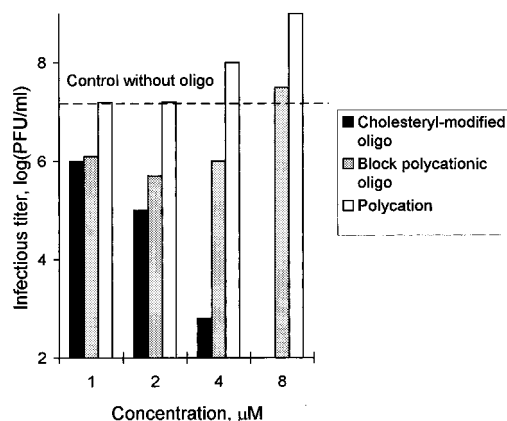


Figure 2. Effects of various concentrations of block polycationic oligo **1**, cholesteryl-modified oligo, and polycation on the HSV-1 reproduction in Vero cells.

the difference between experimental (≈ 11.2) and theoretical (≈ 9) concentration of the amino groups in the copolymer **1** accounts for the change of molar extinction of oligo after modification with the polycation. The straight polycation, which was used in the control experiment on virus reproduction, was obtained via solid support synthesis without growing the oligo chain (Scheme 1), purified as a DMT derivative by reverse phase HPLC, and analyzed using the DMT release test and 2,4,6-TNBS titration as described above.

The copolymer **1** formed duplexes with the complementary 14-mer oligo, which contained one desoxythymidine unit at both 3'- and 5'-ends [0.05 M phosphate buffer (pH 7.0) containing 0.01 M EDTA and 0.15 M NaCl; oligo and copolymer concentrations were 1 μ M]. The stability of these duplexes was not statistically different from the stability of the duplexes formed by the unmodified 12-mer with the same nucleotide structure (T_m equaled 44 and 45 $^{\circ}$ C, respectively). Furthermore, the stability of the block polycationic oligo against nuclease digestion was significantly increased compared to that of the unmodified oligo. No digestion products were detected when the copolymer was exposed to *Crotalus durissus* phosphodiesterase (Boehringer Mannheim) for 2 h at 37 $^{\circ}$ C [0.1 M sodium acetate buffer (pH 8.0)], while PAGE demonstrated that this treatment resulted in complete digestion of the unmodified oligomer.

The effects of the block polycationic oligo on the reproduction of HSV-1 in the Vero cells were investigated and compared to the effects of (1) the 12-mer oligo with the same polynucleotide sequence, modified at the 5'-end with cholesteryl groups as previously described (19), and (2) the straight polycation synthesized as described above (Scheme 1). The experiment on HSV-1 reproduction was performed as previously described (16). Briefly, monolayers of Vero cells were infected at 0.1 PFU/cell multiplicity with the virus. The oligos or polycation was added to the cells at various concentrations 1 h prior to infection. After 8 h of incubation of the infected cells in the presence of oligonucleotides, the medium was replaced with fresh media containing fetal calf serum. The virus infectious titer (PFU/mL) was determined 24 h postinfection on monolayers of Vero cells (21). All experiments were performed in triplicate. The variations in the infectious titers determined were less than 25%. The results of the experiment are presented in Figure 2. The block polycationic oligo decreased the virus titer in the narrow range of oligo concentration of 1–4 μ M, this decrease approximating 30-fold at 2 μ M. At higher concentrations of oligo, the increase in the virus titer was observed. Conversely, the cholesteryl-modified oligo that

did not contain the polycationic block significantly inhibited the infection in the same concentration range, decreasing the virus titer by more than 10000-fold at 4 μ M. The treatment with the straight polycation unlinked to an oligo chain resulted in an increase in the titer, this increase approximating 100-fold at 8 μ M. Therefore, decreased antiviral activity of the copolymer compared to the activity of the cholesteryl-modified oligo is probably explained by the polycation-induced stimulation of the virus infection. In this case, the net effect of the copolymer was a sum of the effects of the oligo chain that inhibited the virus reproduction and the polycation chain that enhanced the infection. The promotion of the infection by polycations has previously been described for various polycations and viruses (22–24). Although the mechanism of this phenomena is not completely understood, it was commonly observed when the polycations were added prior to or simultaneously with the infection, suggesting the polycation effects on the virus adsorption and uptake. We also observed the promotion of HSV-1 infection in Vero cells by short-chain polyspermines added prior to infection (data not presented). One previous observation suggests that polylysine and polyarginine inhibit infection of the cells with HSV-1, by interfering with the virus interactions with cell receptors (25). However, according to Langeland et al. (25), the inhibitory effect decreased with the decrease of the polycation length, and it was observed with polycations of much higher molecular mass than the molecular mass of polycations studied in our work. Because of the polycation effects on infection, the HSV-1 system described in this work may not be suitable as a virus model for investigation of the biological activity of the block polycationic oligos. However, these oligos may become useful for inhibition of reproduction of other viruses and expression of genes in the target cells. The automated synthesis is convenient and allows blocks of varying lengths and molecular masses to be synthesized precisely. Furthermore, it can also be used for introduction of various functional moieties in the block polycationic oligo such as, for example, other hydrophobes (5, 16), polyoxyethylene chains (26, 27), and polypeptides (28, 29).

LITERATURE CITED

- (1) Helene, C. (1991) Rational design of sequence-specific oncogene inhibitors based on antisense and antigene oligonucleotides. *Eur. J. Cancer* 27, 1466–1471.
- (2) Crooke, R. M. (1991) In vitro toxicology and pharmacokinetics of antisense oligonucleotides. *Anti-Cancer Drug Des.* 6, 609–646.
- (3) Stein, C. A., and Cheng, Y.-C. (1993) Antisense oligonucleotides as therapeutic agents - is the bullet really magical? *Science* 261, 1004–1012.
- (4) Letsinger, R. L., Zhang, X., Sun, D. K., Ikeuchi, T., and Sarin, P. S. (1989) Cholesteryl-conjugated oligonucleotides: synthesis, properties and activity as inhibitors of replication of human immunodeficiency virus in cell culture. *Proc. Natl. Acad. Sci. U.S.A.* 86, 6553–6556.
- (5) Kabanov, A. V., Vinogradov, S. V., Ovcharenko, A. V., Krivonos, A. V., Melik-Nubarov, N. S., Kiselev, V. I., and Severin, E. S. (1990) A new class of antivirals: antisense oligonucleotides combined with a hydrophobic substituent effectively inhibit influenza virus reproduction and synthesis of virus-specific proteins in MDCK cells. *FEBS Lett.* 259, 327–330.
- (6) Shea, R. G., Marsters, J. C., and Bischofberger, N. (1990) Synthesis, hybridization properties and antiviral activity of lipid-oligodeoxynucleotide conjugates. *Nucleic Acids Res.* 18, 3777–3783.
- (7) Miller, P. S. (1989) Non-ionic antisense oligonucleotides. In *Antisense Inhibitors of Gene Expression* (J. Cohen, Ed.) CRC Press, Boca Raton, FL.

- (8) Fathi, R., Huang, Q., Coppola, G., Delaney, W., Teasdale, R., Krieg, A. M., and Cook, A. F. (1994) Oligonucleotides with novel, cationic backbone substituents: aminoethylphosphonates. *Nucleic Acids Res.* 22, 5416–5424.
- (9) Leonetti, J.-P., Degols, G., and Lebleu, B. (1990) Biological Activity of Oligonucleotide–Poly(L-Lysine) Conjugates: Mechanism of Cell Uptake. *Bioconjugate Chem.* 1, 149–153.
- (10) Wei, Z., Tung, C. H., Zhu, T., Stein, S. (1994) Synthesis of Oligoarginine–Oligonucleotide Conjugates and Oligoarginine-Bridged Oligonucleotide Pairs. *Bioconjugate Chem.* 5, 468–474.
- (11) Kabanov, A. V., and Kabanov, V. A. (1995) DNA Complexes with Polycations for Delivery of Genetic Material into Cells. *Bioconjugate Chem.* 6, 7–20.
- (12) Kabanov, A. V., Astafieva, I. V., Maksimova, I. V., Lukandin, E. M., Georgiev, G. P., and Kabanov, V. A. (1993) Efficient Transformation of Mammalian Cells Using DNA Interpolyelectrolyte Complexes with Carbon Chain Polycations. *Bioconjugate Chem.* 4, 448–454.
- (13) Kabanov, A. V., Astafieva, I. V., Chikindas, M. L., Rosenblatt, G. F., Kiselev, V. I., Severin, E. S., and Kabanov, V. A. (1991) DNA interpolyelectrolyte complexes as a tool for efficient cell transformation. *Biopolymers* 31, 1437–1443.
- (14) Kulka, M., Smith, C. C., Aurelian, L., Fischelevich, R., Meade, K., Miller, P., and Ts'o, P. O. P. (1989) Site specificity of the inhibitory effects of oligo(nucleoside methylphosphonate)s complementary to the acceptor splice junction of herpes simplex virus type 1 immediate early mRNA 4. *Proc. Natl. Acad. Sci. U.S.A.* 86, 6868–6872.
- (15) Kulka, M., Smith, C. C., Levis, J., Fischelevich, R., Hunter, J. C. R., Cushman, C. D., Miller, P. S., Ts'o, P. O. P., and Aurelian, L. (1994) Synergistic antiviral activities of oligonucleotide methylphosphonates complementary to herpes simplex virus type 1 immediate-early mRNAs 4,5 and 1. *Antimicrob. Agents Chemother.* 38, 675–680.
- (16) Vinogradov, S. V., Suzdaltseva, Yu., Alakhov, V. Yu., and Kabanov, A. V. (1994) Inhibition of Herpes simplex virus 1 reproduction with hydrophobized antisense oligonucleotides. *Biochem. Biophys. Res. Commun.* 203, 959–966.
- (17) Kabanov, A. V., Vinogradov, S. V., Suzdaltseva, Yu. G., and Alakhov, V. Yu. (1995) Water-Soluble Block Polycations as Carriers for Oligonucleotide Delivery, *Bioconjugate Chem.* 6, 639–643.
- (18) Agrawal, S. (1994) Functionalization of oligonucleotides with amino groups and attachment of amino specific reporter groups. In *Protocols for Oligonucleotide Conjugates* (S. Agrawal, Ed.) pp 93–120, Humana Press, Totowa, NJ.
- (19) Atkinson, T., and Smith, M. (1984) Solid-phase synthesis of oligodeoxyribonucleotides by the phosphite-triester method. In *Oligonucleotide Synthesis. A Practical Approach* (M. J. Gait, Ed.) pp 35–82, IRL Press, Oxford, Washington, DC.
- (20) Friser, H. (1975) An improved 2,4,6-trinitrobenzenesulfonic acid method for the determination of amines. *Anal. Biochem.* 64, 284–288.
- (21) Freshney, R. I. (1994) *Culture of Animal Cells*, 3rd ed., John Wiley & Sons, Inc., New York.
- (22) Bailey, C. A., Miller, D. K., and Lenard, J. (1984) Effects of DEAE-dextran on infection and homolysis by VSV. Evidence that nonspecific electrostatic interactions mediate effective binding of VSV to cells. *Virology* 133, 111–118.
- (23) Prakash, J., and Foxe, M. J. (1985) Optimization of conditions for infection of isolated potato protoplasts with potato virus X. *Arch. Virol.* 85, 269–279.
- (24) Rousculp, M. D., Goldsmith, K. T., and Garver, R. I. (1992) Quantative evaluation of retroviral gene transduction efficiency in human lung cancer cells. *Hum. Gene Ther.* 3, 471–477.
- (25) Langeland, N., Moore, L. J., Holmsen, H., and Haarr, L. (1988) Interaction of polylysine with the cellular receptor for herpes simplex virus type 1. *J. Gen. Virol.* 69, 1137–1145.
- (26) Jasschke, A., Furste, J. P., Nordorf, E., Hillenkamp, F., Cech, D., and Erdmann, V. A. (1994) Synthesis and properties of oligodeoxyribonucleotide–polyethylene glycol conjugates. *Nucleic Acids Res.* 22, 4810–4817.
- (27) Jones, D. S., Hachmann, J. P., Osgood, S. A., Hayag, M. S., Barstad, P. A., Iversen, G. M., and Couttus, S. M. (1994) Conjugates of Double-Stranded Oligonucleotides with Poly(ethylene glycol) and Keyhole Limpet Hemocyanin: A Model for Treating Systemic Lupus Erythematosus. *Bioconjugate Chem.* 5, 390–399.
- (28) Soukchareun, S., Tregear, G. W., and Haralambidis, J. (1995) Preparation and Characterization of Antisense Oligonucleotide–Peptide Hybrids Containing Viral Fusion Peptides. *Bioconjugate Chem.* 6, 43–53.
- (29) Arar, K., Aubertin, A.-M., Roche, A.-C., Monsigny, M., and Mayer, R. (1995) Synthesis and Antiviral Activity of Peptide–Oligonucleotide Conjugates Prepared by Using N α -(Bromoacetyl)peptides. *Bioconjugate Chem.* 6, 573–577.

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ARTICLES

Boronated Epidermal Growth Factor as a Potential Targeting Agent for Boron Neutron Capture Therapy of Brain Tumors

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In order for boron neutron capture therapy (BNCT) to be successful, a large number ($\sim 10^9$) of ^{10}B atoms must be delivered to each cancer cell in order to sustain a lethal $^{10}\text{B}(\text{n}, \alpha)^7\text{Li}$ reaction. The majority of high grade gliomas express an amplified epidermal growth factor receptor (EGFR) gene, and increased numbers of EGFR are found on the cell surface. If a sufficiently large number of ^{10}B atoms could be attached to EGF, the resulting bioconjugates might be useful for targeting brain tumors. In order to accomplish this, we have boronated a fourth-generation starburst dendrimer (SD) using an isocyanato polyhedral borane, $\text{Na}(\text{CH}_3)_3\text{NB}_{10}\text{H}_8\text{NCO}$. For conjugation, reactive thiol groups were introduced into the boronated SD using *N*-succinimidyl 3-(2-pyridyldithio)propionate (SPDP), and EGF was derivatized with *m*-maleimidobenzoyl-*N*-hydroxysulfosuccinimide ester (sMBS). Subsequent reaction of thiol groups of derivatized BSD with maleimide groups of derivatized EGF produced stable BSD–EGF bioconjugates containing ~ 960 atoms of boron per molecule of EGF. As determined by electron spectroscopic imaging, the BSD–EGF initially was bound to the cell surface membrane and then was endocytosed, which resulted in accumulation of boron in lysosomes. The favorable *in vitro* properties of these bioconjugates suggest that they may be useful for the *in vivo* targeting of EGFR positive brain tumors.

INTRODUCTION

Boron neutron capture therapy (BNCT), which has been the subject of several recent reviews (Barth et al., 1992; Hawthorne, 1993; Carlsson et al., 1994) and monographs (Allen et al., 1992; Gabel and Moss, 1992; Soloway et al., 1993), is based on the nuclear capture reaction that occurs when a stable isotope, boron-10, is irradiated with low energy (thermal) neutrons to yield high LET α particles and lithium-7 nuclei. These particles have short path lengths ($< 10 \mu\text{m}$) and high kinetic

energy (2.33 MeV), which is deposited within a few microns of the reaction site. The success of BNCT depends upon delivery of a critical number of ^{10}B atoms ($\sim 10^9$ atoms/cell) and a sufficient number of thermal neutrons to each tumor cell to sustain a lethal $^{10}\text{B}(\text{n}, \alpha)^7\text{Li}$ reaction. One of the major challenges in treating high grade brain tumors with BNCT is the targeted delivery of ^{10}B to individual tumor cells. A number of different delivery systems are under investigation, including monoclonal antibodies, peptides, low density lipoproteins, porphyrins, synthetic liposomes, boron-containing amino acids, DNA ligands, modified nucleosides and nucleotide analogs, and epidermal growth factor (EGF), which is the subject of the present report.

EGF is a single-chain, 53-residue polypeptide (Figure 1) that binds to a transmembrane glycoprotein with tyrosine kinase activity (Carpenter and Cohen, 1979). Rapid binding of EGF to its receptors on intact cells triggers dimerization and the immediate clustering of EGF–EGFR complexes in coated pits, which are internalized and form clathrin coated vesicles. These are then uncoated and acidified and subsequently enter early endosomes where recycling receptors are separated from the EGFR (Warren, 1990). Although certain cells may recycle EGFR at low levels (Sorkin et al., 1991), most cells transport internalized EGF and its receptor to lysosomes where both molecules are degraded (Carpenter, 1987; Todderud and Carpenter, 1989). The predominant physiologic response of cells to EGF and EGF-like growth factors (e.g., transforming growth factor α) is increased cell proliferation (Carpenter and Cohen, 1975; Westermarck, 1977; Aakwaag et al., 1990), although for some cell

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¹ Abbreviations: B-EGF, boronated epidermal growth factor; BNCT, boron neutron capture therapy; BSD, boronated starburst dendrimers; DTT, dithiothreitol; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; FBS, fetal bovine serum; LET, linear energy transfer; mAb, monoclonal antibody; PBS, phosphate-buffered saline; SD starburst dendrimer; sMBS, *m*-maleimidobenzoyl-*N*-hydroxysulfosuccinimide ester; SPDP, *N*-succinimidyl 3-(2-pyridyldithio)propionate; ^3H -TdR, tritiated thymidine.

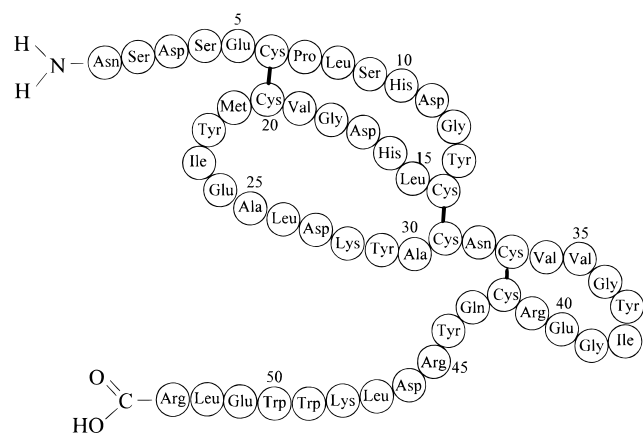


Figure 1. Schematic representation of the structure of human EGF. The thick black lines represent three disulfide bonds (George-Nascimento et al. 1988).

lines with highly elevated numbers of EGFR, growth inhibitory effects have been reported (Werner et al., 1988; Lund-Johansen et al., 1990; Pollak et al., 1990).

The EGF receptor (EGFR) gene is homologous to the *c-erb B* oncogene (Downward et al., 1984) and is often amplified in human glioblastomas. Studies by Bigner et al. (1990) revealed that in a series of 33 human glioma biopsies, 15 showed amplification of the EGFR gene. Similar or even higher frequencies of amplification have been observed by others (Ekstrand et al., 1991; Tuzi et al., 1991), and this is often associated with increased cell surface receptor expression (Torp et al., 1991; Bigner et al., 1990). The distribution of EGFR in high grade gliomas is variable, which probably reflects the cellular heterogeneity of these tumors. However, in 25 of 42 human gliomas studied by Torp et al., more than 50% of the tumor cells bound biotinylated EGF. The number of EGFR on tumor cells may be 100 times greater than that on normal, untransformed cells. The EGFR therefore has been considered as a potential target for specific delivery of therapeutic agents (Mendelsohn, 1988; Sato and Sato 1989; Modjtahedi and Dean, 1991) and monoclonal antibodies against the EGFR or their fragments, as well as EGF or EGF-based bioconjugates, could potentially be used as targeting agents. The purpose of the present study was to develop methodology for the boronation of EGF and to characterize the resulting bioconjugates *in vitro*. Using two heterobifunctional reagents: sMBS and SPDP, we have linked EGF to boronated starburst dendrimers, characterized the affinity of the resulting B-EGF bioconjugates for glioma cells, and determined biological activity and intracellular distribution of boron following internalization of B-EGF.

EXPERIMENTAL PROCEDURES

Reagents. Human recombinant EGF, which previously was shown to bind to the EGFR on human glioma U-343MG cells with high affinity ($K_A = 2.1 \times 10^9 \text{ M}^{-1}$; Capala et al., 1993) was purchased from Upstate Biotechnology, Inc., Lake Placid, NY. Fourth-generation starburst dendrimers (Figure 2a), which are composed of repetitive polyamidoamino groups arranged in a starburst pattern with 48 reactive amino-terminal groups, were kindly provided by Dr. D. Tomalia, Michigan Molecular Institute, Midland, MI. The isocyanato polyhedral borane, $\text{Na}(\text{CH}_3)_3\text{NB}_{10}\text{H}_8\text{NCO}$ (Figure 2b), was prepared in our laboratories as previously described (Alam et al., 1989). Other reagents were purchased from commercial sources as indicated: dithiothreitol, Sigma, St. Louis, MO; the heterobifunctional cross-linking re-

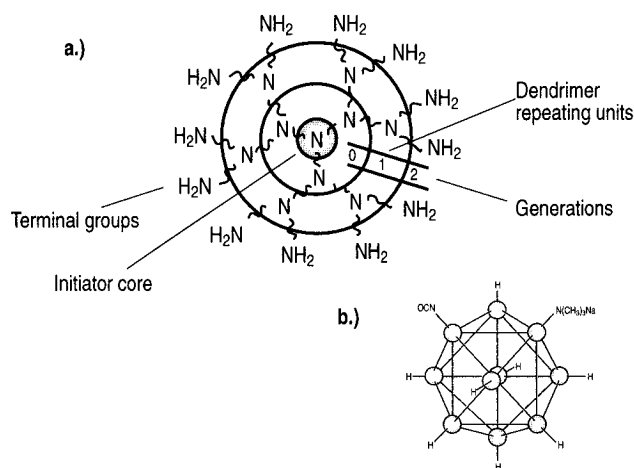


Figure 2. Schematic diagram of a starburst dendrimer (a) and chemical structure of methyl isocyanato polyhedral borane (b).

agents, *m*-maleimidobenzoyl-*N*-hydroxysulfosuccinimide ester and *N*-succinimidyl 3-(2-pyridyldithio)propionate, Pierce, Rockford, IL; Sephadex G-50, Pharmacia Biotech, Piscataway, NJ; [^3H]thymidine, Amersham, Arlington Heights, IL, and [^{125}I]sodium iodide, Dupont NEN, Boston, MA.

Cells. The human malignant glioma U-343MG cell line, expressing 2.5×10^5 high and 8.6×10^5 low affinity receptors (Werner et al., 1988), was kindly provided by Dr. B. Westermark, Department of Pathology, University of Uppsala, Sweden, and was previously described in detail by Westermark et al. (1982) and Nistér et al. (1987). It has been used by one of us in several recent studies on targeting EGFR *in vitro* (Capala et al., 1990, 1993; Capala and Carlsson, 1991; Andersson et al. 1992). Rat C6_{EGFR} glioma cells were produced by transfecting the C6 rat glioma (American Type Culture Collection, Rockville, MD) with the gene encoding EGFR (Fenstermaker et al., 1995). As determined by a radioligand binding assay and Scatchard analysis, the parental C6 cells did not express EGFR and the transfected cells, C6_{EGFR}, expressed 3×10^6 receptors per cell (Fenstermaker et al., 1995). All cell lines were grown in DMEM medium (Gibco, Grand Island, NY) supplemented with 10% FBS (Sigma, St. Louis, MO), L-glutamine (2 mM), solution I (1 mM oxalacetic acid, 0.2 UI/mL crystalline insulin, 0.5 mM sodium pyruvate), streptomycin (100 mg/mL), and penicillin (100 UI/mL) in humidified atmosphere at 37 °C. For tests of biological activity of boronated EGF, cells were grown in serum free hybridoma HyQ-CCM1 medium (HyClone Laboratories, Inc., Logan, UT).

Radiolabeling of EGF. EGF was radioiodinated by means of the chloramine-T method, as previously described (Capala et al., 1990). Briefly, 10 μg of lyophilized EGF was dissolved in 100 μL of 0.25 M potassium phosphate buffer, pH 7.5 and incubated for 1 min with 1 mCi of [^{125}I]sodium iodide and 20 μL of freshly prepared chloramine-T solution (2 mg/mL in 0.5 M phosphate buffer, pH 7.5). The reaction was quenched by adding 50 μL of freshly prepared sodium metabisulfite solution (2 mg/mL in 0.5 M phosphate buffer, pH 7.5). Unbound [^{125}I] was separated from [^{125}I]-EGF using a PD-10 column (Pharmacia Biotech, Piscataway, NJ), eluted with PBS, pH 7.2.

Boronation of EGF. Fourth-generation starburst dendrimers (Figure 2a; Tomalia et al., 1990) were boronated with $\text{Na}(\text{CH}_3)_3\text{NB}_{10}\text{H}_8\text{NCO}$ (Figure 2b), as described in detail in a recent publication (Barth et al., 1994). The synthetic scheme for conjugation of BSD to

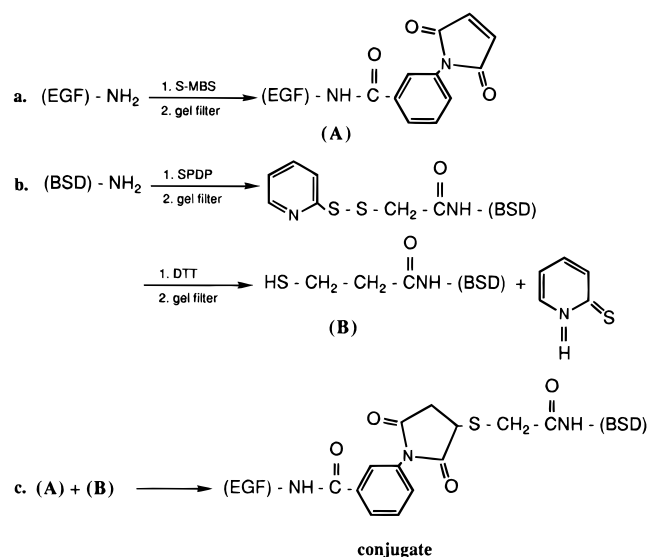


Figure 3. Conjugation scheme for linkage of boronated starburst dendrimer (BSD) to epidermal growth factor (EGF) using the heterobifunctional reagents sMBS and SPDP.

EGF is summarized in Figure 3. First, reactive amino groups of EGF were modified with sMBS (10× molar excess in 0.1 M phosphate buffer, pH 7.2, at room temperature for 1 h) to introduce maleimide groups, and BSD was derivatized with SPDP (10× molar excess in 0.1 M carbonate buffer, pH 9.0, at 37 °C for 2 h) to introduce thiol groups by subsequent reduction of SPDP's disulfide bond using DTT (3 mg/mL at room temperature for 1 h). Then, maleimido groups on EGF were reacted with free sulfhydryls on BSD to form stable covalent bonds between EGF and BSD. To minimize the volume of the reaction mixture, both the BSD and derivatized EGF were concentrated using Centricon 3 microconcentrators (Amicon, Danvers, MA). After overnight reaction at room temperature, the mixture was passed through a Sephadex G-50 column and eluted with 0.1 M Tris, 0.2 M NaCl buffer (pH 8.5). One mL fractions were collected, and protein concentrations were determined spectrophotometrically by measuring absorbance at 280 nm using a Beckman DU-6 spectrophotometer (Beckman Instruments, Inc., Irvine, CA). Boron was quantified by means of direct current plasma atomic emission spectroscopy using a Spectraspan VB spectrometer (Applied Research Laboratories, La Brea, CA) as described in detail by Barth *et al.* (1991). Fractions containing peak concentrations of both protein and boron were pooled and used in the studies described in the following sections. The final concentration of EGF was precisely determined using Water's pico-TAG system (Cohen *et al.*, 1987; Bidlingmayer *et al.*, 1984) for amino acid analysis of the B-EGF. To calculate the number of boron atoms per molecule of EGF in pooled fractions, the molar concentration of boron was divided by molar concentration of EGF. Fractions obtained by gel chromatography, which contained B-EGF, were also analyzed by means of gel electrophoresis (12% polyacrylamide) using a Mini-Protein II Dual Slab Cell (BioRad, Hercules, CA) in order to assess purity of the bioconjugate. The same procedure was used to conjugate radioiodinated EGF to BSD. In this case, however, ¹²⁵I radioactivity in each fraction was measured using a liquid scintillation counter (1214 RackBeta "Excel", LKB Wallac Oy, Turku, Finland).

Receptor Binding Assays. Reactivity of boronated and native EGF with C6_{EGFR} cells was studied by means of a competitive binding assay. C6_{EGFR} cells (~5 × 10⁵ cells/well) were seeded into 24-well flat-bottomed plates

(Corning, Corning, NY) and incubated at 4 °C for 90 min in medium containing 0.185 nM of [¹²⁵I]-EGF and varying concentrations of native (0–83 nM) or boronated (0–410 nM) EGF. The cells then were washed three times with cold PBS (pH = 7.2), and cell-associated radioactivity was determined by γ scintillation counting (Model 1185, Tm Analytic, Elk Grove Village, IL). Affinity constants (K_A) were calculated using a four-parameter logistic equation for fitting the competition curve to the experimental displacement data (Munson, 1984). The same procedure was employed to define the reactivity of B-EGF with U-343MG cells, which subsequently were used in subcellular boron distribution studies. In this case, however, ~10⁵ cells/well were seeded into 24 well plates, and concentrations of [¹²⁵I]-EGF, native EGF, and B-EGF were 0.16 nM, 0–166 and 0–166 nM, respectively. In another set of experiments, U-343MG cells (2 × 10⁵ cells/well) were incubated with increasing concentrations (0–166 nM) of [¹²⁵I]-EGF-BSD bioconjugate at 4 °C for 90 min in order to directly measure its binding to cells as a function of its concentration in the medium. Cells, whose receptors were saturated with native EGF (1.6 μM), were used as background controls to quantify nonspecific binding of the bioconjugate.

Biologic Activity. The effects of increasing concentrations of native and boronated EGF on cell proliferation were studied as follows. C6_{EGFR} cells (~10⁴ cells/well) were seeded into 96-well flat-bottom plates (Corning, Corning, NY) and allowed to grow for 2 days. Then, they were washed and incubated at 37 °C in HyQ-CCM1 serum-free medium containing 21.7 nM of [³H]-TdR (specific activity 46 Ci/mmol) and increasing concentrations of native (0–16.6 nM) or boronated (0–500 nM) EGF for 24 h (six replicates of each). The cells were harvested using a Skatron cell harvester (Skatron Inc., Sterling, VA), and cell-associated radioactivity was measured using a LS 7800 liquid scintillation counter (Beckman, Irvine, CA).

Subcellular Localization. The subcellular localization of B-EGF was determined by means of electron spectroscopic imaging (Bendayan *et al.*, 1989). U-343MG cells first were washed and incubated in serum-free medium for at least 2 h to remove all EGF that might have been present in the medium or bound to the cells. Following this, the cells were rinsed again with serum-free medium and exposed to 0.1 μM of B-EGF at 4 °C for 90 min or at 37 °C for either 30 min or 180 min. To determine the specificity of B-EGF uptake by competitive inhibition, cells were incubated with 0.1 μM of B-EGF at 37 °C for 30 min in the presence of native EGF (3.3 μM). Control cells were treated in the same way, but in media without B-EGF. At the end of the incubation period, the medium containing B-EGF was removed. The cells were rinsed once with PBS, fixed in 1% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2), and then transferred to Eppendorf tubes. Subsequently, the cells were post-fixed with 1% osmium tetroxide and, upon dehydration in graded ethanol solutions, embedded in Epon. Ultrathin sections, <30 nm in thickness, were then examined without further staining with heavy metals, using a Zeiss CEM-902 electron microscope (Carl Zeiss, Oberkochen, Germany). This microscope is equipped for electron energy spectroscopy imaging (ESI) and for spectrum analysis having an electron energy loss spectrophotometer (EELS) and the CEM-902 computer for image processing and analysis. This system has been described in detail previously (Ottensmeyer, 1984; Ottensmeyer and Andrew, 1980). For the detection of boron and for obtaining the net distribution of this element, the image generated at any energy loss below the element

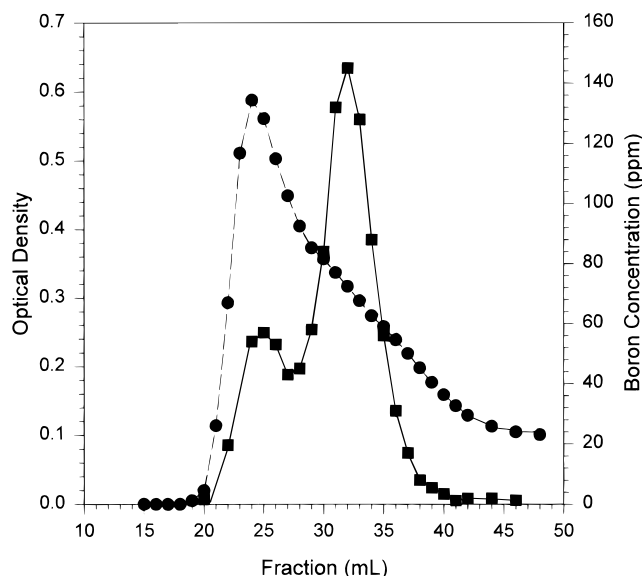


Figure 4. Sephadex G-50 column chromatographic profile of EGF-BSD bioconjugate. SPDP-derivatized BSD was combined with sulfo-MBS-treated EGF, allowed to incubate for 24 h, loaded onto a Sephadex G-50 column (0.9 $\times$ 45 cm), and eluted with 0.1 M Tris-HCl buffer, pH 8.5, containing 0.2 M NaCl. Fractions of $\sim$ 1 mL each were collected, absorbance ($\bullet$) was measured at 280 nm, and boron concentration ($\blacksquare$) values were determined by DCP-AES.

specific absorption edge was subtracted from that obtained with an electron energy loss above that edge and superimposed on the transmission electron micrograph of the same area as described previously (Bendayan et al., 1989). Since the absorption edge for boron is at $\Delta E = 188$ eV, in the present study the net image was generated by subtracting the image obtained at $\Delta E = 200$ eV from that obtained at $\Delta E = 180$ eV. The electron energy loss spectrum was also generated (Bendayan et al., 1989).

RESULTS

Boronation of EGF. Following conjugation of BSD and EGF, chromatographic separation on Sephadex G-50 yielded two boron peaks (fractions 20–27 and 28–38), and a single protein peak was detected (fractions 20–27), indicating that these fractions (20–27) also contained EGF (Figure 4). In order to obtain accurate data on the K_A , it was essential to remove EGF from fractions containing the bioconjugate, which subsequently was used in binding studies. To prove that good separation between conjugated and unconjugated EGF had been achieved by means of Sephadex G-50 column chromatography, [^{125} I]-EGF was conjugated to BSD and the elution profile of the reaction mixture was compared to the elution profiles of unconjugated [^{125} I]-EGF and unbound [^{125} I]. As shown in Figure 5, unconjugated [^{125} I]-EGF was eluted from the column a number of fractions later than the conjugate, and it was concluded that no native EGF was present in the bioconjugate-containing fractions. However, due to the small differences in molecular weights, separation of EGF-BSD bioconjugate from BSD-BSD dimers present in the reaction mixture was difficult. The molecular weights of the bioconjugate and of dimers, which resulted from the formation of disulfide bonds between thiols, were 28–39 and 22 kD, respectively. Therefore, it was possible that the pooled fractions contained dimers, but not unconjugated EGF. SDS gel profiles of the BSD-EGF bioconjugate containing fractions confirmed that there was no unbound EGF in these fractions, which was important for the determi-

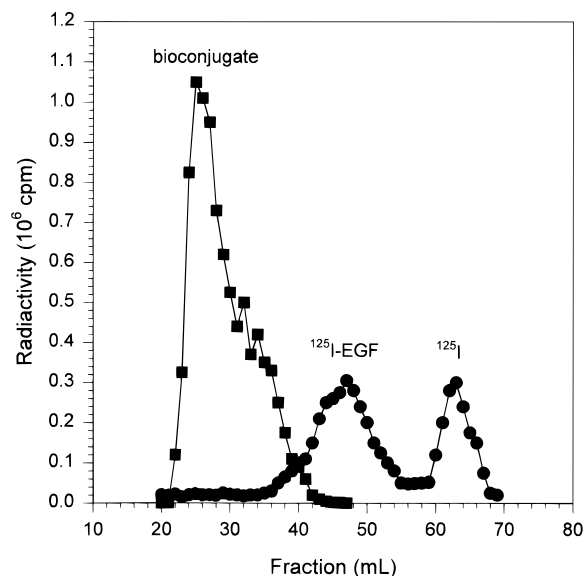


Figure 5. Sephadex G-50 column chromatographic profile of [^{125} I]-EGF-BSD bioconjugate ($\blacksquare$). EGF was labeled with [^{125} I] using the chloramine-T method, treated with sulfo-MBS, and combined with SPDP-derivatized BSD. The reaction mixture was allowed to incubate for 24 h and then loaded onto a Sephadex G-50 column (0.9 $\times$ 45 cm) and eluted with 0.1 M Tris-HCl buffer, pH 8.5, containing 0.2 M NaCl. Fractions of $\sim$ 1 mL each were collected, and [^{125} I] radioactivity in each was determined by scintillation counting. ($\bullet$) represent elution profiles of [^{125} I]-EGF and [^{125} I], indicating good separation of conjugates from unbound EGF.

nation of boron/EGF ratio, as well as biological activity and affinity of the conjugate. As measured by DCP-AES, there were $\sim$ 175 μ g (15.9 μ mol) of boron in pooled fractions (22–26). The amount of EGF in the same fractions, evaluated by amino acid analysis of the B-EGF, was $\sim$ 100 μ g (16.5 nmol). On the basis of these protein and boron determinations, it was calculated that the bioconjugates contained $\sim$ 960 atoms of boron per molecule of EGF.

Binding of EGF-BSD Conjugates to EGFR. As determined by competitive binding assays (Figure 6), approximately 5 times more B-EGF (410 nM) than native EGF was required to produce a 96% reduction in the binding of [^{125} I]-EGF. The calculated K_A for native EGF was $(9.1 \pm 3.3) \times 10^8$ M $^{-1}$, compared to $(8.55 \pm 0.9) \times 10^7$ M $^{-1}$ for B-EGF, indicating that linkage of the boronated starburst dendrimer to EGF produced a 10-fold decrease in the K_A of the bioconjugate. Direct binding of the radiolabeled conjugate to U-343MG cells as a function of its concentration in the medium was assessed by measurement of the [^{125} I] radioactivity associated with these cells following incubation with varying concentrations of [^{125} I]-EGF-BSD. As shown in Figure 7, increased concentrations of the bioconjugate resulted in higher cell-bound radioactivity, which plateaued at 23000–24000 cpm/ 10^6 cells for bioconjugate concentrations $>$ 16.5 nM. Nonspecific binding of the bioconjugate to cells, which were presaturated with native EGF, was several times lower ($<$ 4000 cpm/ 10^6 cells). These results indicated that the binding of the bioconjugate to cells was EGFR-specific.

Biologic Activity of Bioconjugates. Cell proliferation, as determined by uptake of [^{3} H]-TdR by C6_{EGFR} cells, was measured following incubation with increasing concentrations of native (1.66×10^{-3} to 16.6 nM) or boronated (5×10^{-3} to 500 nM) EGF. As shown in Figure 8, native and B-EGF had similar mitogenic activity, as determined by the uptake of [^{3} H]-TdR, but because of

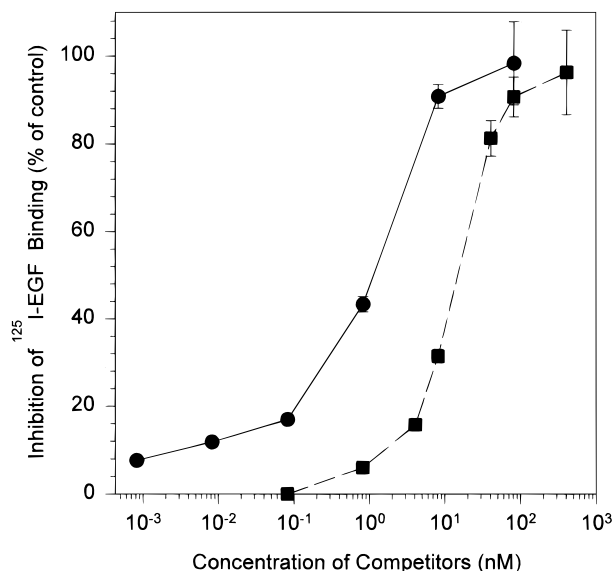


Figure 6. Inhibition of ^{125}I -EGF binding when increased concentrations of native (●) or boronated (■) EGF were used as competitors. C6_{EGFR} cells ($\sim 5 \times 10^5$ cells/well) were incubated with medium containing 0.185 nM of ^{125}I -EGF and varying concentrations of native (0–83 nM) or boronated (0–410 nM) EGF at 4 °C for 90 min. Then, the cells were washed, and cell-associated radioactivity in three samples for each concentration was determined by γ scintillation counting. The mean value and the maximal variation are shown for each point.

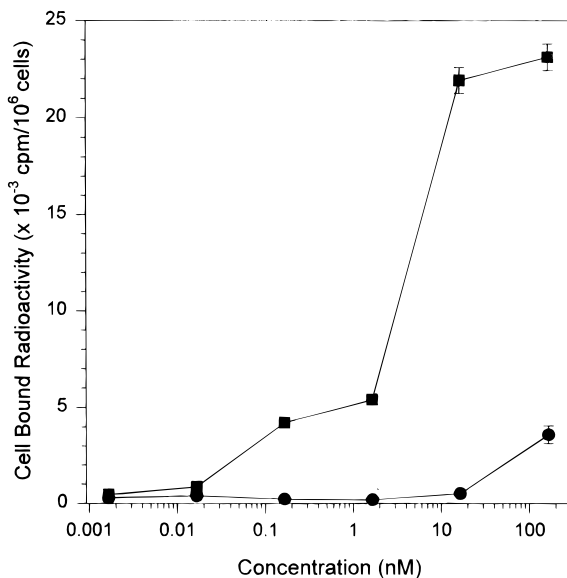


Figure 7. Cell associated ^{125}I radioactivity following incubation of U-343MG cells (2×10^5 cells/well) with increasing concentrations (0–166 nM) of ^{125}I -EGF-BSD bioconjugate at 4 °C for 90 min (■). The cells were then washed, and cell-associated radioactivity in two samples for each concentration was determined by β scintillation counting. (●) represent the background values due to nonspecific binding, obtained from controls—cells whose receptors were saturated with native EGF. The mean value and the maximal variation from measurements are shown for each point.

overlapping standard deviations, it could not be concluded that there was dose dependency. More recent studies, however, indicate that there was a marked dose-dependent increase in ^3H -TdR uptake with increasing concentrations (0.1–1 mM) of either EGF or B-EGF.

Subcellular Distribution of Boron Following Incubation with EGF-BSD. Following incubation of U-343MG cells with B-EGF at 4 °C for 30 min, the boron signal, obtained by electron energy loss spectroscopy, was

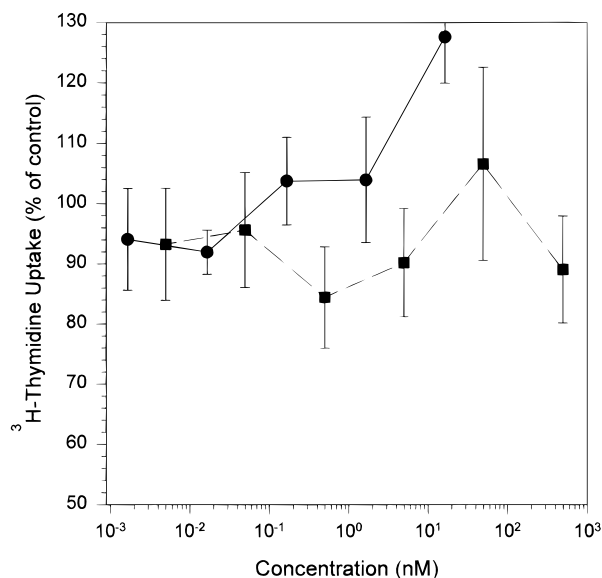


Figure 8. Mitogenic activity of native (●) or boronated (■) EGF. C6_{EGFR} cells, grown in 96-well plates ($\sim 10^4$ cells/well), were washed and incubated at 37 °C in HyQ serum-free medium containing ^3H -TdR (1 $\mu\text{Ci/mL}$) and increasing concentrations of native (0–16.6 nM) or boronated (0–500 nM) EGF for 24 h. Then the cells were harvested, and cell-associated ^3H radioactivity in six samples for each point was measured using a β scintillation counter. Mean values and standard deviations are shown for each point.

found to be mainly associated with the cell plasma membrane. In certain areas the signal followed closely the plasma membrane, with very little intracellular labeling (Figure 9a). In contrast, when cells were analyzed 30 min after exposure to the boronated-EGF at 37 °C, the signal was associated not only with the plasma membrane but also with the cytoplasm (Figure 9b). This finding was present in early and late endosomal structures, as well as in lysosomes displaying encircled membrane profiles with myelin-like figures. Little labeling was associated with other cellular compartments such as mitochondria. At 180 min of exposure at 37 °C, the distribution of the signal appeared similar to that obtained at 30 min, with the difference being in intensity of the signal, which was lower (Figure 9c). EELS analysis made either at the plasma membrane or lysosomal structures confirmed the presence of boron, since its specific absorption edge at $\Delta E = 188$ eV was recorded at those sites (Figure 9d). Under control conditions, when cells had been incubated with excess of native EGF, little specific cell-associated signals were detected (results not shown). Unfortunately, lower magnification of the cells (i.e., a larger field) applied to the detection of B-EGF, which could illustrate how representative these data are, is not possible since it would result in a loss of the boron signal.

DISCUSSION

In the present study, we have described a method for the boronation of EGF using BSD as a carrier for multiple polyhedral borane anions. The procedure used differs from that described by Barth *et al.* (1994) for the boronation of mAbs, where BSD was modified by SMBS, and the protein by SPDP. Chemical reactions required to introduce thiol groups into proteins by SPDP include a reduction step, which could cause breaks of internal disulfide bonds between EGF's cysteines and compromise the stability of its three-dimensional structure. Therefore, in the conjugation scheme described in the present

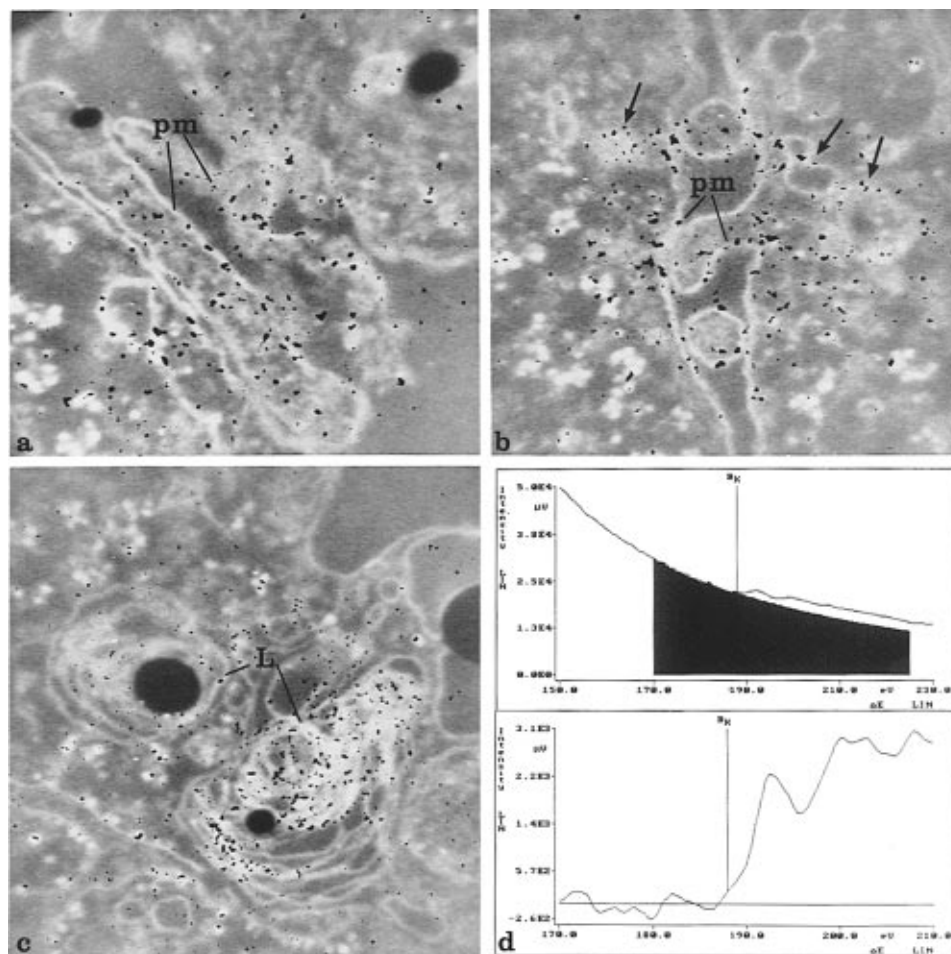


Figure 9. B-EGF-treated Glioma U-343 MG cells examined by electron spectroscopic imaging (ESI). Boron net distribution ($\Delta E = 200\text{--}180\text{ eV}$), revealed as black dots, has been superimposed on high contrasted images ($\Delta E = 250\text{ eV}$). Boron distribution varies according to the experimental protocol: (a) cells incubated with B-EGF for 90 min at 4°C . The signal is associated with the plasma membrane (pm) of the cells with very little internalization. (b) Cells incubated with B-EGF for 30 min at 37°C . The signal was associated with the plasma membrane (pm) as well as with vesicular structures (arrows) of the endosomal compartment. (c) Cells incubated with B-EGF for 180 min at 37°C . The labeling was mainly concentrated at the level of lysosomal structures (L). These structures display accumulations of membranes having a myelin-like appearance. (d) The spectrum of electron energy loss spectrometry (EELS) taken from a lysosomal-like structure of the Glioma U-343MG cells, reveals the presence of the boron edge at $\Delta E = 188\text{ eV}$. Stripped background spectrum shows the real increasing at $\Delta E = 188\text{ eV}$, confirming the boron (B_K) localization. Original magnification $\times 30\,000$; the original photograph has been reduced to 75% of its original size for publication purposes.

study, the EGF was modified by sMBS under mild conditions in order to minimize changes in the tertiary structure and to preserve its ability to bind to EGFR on target cells. Preincubation of cells with native EGF in order to saturate EGFR resulted in a 94% decrease in cell-associated radioactivity following exposure to [^{125}I]-EGF-BSD, indicating high specificity of B-EGF for EGFR. The BSD was modified with SPDP in order to introduce reactive thiol groups, which produced covalent bonds with maleimide groups on modified EGF. Despite mild conditions of reactions involving EGF, data from the competitive binding assays indicated that linkage of BSD to EGF produced a ~ 10 -fold decrease in the K_A of the bioconjugate, but no significant reduction in mitogenic activity. In the conjugation procedure that we employed, MBS reacted with the α or ϵ amino groups of EGF. Although the K_A for MBS-treated EGF was not determined by us, it has been reported that derivatization of the α - or ϵ - NH_2 groups of EGF had little effect on EGF receptor binding compared to native EGF. EGF which had been biotinylated using *N*-biotinyl- ϵ -aminocaproyl-*N*-hydroxysuccinimide (BioNHS) showed almost identical binding to the EGFR as native EGF (Spitzer et al., 1989; Reeves et al., 1994). Therefore, it seems more likely that the reduction in K_A of the bioconjugate was due to steric hindrance by bulky BSD groups rather than modification

of the EGF molecules. The reduction in K_A might have been due to steric hindrance caused by attachment of the dendrimers not only to the derivatized aminoterminal groups but also to amino groups of lysine residues that are constituents of human EGF. This could be avoided by using recombinant mouse EGF, which also binds to EGFR on human tumor cells but does not contain lysine. However, only one BSD could be linked to each EGF molecule, which would result in a decreased number of boron atoms per molecule of bioconjugate. Furthermore, mouse EGF might be immunogenic in humans, and this would be a major disadvantage, if the bioconjugate was to be given intermittently over an interval of weeks or months. Nevertheless, the K_A of our conjugate compared favorably to the K_A of mAbs that have been used for *in vivo* targeting of radionuclides and toxins (Oredipe et al., 1992). Alternative methods of boronation of EGF, using dextran as a boron carrier, have been described by Carlsson et al. (1994). In one of them, dextran chains of 20 kD were activated by 1-cyano-4-(dimethylamino)-pyridinium tetrafluoroborate (CDAP) and linked to EGF and racemic *o*-carboranylalanine (CA) or 7-(3-aminopropyl)-*nido*-carborane (ANC-1). However, EGFR binding experiments carried out with [^{125}I]-EGF-dextran-CA or [^{125}I]-EGF-dextran-ANC-1 showed that only 25–45% of

the cell-associated radioactivity was bound to the glioma cells specifically via EGFR.

Amplification of the EGFR gene in tumor cells may increase cell surface receptor expression to 10^5 – 10^6 sites per cell (Mendelsohn, 1988; Bigner et al., 1990). Moreover, expression of the EGFR increases as tumors progress from low grade astrocytomas to high grade gliomas and glioblastomas. On the basis of protein and boron determinations, it was calculated that our EGF bioconjugates obtained ~960 atoms of boron per EGF molecule. The presence of BSD dimers, however, would have given somewhat higher boron values and therefore the calculated number of boron atoms per molecule of bioconjugate might have been somewhat lower than the number we have estimated. Nevertheless, assuming that the EGFR on each tumor cell could be saturated with boronated EGF, this would not have been of great significance since $\sim 10^8$ boron atoms would still be delivered to each cell.

As shown by ESI, B-EGF, when bound to its specific receptor, was internalized by the cells through the endosomal compartment. Boron was directed toward the lysosomal structures where it was retained and accumulated for periods of time up to 3 h. This suggests either that linkage to BSD prevented degradation of EGF or that the boron was cell-associated, even after EGF had been degraded. This would not only enhance the effectiveness of tumor cell killing by the $^{10}\text{B}(\text{n},\alpha)^7\text{Li}$ fission reaction products, but it would also increase the therapeutic effect *in vivo* because boron concentration in the cells might remain constant, while boron concentration in the blood would decline as a function of time.

Attempts have been made to use EGFR as a target for radioimmunotherapy, and in one series of patients with recurrent glioblastomas, 50% showed some evidence of a clinical response with no significant toxicity (Epenetos et al., 1985; Kalofonos et al., 1989), although there was no followup report indicating improved survival. Recently, Snelling et al. recently have reported on a series of 59 patients with primary high grade gliomas who, following surgery, were treated with ^{125}I -labeled mAb-425, which binds specifically to human EGFR (Snelling et al., 1995). Although no definitive conclusion can yet be made about therapeutic efficacy, no significant life-threatening toxicities were observed and, at the time of writing, 34 of the 59 patients were still alive and the median overall survival time was 13.5 months. Nevertheless, the EGFR remains one of the more attractive targets on high grade gliomas (Harris, 1990). Targeting the EGFR by means of boronated EGF may be easier than using boronated mAbs directed against EGFR, since EGF has a relatively low molecular weight (~6 kD) compared to that of mAbs, and EGF-based conjugates would diffuse more rapidly into poorly vascularized regions of the tumor (Jain, 1989; Barth, 1995). The rapid internalization and degradation of EGF-EGFR complexes, as well as their high affinity constant, are further advantages, if the boron persisted intracellularly.

In vitro studies on targeting EGFR on glioma cells with ^{131}I -radiolabeled EGF bioconjugates have shown that tumor cells could be killed (Andersson et al., 1992; Capala et al., 1990; Capala and Carlsson, 1991). In one series of experiments the activity of $0.086\ \mu\text{Ci}$ ($3.2\ \text{kBq}$)/ 10^6 cells was delivered to glioma cells by EGF-dextran- ^{131}I . The radioactivity remained cell-associated for much longer periods of time (>24 h) than when the radioactivity was delivered by only EGF. Clonogenic survival was reduced to 51%, which corresponded to 3.0 Gy of external ^{60}Co irradiation. Conjugation of EGF to dextran also changed its pharmacokinetics. The biological half-life of [^{125}I]-EGF in rats was 2.0–2.3 h (Scott Robson et al., 1991), while EGF-dextran- ^{131}I had a 6–10 h half-life in mice

(Lindstöm et al., 1993). Furthermore, the uptake by the liver and kidneys was different for [^{125}I]-EGF and EGF-dextran- ^{131}I .

One major consideration is the route by which boronated EGF would be administered to brain tumor-bearing animals or humans. There is a number of possible alternatives, which briefly are summarized as follows, but interested readers are referred to a recent monograph on this subject (Flanagan et al. 1994). Intravenous injection of the bioconjugate would not be feasible due to the very small amount (<0.001%) that would localize in the brain tumor (Vaughan et al., 1987; Bradwell et al., 1985; Thomas et al., 1990). Intracarotid injection could significantly increase the amount delivered to the brain and tumor (Lee et al., 1987), but this still might not be adequate due to the blood-brain barrier (BBB), which is formed by the tight endothelial cell junctions of capillaries (Brightman, 1989) within the brain and the blood tumor barrier (Jain, 1989). The intracarotid infusion of a hyperosmotic solution of mannitol can disrupt the BBB (Neuwelt et al., 1986), and this approach has been shown to significantly increase the tumor uptake of both intact mAbs (Neuwelt et al., 1994) and their F(ab) and F(ab')₂ fragments in brain tumor-bearing mice (Neuwelt et al., 1988). Finally, direct, stereotactic intracerebral injection of the boronated EGF into either the tumor or the site from which the tumor had been surgically resected would result in the highest concentrations. This approach has been used experimentally in mice to deliver antimelanoma antibodies (Liebert et al., 1990) and clinically for the delivery of ^{131}I -labeled antitenascin monoclonal antibody for targeted radiotherapy of patients with glioblastoma who had recurrences of their tumors (Riva et al., 1994). Alternatively, B-EGF might be delivered intratumorally, using B-EGF-containing polymers (Brem et al., 1991) or implantable pumps (Kroin and Penn, 1989; Madrit et al., 1991). Studies currently in progress in our laboratory on intracarotid injection with or without BBB disruption would provide relevant information the optimal route for the administration of boronated EGF to brain tumor-bearing rodents (Yang et al., 1996).

In order for boronated EGF to be useful as a targeting agent for BNCT of gliomas, the following criteria must be met: (1) the conjugates must retain their high affinity for EGF receptors *in vivo*; (2) following either systemic or local administration, sufficient amounts must reach glioma cells dispersed within normal brain; (3) the boron must persist intracellularly within glioma cells for a longer time than that required to clear from normal brain. Since there may be considerable variability in the cellular expression of EGFR within any given glioma, combinations of low and high molecular weight delivery systems could be used to target different subpopulations of tumor cells. In order to determine the potential usefulness of EGF as a targeting agent for BNCT, further *in vitro* and *in vivo* studies must be carried out to define binding of B-EGF to various tumors known to have elevated number of EGFR, as well as its biodistribution and pharmacokinetics following different routes of administration. Such studies currently are in progress in our laboratory.

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NOTE ADDED IN PROOF

Further studies on the affinity constants (K_A) of native and B-EGF recently have been carried out with C6_{EGFR} cells grown in the presence of 1 μ M dexamethasone for ~24 h prior to determination of K_A . The K_A values, determined at ambient temperature rather than at 4 °C, for high- and low-affinity receptor sites for native were much closer than those that we have presented in the Results section of this report.

LITERATURE CITED

- Aakwaag, S., Utaaker, E., Lea, O. A., and Lahooti, H. (1990) Growth Control of Human Mammary Cancer Cells (MCF-7 Cells) in Culture: Effect of Estradiol and Growth Factors in Serum-containing Medium. *Cancer Res.* 50, 7806–7810.
- Alam, F., Barth, R. F., and Soloway, A. H. (1989) Boron Containing Immunoconjugates for Neutron Capture Therapy and for Immunocytochemistry. *Antibody, Immunoconjugates, Radiopharm.* 2, 145–163.
- Allen, B. J., Moore, D. E., and Harrington, B. V., Eds. (1992) *Progress in Neutron Capture Therapy for Cancer*, Plenum Press, New York and London.
- Andersson, A., Capala, J., and Carlsson, J. (1992) Effects of EGF-dextran-thyrosine-¹³¹I Conjugates on the Clonogenic Survival of Cultured Glioma Cells. *J. Neuro-Oncology* 14, 213–223.
- Barth, R. F. (1996) Biologic Considerations in Targeting Brain Tumors for Boron Neutron Capture Therapy. *Cancer Boron Neutron Capture Therapy* (Y. Mishima, Ed.), Plenum Press, New York (in press).
- Barth, R. F., Adams, D. M., Soloway, A. H., Mechetner, E. B., Alam, F., and Anisuzzaman, A. K. M. (1991) Determination of Boron in Tissues and Cells Using Direct-Current Plasma Atomic Emission Spectroscopy. *Anal. Chem.* 63, 890–893.
- Barth, R. F., Soloway, A. H., Fairchild, R. G., and Brugger, R. M. (1992) Boron Neutron Capture Therapy for Cancer. Realities and Prospects. *Cancer* 70, 2995–3007.
- Barth, R. F., Adams, D., Soloway, A. H., Alam, F., and Darby, M. V. (1994) Boronated Starburst Dendrimer-Monoclonal Antibody Immunoconjugates: Evaluation as a Potential Delivery System for Neutron Capture Therapy. *Bioconjugate Chem.* 5, 58–66.
- Bendayan, M., Barth, R. F., Gingras, D., Londoño, I., Robinson, P. T., Alam, F., Adams, D. M., and Mattiazzi, L. (1989) Electron Spectroscopic Imaging for High-resolution Immunocytochemistry: Use of Boronated Protein A. *J. Histochem. Cytochem.* 37, 573–580.
- Bidlingmeyer, B. A., Cohen, S. A., and Tarvin, T. L. (1984) Rapid Analysis of Amino Acids Using Pre-column Derivatization. *J. Chromatogr.* 336, 93–104.
- Bigner, S. H., Humphrey, P. A., Wong, A. J., Vogelsein, B., Mark, J., Friedman, H. S., and Bigner, D. D. (1990) Characterization of the Epidermal Growth Factor Receptor in Human Glioma Cell Lines and Xenografts. *Cancer Res.* 50, 8017–8022.
- Bradwell, A. R., Fairweather, D. S., Dykes, P. W., Keeling, A., Vaughan, A., and Taylor, J. (1985) Limiting factors in the Localization of Tumours with Radiolabeled Antibodies. *Immunol. Today* 6, 163–170.
- Brem, H., Mahaley, M. S., Jr., Vick, N. A., Black, K. L., Schold, S. C., Jr., Burger, P. C., Friedman, A. H., Ciric, I. S., Eller, T. W., Cozzenes, J. W., and Kenealy, J. N. (1991) Interstitial Chemotherapy with Drug Polymer Implants for the Treatment of Recurrent Gliomas. *J. Neurosurg.* 74, 441–446.
- Brightman, M. W. (1989) The Anatomic Basis of the Blood-Brain Barrier. *Implications of the Blood-Brain Barrier and Its Manipulation* (E. A. Neuwelt, Ed.) Vol. 1, pp 53–83, Plenum Publishing Corp., New York.
- Capala, J., and Carlsson, J. (1991) Influence of Chloroquine and Lidocaine on the Therapeutical Effects of ¹³¹I-EGF. Studies on Cultured Glioma Cells. *Int. J. Rad. Biol.* 60, 497–510.
- Capala, J., Pråhl, M., Scott Robson, S., Pontén, J., Westermark, B., and Carlsson, J. (1990) Effects of ¹³¹I-EGF on Cultured Human Glioma Cells. *J. Neuro-Oncol.* 9, 201–210.
- Capala, J., Barth, R. F., Adams, D. M., Soloway, A. H., and Carlsson, J. (1993) Epidermal Growth Factor as a Potential Targeting Agent for Delivery of ¹⁰B to Malignant Gliomas. *Advances of Neutron capture Therapy* (A. H. Soloway, R. F. Barth, and D. E. Carpenter, Eds.) pp 371–375, Plenum Press, New York.
- Carlsson, J., Gedda, L., Grönvik, C., Hartman, T., Lindström, A., Lindström, P., Lundqvist, H., Löfqvist, A., Malmqvist, J., Olsson, P., Essand, M., Pontén, J., Sjöberg, S., and Westermark, B. (1994) Strategy for Boron Neutron Capture Therapy Against Tumor Cells with Over-Expression of the Epidermal Growth Factor-Receptor. *Int. J. Radiat. Oncol. Biol. Phys.* 30, 105–115.
- Carpenter, G. (1987) Receptors for Epidermal Growth Factor and Other Polypeptide Mitogens. *Ann. Rev. Biochem.* 56, 881–914.
- Carpenter, G., and Cohen, S. (1975) ¹²⁵I-Labelled Epidermal Growth Factor. Binding, Internalization, and Degradation in Human Fibroblasts. *J. Cell Biol.* 71, 159–171.
- Carpenter, G., and Cohen, S. (1979) Epidermal Growth Factor. *Ann. Rev. Biochem.* 48, 193–216.
- Cohen, S. A., Tarvin, T. L., and Bidlingmeyer, B. A. (1987) Amino Acid Analysis Using Pre-column Derivatization With Phenylisothiocyanate: Matrix Effects and Tryptophan Analysis. *Proteins: Structure and Function* (J. J. L'Italien, Ed.) pp 207–213, Plenum Press, New York.
- Downward, J., Yarden, Y., Mayes, E., Scare, G., Totty, P., Stockwell, A., Ullrich, A., Schlessinger, J., and Waterfield, M. D. (1984) Close Similarity of Epidermal Growth Factor Receptor and v-erb-B Oncogene Protein Sequences. *Nature* 307, 521–527.
- Ekstrand, A. J., James, C. D., Cavenee, W. K., Selinger, B., Petterson, R. E., and Collins, V. P. (1991) Genes for Epidermal Growth Factor Receptor, Transforming Growth Factor α , and Epidermal Growth Factor and Their Expression in Human Gliomas *in Vivo*. *Cancer Res.* 51, 521–527.
- Epenetos, A. A., Courtney-Luck, N., Pickering, D., Hooker, G., Durbin, H., Lavender, J. P., and McKenzie, C. G. (1985) Antibody Guided Irradiation of Brain Glioma By Arterial Infusion of Radioactive Monoclonal Antibody Against Epidermal Growth Factor Receptor and Blood Group A Antigen. *Br. Med. J.* 290, 1463–1466.
- Fenstermaker, R. A., Capala, J., Barth, R. F., Hujer, A., Kung, H.-J., and Kaetzer, D. M., Jr. (1995) The Effect of Epidermal Growth Factor Receptors (EGFR) Expression on *in Vivo* Growth of Rat C6 Glioma Cells. *Leukemia* 9 (Suppl. 1), S106–S112.
- Flanagan, T. R., Emerich, D. F., and Winn, S. R., Eds. (1994) Providing Pharmacological Access to the Brain. *Methods in Neurosciences*, Vol. 21, Academic Press, Inc., New York.
- Gabel, D., and Moss, R., Eds. (1992) *Boron neutron Capture Therapy. Toward Clinical Trials of Glioma Treatment*, Plenum Press, New York and London.
- George-Nascimento, C., Gyenes, A., Halloran, S. M., Merryweather, J., Valenzuela, P., Steimer, K. S., Masiarz, F., and Randolph, A. (1988) Characterization of Recombinant Human Epidermal Growth Factor Produced in Yeast. *Biochemistry* 27, 792–802.
- Harris, A. L. (1990) The Epidermal Growth Factor Receptor as a Target for Therapy. *Cancer Cells* 2, 321–323.
- Hawthorne, M. F. (1993) The Role of Chemistry in the Development of Boron Neutron Capture Therapy of Cancer. *Angew. Chem., Int. Ed. Engl.* 32, 950–984.
- Jain, R. K. (1989) Delivery of Novel Therapeutic Agents in Tumors: Physiological Barriers and Strategies. *J. Natl. Cancer Inst.* 81, 570–576.
- Kalofonos, H. P., Pawlikowska, T. R., Hemingway, A., Courtenay-Luck, N., Dhokia, B., Snook, D., Sivolapenko, G. B., Hooker, G. R., McKenzie, C. G., Lavender, P. J., Thomas, D. G. T., and Epenetos, A. A. (1989) Antibody Guided Diagnosis and Therapy of Brain Gliomas Using Radiolabeled Monoclonal Antibodies Against Epidermal Growth Factor Receptor and Placental Alkaline Phosphatase. *J. Nucl. Med.* 30, 1636–1645.

- Kroin, J. F., and Penn, R. D. (1989) Implantable Pumps to Deliver Drugs Directly into CNS. *Implications of The Blood Brain Barrier and Its Manipulation* (E. A. Neuwelt, Ed.) Vol. 2, pp 107–261, Plenum Medical Book Co., New York and London.
- Lee, Y., Bullard, D. E., Wikstrand, C. J., Zalutsky, M. R., Muhlbaier, L. H., and Bigner, D. B. (1987) Comparison of Monoclonal Antibody Delivery to Intracranial Glioma Xenografts by Intravenous and Intracarotid Administration. *Cancer Res.* 47, 1941–1946.
- Liebert, M., Wahl, R. L., Lawless, G., McKeever, P. E., Taren, J. A., Beierwalters, W. H., and Brasswell, R. (1990) Direct Stereotactic Intracerebral Injection of Monoclonal Antibodies and Their Fragments: A Potential Approach to Brain Tumor Immunotherapy. *Am. J. Physiol. Imaging* 5, 55–59.
- Lindström, A., Lundqvist, H., and Carlsson, J. (1993) Distribution of ^{125}I after Administration of ^{125}I -Labelled Epidermal Growth Factor-Dextran Conjugates. *Comprehensive Summaries of Uppsala Dissertations from the Faculty of Science*, Chapter V, p 428, Acta Univ. Upsaliensis, Uppsala, Sweden.
- Lund-Johansen, M., Bjerkvig, R., Humphrey, P. A., Bigner, S. H., Bigner, D. D., and Laerum, O.-D. (1990) Effects of Epidermal Growth Factor on Glioma Cell Growth, Migration and Invasion *in Vitro*. *Cancer Res.* 50, 6039–6044.
- Madrit, Y., Feigenbaum Langer, L., Brem, H., and Langer, R. (1991) New Directions if the Delivery of Drugs and Other Substances to the Central Nervous System. *Semin. Pharmacol.* 22, 299–324.
- Mendelsohn, J. (1988) Growth Factory Receptors as Targets for Antitumor Therapy with Monoclonal Antibodies. *Prog. Allergy* 45, 147–160.
- Modjtahedi, H., and Dean, C. (1991) The Receptor for EGF and Its Ligands: Expression, Prognostic Value and Target for Therapy in Cancer (Review). *Int. J. Oncol.* 4, 227–296.
- Munson, P. J. (1984) Heterogeneous Receptors and Binding Curve Analysis in Neurobiology. *Brain Receptor Methodologies, Part A: General Methods and Concepts. Amines and Acetylcholine* (P. J. Marangos, I. C. Campbell, and R. M. Cohen, Eds.) pp 33–48, Academic Press, Inc., New York.
- Neuwelt, E. A., Howieson, J., Frenkel, E. P., Specht, H. D., Weigel, R., Buchman, C. G., and Hill, S. A. (1986) Therapeutic Efficacy of Multiagent Chemotherapy with Drug Delivery Enhancement by Blood-Brain Barrier Modification in Glioblastoma. *Neurosurgery* 19, 573–582.
- Neuwelt, E. A., Barnett, P. A., Hellström, I., Hellström, K. E., Beaumier, P., McCormick, C. I., and Weigel, R. M. (1988) Delivery of Melanoma-associated Immunoglobulin Monoclonal Antibody and Fab Fragments to Normal Brain Utilizing Osmotic Blood-Brain Barrier Disruption. *Cancer Res.* 48, 4725–4729.
- Neuwelt, E. A., Barnett, P. A., Hellström, K. E., Hellström, I., McCormick, C. I., and Ramsey, F. L. (1994) Effect of Blood-Brain Barrier Disruption on Intact and Fragmented Monoclonal Antibody Localization in Intracerebral Human Lung Carcinoma Xenografts. *J. Nucl. Med.* 35, 1831–1841.
- Nistér, M., Wedell, B., Betsholtz, C., Bywater, M., Pettersson, M., Westermarck, B., and Mark, J. (1987) Evidence for Progressional Changes in The Human Malignant Glioma Line U-343MGa: Analysis of Karyotype and Expression of Genes Encoding the Subunit Chains of Platelet-derived Growth Factor. *Cancer Res.* 47, 4953–4960.
- Oredipe, O. A., Barth, R. F., Rotaru, J. H., and Stepleski, Z. (1992) Lack of Effect of Recombinant Human Interferon- $\alpha 2\text{b}$ on Expression of 17-1A Antigen on Human Colon Cancer Cells. *Hybridoma* 11, 607–615.
- Ottensmeyer, F. P. (1984) Electron Spectroscopy Imaging: Parallel Energy Filtering and Microanalysis in the Fixed-beam Electron Microscope. *J. Ultrastruct. Res.* 88, 121–134.
- Ottensmeyer, F. P., and Andrew, J. W. (1980) High Resolution Microanalysis of Biological Specimens by Electron Energy Loss Spectroscopy and by Electron Spectroscopic Imaging. *J. Ultrastruct. Res.* 72, 336–348.
- Pollak, I. F., Randal, L. S., Kristofik, M. P., Kelly, R. H., Selker, R. G., and Vertosich, F. T., Jr. (1990) Response of Malignant Glioma Cell Lines to Epidermal Growth Factor and Platelet-Derived Growth Factor in a Serum-free Medium. *J. Neurosurg.* 73, 106–112.
- Reeves, J. R., Cooke, T. G., Fenton-Lee, D., McNicol, A. M., Ozanne, B. W., Richards, R. C., and Walsh, A. (1994) Localization of EGF Receptors in Frozen Tissue Sections by Antibody and Biotinylated EGF-based Techniques. *J. Immunocytochem. Cytol.* 42, 307–314.
- Riva, P., Arista, A., Tison, V., Sturiale, C., Franceschi, G., Spinelli, A., Riva, N., Casi, M., Moscatelli, G., and Frattarelli, M. (1994) Intralesional Radioimmunotherapy of Malignant Gliomas. An Effective Treatment in Recurrent Tumors. *Cancer* 73, 1076–1082.
- Sato, G. H., and Sato, J. D. (1989) Growth Factor Receptor Monoclonal Antibodies and Cancer Immunotherapy. *J. Natl. Cancer Inst.* 81, 1600–1601.
- Scott Robson, S., Capala, J., Carlsson, J., Malmberg, P., and Lundqvist, H. (1991) Distribution and Stability in the Rat of a $^{76}\text{Br}/^{125}\text{I}$ -labelled Polypeptide, Epidermal Growth Factor. *Nucl. Med. Biol.* 18, 2451–246.
- Snelling, L., Miyamoto, C. T., Bender, H., Brady, L. W., Stepleski, Z., Class, R., Emrich, J., and Rackover, M. A. (1995) Epidermal Growth Factor Receptor 425 Monoclonal Antibodies Radiolabelled with Iodine-125 in the Adjuvant Treatment of High-Grade Astrocytomas. *Hybridoma* 14, 111–114.
- Soloway, A. H., Barth, R. F., and Carpenter, D. E., Eds. (1993) *Advances of Neutron Capture Therapy*, Plenum Press, New York.
- Sorkin, A., Krolenko, S., Kurjavtceva, N., Lazebnik, J., Teslenko, L., Mangelsdorf Soderquist, A., and Nikolsky, N. (1991) Recycling of Epidermal Growth Factor Receptor Complexes in A431 Cells: Identification of Dual Pathways. *J. Cell. Biol.* 112, 55–63.
- Spitzer, E., de Los Angeles, M., Perez, R., and Grosse, R. (1989) Binding Properties of Biotinylated Epidermal Growth Factor to Its Receptor on Cultured Cells and Tissue Sections. *J. Cell. Biochem.* 41, 47–56.
- Thomas, G. D., Chappell, M. J., Dykes, P. W., Ramsden, D. B., Godfrey, K. R., Ellis, J. R. M., and Bradwell, A. R. (1990) Theoretical Considerations for Improving Tumor Targeting. *Br. J. Cancer Suppl.* 10, 70–73.
- Todderund, G., and Carpenter, G. (1989) Epidermal Growth Factor: the Receptor and Its Function. *BioFactors* 2, 11–15.
- Tomalia, D. A., Naylor, A. M., and Goddard, W. A., III. (1990) Starburst Dendrimers: Molecular-Level Control of Size, Shape, Surface Chemistry, Topology, and Flexibility from Atoms to Macroscopic Matter. *Angew. Chem., Int. Ed. Engl.* 29, 138–175.
- Torp, S. H., Helseth, E., Dalen, A., and Unsgaard, G. (1991) Epidermal Growth Factor Receptor Expression in Human Gliomas. *Cancer Immunol. Immunother.* 33, 61–64.
- Tuzi, N. L., Venter, D. J., Kumar, S., Staddon, S. L., Lemoine, N. R., and Gullick, W. J. (1991) Expression of Growth Factor Receptors in Human Brain Tumors. *Br. J. Cancer* 63, 227–233.
- Vaughan, A. T. M., Anderson, P., Dykes, P. W., Chapman, C. E., and Bradwell, A. R. (1987) Limitations to the Killing of Tumors Using Radiolabelled Antibodies. *Br. J. Radiol.* 60, 567–578.
- Warren, G. (1990) Trawling for Receptors. *Nature* 346, 318–319.
- Werner, M. H., Humphrey, P. A., Bigner, D. D., and Bigner, S. H. (1988) Growth Effects of Epidermal Growth Factor (EGF) and a Monoclonal Antibody Against the EGF Receptor on Four Glioma Cell Lines. *Acta Neuropathol.* 77, 196–201.
- Westermarck, B. (1977) Local Starvation for EGF Cannot Explain Density-Dependent Inhibition of Normal Human Glial Cells. *Proc. Natl. Acad. Sci. U.S.A.* 74, 1619–1621.
- Westermarck, B., Magnusson, A., Heldin, C. H. (1982) Effect of Epidermal Growth Factor on Membrane Mobility and Cell Locomotion in Cultures of Human Clonal Glioma Cells. *J. Neurosci. Res.* 8, 491–507.
- Yang, W., Barth, R. F., Carpenter, D. E., and Goodman, J. H. (1996) Enhanced Delivery of Boronophenylalanine to Brain Tumors for Neutron Capture Therapy by Means of Carotid Injection and Blood-Brain Barrier Disruption. *Neurosurgery* (in press).

Rapid Characterization of Chemically-Modified Proteins by Electrospray Mass Spectrometry

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Electrospray mass spectrometry (ESI-MS) has been used to examine monoclonal antibodies (MAbs), antibody fragments (F_{ab} and F_c), modified fragments, and a range of other chemically-modified proteins as part of a study aimed at establishing ESI-MS as a method for the characterization of radioimmunoconjugates. This has been approached from two angles. Firstly, ESI-MS of complexes formed between chelators and other small molecules conjugated to hen egg white lysozyme (HEL) (14 kDa) demonstrate the considerable advantages of this powerful new technique compared with existing methods for the characterization of chemically-conjugated proteins. Molecular weights can be determined rapidly to within 0.01–0.05% and with good sensitivity (10–50 pmol total), thus providing specific structural information and opening the way for ESI-MS to be applied widely for the structural characterization of radioimmunoconjugates. Secondly, the conditions for ESI-MS of intact antibodies and antibody fragments have been examined in detail, and we have shown that the addition of up to 10 biotin molecules to the 50 kDa F_{ab} fragment can be easily detected in ESI mass spectra, thus demonstrating the potential for the characterization of modified MAb fragments and metabolites. Finally, the strengths and limitations of ESI-MS of intact antibodies are discussed, and these results indicate that it may only be possible to detect average shifts in the mass of intact antibodies following modification.

INTRODUCTION

Radioimmunospecific pharmaceuticals, in which radionuclides are incorporated into a monoclonal antibody (MAb) or genetically engineered proteins, are contemporary approaches to diagnostic and therapeutic treatments of cancer (1, 2). Methods for labeling antibodies include the following: (i) direct labeling for isotopes such as iodine-123, iodine-131 (3), and technetium-99m (4) and (ii) indirect labeling with the use of bifunctional chelators for isotopes including indium-111, yttrium-90 (3), and rhenium-188 (4). Many of the factors limiting clinical applications of radioimmunoconjugates, for example, problems with the human anti-mouse antibody (HAMA) response, have been overcome by the use of antibody fragments and the development of recombinant forms of MAbs (5). However, the chemistry involved in modification of MAbs remains a significant limiting factor in the development of radioimmunoconjugates for clinical use. For the ongoing development of radioimmunoconjugates, it is vital to establish loading values of chelators on the proteins (*i.e.*, average numbers of moles of chelator bound to the protein), to characterize the nature of the ligand binding, and to have a method for determining the purity of the resulting radiopharmaceutical.

Current techniques available for assessment of loading conditions of MAbs are most commonly based on UV spectroscopy (6) and radioactivity trace assays (7). The accuracy of the UV spectroscopy assays depends on the relative absorptivities of the conjugate and the protein. Radioassays measuring radioisotope bound in an anti-

body–ligand conjugate may be subject to error if there are noncovalent interactions of the isotopes with the protein. Further, both UV and radioassays only produce an average measurement of ligand binding and yield no information concerning the sites and nature of the binding. Hillenkamp *et al.* (8) have shown matrix-assisted UV laser desorption ionization (MALDI) mass spectrometry to be a useful tool for the determination of loading values of chelators bound to proteins. With this technique, average molecular masses of proteins up to 200 kDa can be determined using only very small amounts of protein (1–10 pmol). A complementary technique to MALDI-MS is electrospray mass spectrometry (ESI-MS) (9). The utility of ESI-MS for the characterization of proteins and large biomolecules is now well established (9–11). ESI-MS offers higher resolution and mass accuracy than MALDI. Ionization of the protein is achieved by evaporation of the solution under the influence of an electric field to yield a series of multiply-charged protonated molecular ions. For proteins, an envelope of multiply charged ions is normally observed with mass-to-charge ratios (m/z) within the range of 500–4000. The ionization process is very gentle so little or no fragmentation of the large molecules occurs.

While ESI-MS of monoclonal antibodies, antibody fragments, and recombinantly engineered antibodies have been published (8, 11–18), there have been no mass spectra reported for chemically-modified antibodies. We report here the development of techniques using ESI-MS for the characterization of coupled chelators and other labels using hen egg white lysozyme (HEL) (14 kDa) as a model to demonstrate the potential of ESI-MS for assessment of chemical protein conjugates. In addition, we have optimized conditions for ESI-MS of the murine B72.3 MAb (19), F_{ab} , and F_c fragments, and we show the ESI-MS of the biotinylated B72.3 F_{ab} fragment. Finally,

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the strengths and limitations of ESI-MS for characterization of MABs are discussed.

EXPERIMENTAL PROCEDURES

Materials. Hen egg white lysozyme (HEL), poly(propylene glycol) 3000, anhydrous dimethyl sulfoxide (DMSO), 1-(3-(dimethylamino)propyl)-3-ethylcarbodiimide hydrochloride (EDC), mercuripapain ($2 \times$ crystallized suspension in 70% ethanol) were purchased from Sigma Pty. Ltd. (St. Louis, MO), Iodogen and (*N*-hydroxy-succinimido)biotin (NHS-biotin) were purchased from Pierce (Rockford, IL), and murine B72.3 monoclonal antibody was purchased from Bioquest Limited (Sydney, NSW, Australia). $[\text{Co}(\text{diAMSarH}_2)](\text{NO}_3)_2$ (diAMSar = 1,8-diamino-3,6,10,13,16,19-hezaazabicyclo[6.6.6]icosane) was a gift from Dr. Stephen Ralph, Department of Chemistry, University of Wollongong, Australia.

Chemical Modification Reactions. *Biotinylation.* HEL or B72.3 F_{ab} fragment (1 mg/mL) in 50 mM sodium bicarbonate buffer (pH 8.5) was reacted at 2- 5- and 8-fold molar ratios with NHS-biotin dissolved in anhydrous DMSO. Samples were incubated at room temperature for 30 min (20).

Iodination. One hundred μL of Iodogen (0.5 mg/mL) in chloroform was pipetted into a test tube and the solvent evaporated to coat the tube with the oxidative agent. One hundred μL of HEL (5 mg/mL) in 10 mM sodium phosphate-buffered saline (pH 7.2) was pipetted into coated tubes, and varying molar ratios of Na^{127}I were added. Samples were reacted at room temperature for 10 min before the reaction was quenched by decanting the mixture from the tube (21).

Conjugation of Chelators. A 100-fold molar excess of EDC and a 1000-fold molar excess of $[\text{Co}(\text{diAMSarH}_2)](\text{NO}_3)_2$ was added to HEL (10 mg/mL) in 75 mM phosphate buffer (final pH 4.75) and incubated at 37 °C for 60 min. Excess EDC and $[\text{Co}(\text{diAMSarH}_2)](\text{NO}_3)_2$ were removed by ultrafiltration. This procedure was based on the method of Conrad *et al.* (22), although a 10-fold molar excess of EDC was used to conjugate $\text{Co}(\text{diAMSar})^{3+}$ (23) to cytochrome *c*. Samples were incubated at room temperature for 30 min.

Preparation of F_{ab} and F_c Fragments. Papain (4 mg/mL) was diluted in digestion buffer (75 mM NaCl, 2 mM disodium EDTA, and 75 mM sodium phosphate, pH 7.0) and activated for 1–2 min at 37 °C before addition to the MAB. Fragments were prepared by dialyzing B72.3 MAB (10 mg/mL) into the digestion buffer containing 10 mM cysteine–HCl and then digesting with 10% papain (w/w) for 90 min at 37 °C.

Separation of F_{ab} and F_c Fragments. MAB fragments were separated on a FPLC system (Pharmacia, Uppsala, Sweden) by HR 10/10 Mono Q anion exchange with a linear gradient of 5–300 mM sodium phosphate buffer (pH 8) at a flow rate of 2 mL/min. The gradient was generated over 15 min. Conjugated protein samples, *i.e.*, biotinylated HEL or F_{ab} fragment, and iodinated and $\text{Co}(\text{diAMSar})$ -modified HEL were analyzed by ESI-MS without further purification. This is a key feature of the method enabling analysis of reaction mixtures to determine the individual species and their relative abundance. All samples were extensively dialyzed against water or desalted using Centricon-10 and Centricon-30 ultrafiltration tubes (Amicon, Lexington, MA). It is essential for ESI-MS analyses that stabilizing buffer salts and unreacted species are removed prior to analysis. The presence of salt in the sample both lowers ESI ionization efficiency and can reduce the accuracy of the mass measurement. Further, desalting reduces the tendency for excess unreacted materials to form nonspecific non-

covalent adducts often observed by ESI-MS (more below). Dialyzed material was lyophilized in a Speed Vac (Savant, Farmingdale, NY) and stored at –20 °C until required.

Mass Spectrometry. A VG Quattro triple quadrupole mass spectrometer (Fisons Biotech MS, Altrincham, U.K.) was used for all experiments. Protein samples were dissolved in 50% aqueous acetonitrile plus 1% formic acid (pH 2.5). The samples were delivered to the instrument in 50% aqueous methanol via an ISCO SFC-500 syringe pump (Lincoln, NE) at a flow rate of 5–10 $\mu\text{L}/\text{min}$. Ten μL of sample was injected for each analysis. HEL and F_c fragments were dissolved at a concentration of 20 pmol/ μL , the F_{ab} fragment at 10 pmol/ μL , and the MAB required a minimum of 1 pmol/ μL for optimum sensitivity. Similarly, the potential on the first source skimmer also required tuning for each protein. The skimmer potential for the MAB was 150 V, and 70–75 V was used for the other proteins. A dry nitrogen bath gas at atmospheric pressure was employed to assist evaporation of the solvent. The electrospray probe tip potential was 3.5 kV with 0.5 kV on the chicane counter electrode. The mass range was calibrated with HEL or ammoniated poly(propylene glycol) 3000 depending on the m/z range of the protein sample to be analyzed. The m/z scale in the raw spectra were converted to exact molecular weights using the transform function of the MassLynx software (Cambridge, U.K.). The m/z values for individual peaks are determined at the center of each peak at 80% height following smoothing. The uncertainties in the masses reported here are calculated from the range of masses generated by this function. For the modified F_{ab} fragment, molecular weights and uncertainties were determined by deconvoluting the raw spectra using the more sophisticated maximum entropy (MaxEnt) method (24).

RESULTS

Chemically-Modified Proteins. A range of different chemical labeling agents using a small protein as a model have been examined. Figure 1a–c shows the ESI mass spectra of HEL following biotinylation with (*N*-hydroxy-succinimido)biotin (NHS-biotin) in protein to biotin ratios of 1:2 (Figure 1a), 1:5 (Figure 1b), and 1:8 (Figure 1c). In each case the most abundant ions are due to the 9⁺ charge state, and the observed charge states range from 5⁺ to 10⁺. This compares (or contrasts) with the charge states observed for native HEL under the same solution conditions. Figure 2a–c shows the spectra corresponding to Figure 1a–c following transformation from a m/z to a mass scale. The addition of one biotin molecule to a protein increases its overall mass by 226 Da, and this is readily detected in the ESI mass spectra. Figure 2a clearly shows a peak due to the native HEL ($14\,306 \pm 2$ Da) and a peak due to the addition of 1 biotin ($14\,532 \pm 2$ Da) which is the most abundant species under these conditions. Peaks corresponding to the addition of two biotins ($14\,758 \pm 2$ Da), three biotins ($14\,986 \pm 1$ Da), four biotins ($15\,211 \pm 3$ Da), and five biotins ($15\,438 \pm 5$ Da) are also evident in this spectrum. As the ratio of biotin to protein was increased the distribution of molecular species shifted to higher masses. With a 1:5 HEL to biotin ratio, the most abundant species is HEL plus two biotins (Figure 2b), and with a 1:8 ratio, HEL plus four biotins is the most abundant (Figure 2c). In this case there is also a peak due to the addition of six biotins ($15\,665 \pm 3$ Da), and the native protein is no longer evident. There is no indication of more than six biotin molecules being incorporated into HEL even with the 1:8 ratio (Figure 2c). The molar incorporation of biotin into

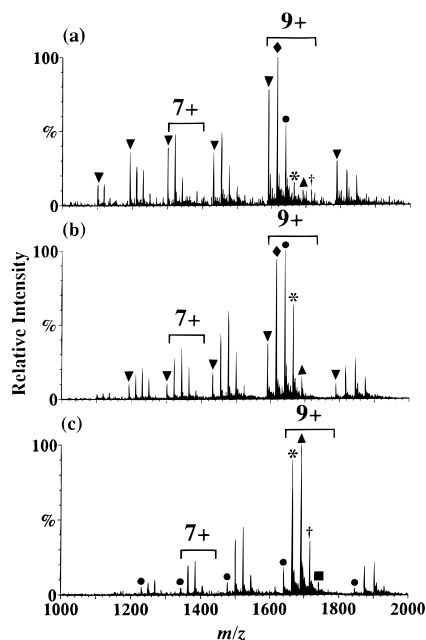


Figure 1. Electrospray mass spectra of biotinylated hen egg white lysozyme (HEL), skimmer potential 70 V, protein concentration 20 pmol/ μ L. Molar ratio of protein to biotin: (a) 1:2, (b) 1:5, (c) 1:8. Key: ∇ , HEL; $\blacklozenge$, HEL + 1 biotin; $\bullet$, HEL + 2 biotin; $*$, HEL + 3 biotin; $\blacktriangle$, HEL + 4 biotin; $\dagger$, HEL + 5 biotin; $\blacksquare$, HEL + 6 biotin.

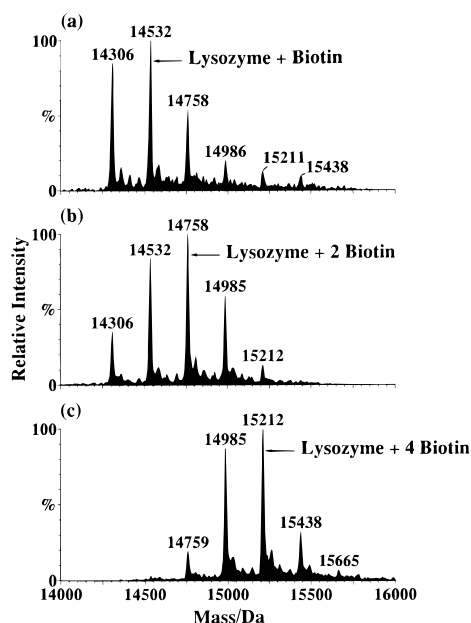


Figure 2. Electrospray mass spectra of biotinylated HEL transformed to a mass scale, skimmer potential 70 V, protein concentration, 20 pmol/ μ L. Molar ratio of protein to biotin: (a) 1:2, (b) 1:5, (c) 1:8.

HEL can be estimated by dividing the relative intensity of each peak in the transformed mass spectrum by the total intensity of the peaks in the spectrum, assuming the ionization efficiency for each species is comparable (25). This approach indicates that when the ratio of HEL to biotin was 1:2, 1.3 molecules of biotin were incorporated per molecule of HEL. This increased to 1.8 and 3.7 for the ratios of 1:5 and 1:8, respectively. Note that the small peaks approximately 50 Da higher than the HEL were also present in the spectrum of the native HEL (not shown) and are thus probably due to nonspecific salt adducts rather than a product arising from reaction with NHS-biotin.

The addition of radioisotopes of iodine to proteins has been well established as a method for direct labeling (26). ESI-MS spectra (not shown) were obtained following the reaction of HEL with mole ratios of protein to Na^{127}I ranging from 1:1 to 1:100. These data, summarized in Table 1, demonstrate that even with a 100-fold molar excess of Na^{127}I there is no evidence of more than four iodine-127 atoms being conjugated to HEL. Figure 3a shows the molar incorporation of iodine-127 versus molar ratio of Na^{127}I added to HEL. These values are calculated from the relative intensities of iodinated HEL in the ESI spectra. The degree of incorporation initially increases logarithmically with increasing Na^{127}I up to 1:10 and thereafter levels off to a average incorporation of 2.5 iodine-127 atoms per molecule of HEL. Figure 3b shows the percentage yield of iodinated HEL as a function of moles of Na^{127}I added to the protein. These data indicate that at a molar ratio of protein to Na^{127}I of 1:50 or greater there is a 96.3% to 98.1% incorporation of iodine-127 into the protein, i.e., the majority of the protein has been modified by iodine-127.

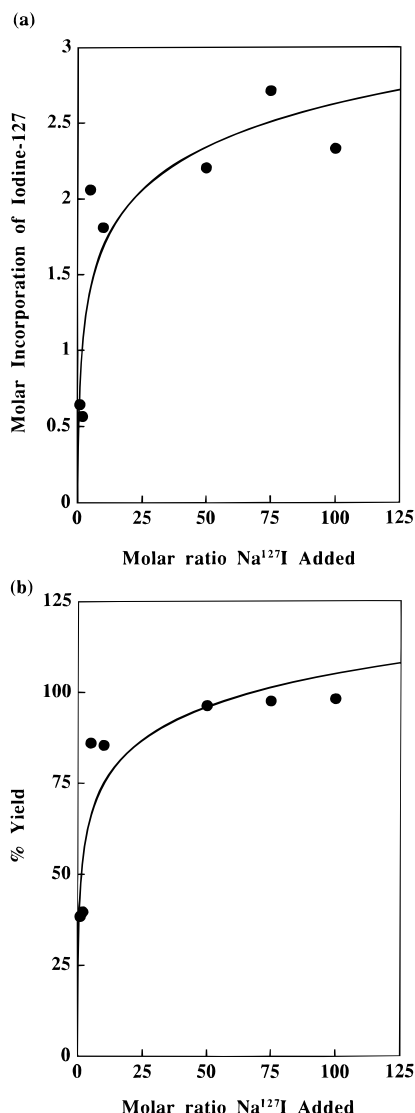
A second method for attachment of a radioisotope or metal to a protein involves the use of a bifunctional chelator or bioconjugate, i.e., indirect labeling. There is increasing interest in using stable macrocyclic cage complexes in nuclear medicine, as retention of the radioisotope or metal within the cage structure is markedly improved *in vivo* (27). Thus, in the present study a readily available cage compound was used to demonstrate the utility of ESI-MS to examine protein conjugates of this type. Covalent attachment of the macrocyclic cage complex $[\text{Co}(\text{diAMSar})]^{3+}$ (23) to acidic protein residues (glutamic and aspartic acid) of HEL is achieved via 1-(3-(dimethylamino)propyl)-3-ethylcarbodiimide hydrochloride (EDC) coupling. The ESI spectrum of HEL coupled to $[\text{Co}(\text{diAMSar})]^{3+}$ via EDC is shown in Figure 4. This shows the addition of one $[\text{Co}(\text{diAMSar})]^{3+}$ moiety to HEL at $14\,662 \pm 3$ Da (theoretical mass 14 661 Da). There are also peaks corresponding to the *N*-acylurea protein adduct at 14 467 Da and to the EDC modified $\text{Co}(\text{diAMSar})$ -HEL conjugate at 14 818 Da.

Analysis of B72.3 Monoclonal Antibody and Antibody Fragments. Illustrated in Figure 5 is the ESI mass spectrum for the intact murine B72.3 monoclonal antibody showing charge states 54^+ to 39^+ . The molecular mass determined for this MAb was $146\,861 \pm 52$ Da, which is approximately 3% higher than the mass of 143 756 Da calculated from the DNA sequence (28, 29). This is consistent with the known degree of glycosylation of the IgG class of MAb (30). Some of the experimental parameters required for observation of these large proteins by ESI differed to those for proteins of lower mass. These include the following: the need for a higher skimmer potential (150 V compared with 75 V for antibody fragments), a sharper dependence on protein concentration with an optimum range of 1–5 pmol/ μ L compared with 10–20 pmol/ μ L for smaller proteins, and a generally higher observed m/z range, being centered around m/z 3000 compared with m/z 1500 for HEL (Figure 1) and the reduced F_c fragment (Figure 6). Further details of B72.3 MAb ESI-MS optimization are reported elsewhere (31).

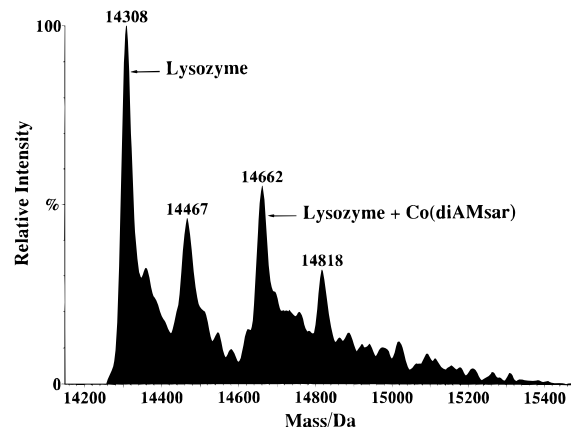
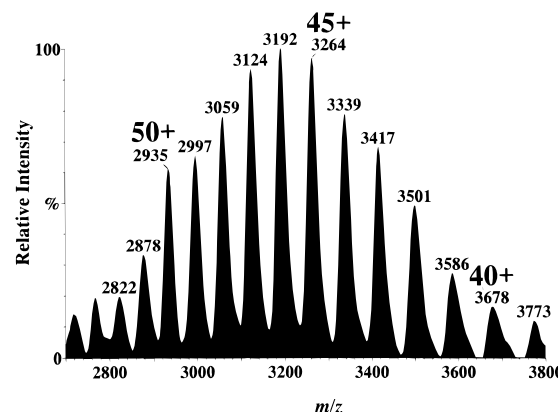
The data processing is also important for these large proteins. The heterogeneity arising from glycosylation (32) causes broadening of the peaks in the ESI mass spectrum of the intact MAb and can result in large uncertainties in mass measurement. There may be also additional peak broadening associated with noncovalent salt adducts (31). The data in the spectrum shown in Figure 5 have been "smoothed" to enable an average

Table 1. Expected and Observed Masses for Iodinated Lysozyme at Varying Molar Ratios of Protein to Sodium Iodide (Na^{127}I)

component	expected mass (Da)	observed mass (Da)						
		1:1	1:2	1:5	1:10	1:50	1:75	1:100
lysozyme	14 306	14 306 $\pm$ 1	14 307 $\pm$ 1	14 306 $\pm$ 2	14 308 $\pm$ 1	14 306 $\pm$ 3	14 307 $\pm$ 3	14 306 $\pm$ 2
+ iodine-127	14 432	14 431 $\pm$ 1	14 433 $\pm$ 2	14 434 $\pm$ 2	14 433 $\pm$ 1	14 434 $\pm$ 1	14 434 $\pm$ 1	14 432 $\pm$ 1
+ 2 iodine-127	14 558	14 558 $\pm$ 4	14 559 $\pm$ 3	14 559 $\pm$ 2	14 559 $\pm$ 2	14 559 $\pm$ 1	14 559 $\pm$ 1	14 555 $\pm$ 2
+ 3 iodine-127	14 684			14 687 $\pm$ 7	14 686 $\pm$ 2	14 684 $\pm$ 1	14 685 $\pm$ 1	14 683 $\pm$ 2
+ 4 iodine-127	14 810			14 814 $\pm$ 6	14 813 $\pm$ 1	14 811 $\pm$ 1	14 811 $\pm$ 2	14 810 $\pm$ 2
+ 5 iodine-127	14 936							
+ 6 iodine-127	15 062							

**Figure 3.** (a) Molar incorporation of iodine into HEL versus molar ratio Na^{127}I added. (b) Percentage yield of iodinated hen egg white lysozyme versus molar ratio Na^{127}I added.

molecular weight to be determined from the data to overcome this problem. It was not possible to derive a molecular weight from unsmoothed data. It should also be noted that the peaks become more asymmetric as the charge state decreases. This has been attributed to nonspecific salt adducts which are present even after extensive dialysis (31). These are more pronounced at higher m/z as the salt adducts appear to block accessible charge states. Thus, we have based the mass calculation on the peak centers at 80% of the peak height to minimize the effect of this asymmetry. Furthermore, only the seven most intense charge states, i.e., 49^+ to 43^+ , were included in the calculation. The same data processing

**Figure 4.** Electrospray mass spectrum of HEL-[Co(diAMsar)] conjugate transformed to a mass scale, skimmer potential 70 V, protein concentration, 20 pmol/ μL .**Figure 5.** Electrospray mass spectrum of intact murine B72.3 monoclonal antibody, skimmer potential 150 V, protein concentration 1 pmol/ μL .

parameters for smaller proteins yield molecular masses to within 0.05%.

The ESI spectrum of the reduced B72.3 F_c subunit is shown in Figure 6a. Charge states 14^+ to 22^+ are given, with an expanded view of charge state 19^+ shown inset. The transformed ESI-MS spectrum shown in Figure 6b indicates the presence of three major components labeled A, B, and C. The predominant peak in the spectrum (component A) has an average molecular mass of $25\,733 \pm 7$ Da, which is consistent with the amino acid sequence of this fragment (heavy chain fragment: Val-225 $\rightarrow$ Lys-438) (28) plus the core carbohydrate portion (2 *N*-acetylglucosamine, 3 mannose, 1 fucose) with the addition of two *N*-acetylglucosamine units (more below). The other major peaks at $25\,895 \pm 6$ Da (component B) and $26\,057 \pm 8$ Da (component C) correspond closely to the addition of 1 and 2 galactose units, respectively.

The ESI mass spectrum of the B72.3 F_{ab} subunit showing charge states 23^+ to 37^+ is illustrated in Figure 7. The average molecular weight calculated from the raw

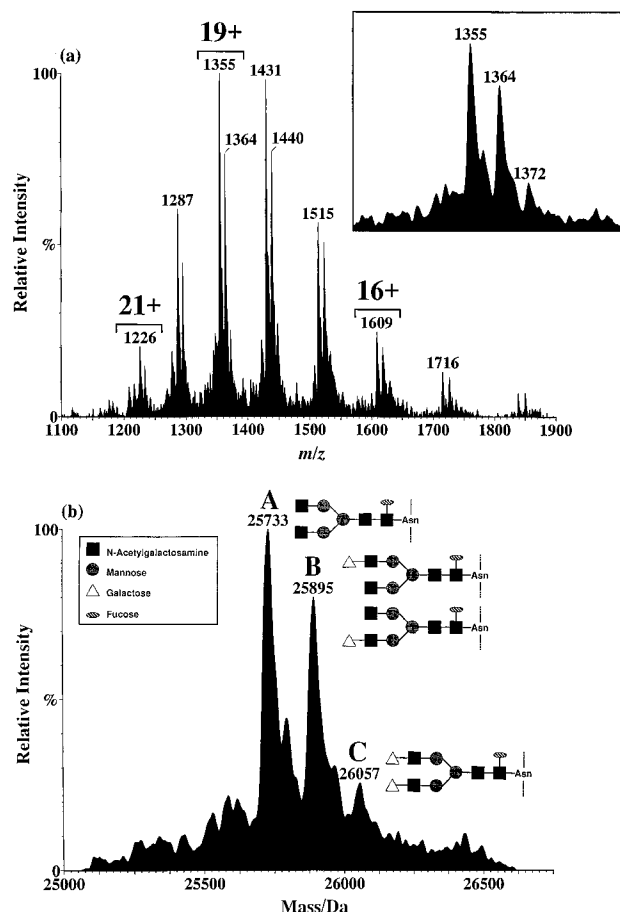


Figure 6. Reduced F_c fragment of the B72.3 monoclonal antibody, skimmer potential 75 V, protein concentration 20 pmol/ μ L. (a) Electrospray mass spectrum. The inset shows the expanded view of 19⁺ charge state. (b) Electrospray mass spectrum transformed to a mass scale. Glycosylation patterns are as indicated.

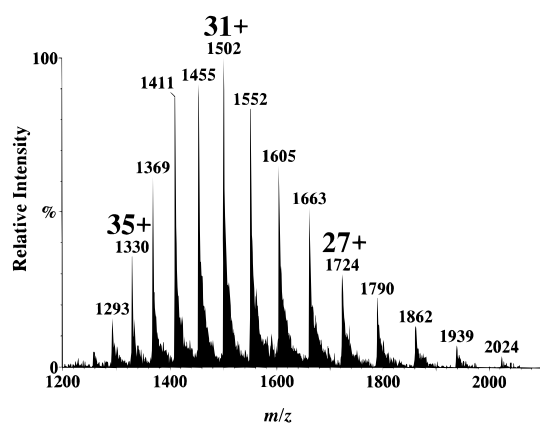


Figure 7. Electrospray mass spectrum of F_{ab} fragment of the B72.3 monoclonal antibody, skimmer potential 75 V, protein concentration 10 pmol/ μ L.

data was $46\,525 \pm 8$ Da. On the basis of the DNA sequence of the B72.3 MAb, the expected mass of the F_{ab} fragment is 46 832 Da (light chain: Asp-1 $\rightarrow$ Cys-214 plus heavy chain fragment: Gln-1 $\rightarrow$ Gly-217) (28, 29). Thus, the lower mass observed by ESI-MS may suggest some additional enzymatic digestion or degradation. The ESI-MS transformed spectra of biotinylated F_{ab} fragment at ratios of 1:2, 1:5, and 1:8 are shown in Figure 8a–c. Successive increases in mass of only 226 Da on the 50 kDa protein fragment are clearly evident in the spectra. An average of 2.1 biotin molecules were incorporated into the B72.3 F_{ab} fragment when the ratio of protein to biotin

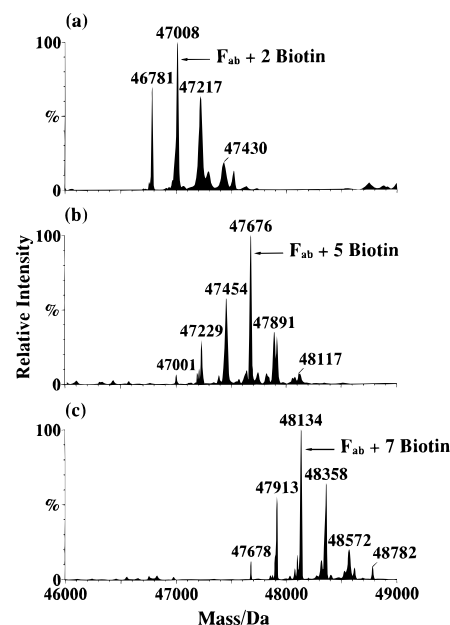


Figure 8. Electrospray mass spectra of biotinylated F_{ab} fragment converted to a mass scale using MaxEnt deconvolution software, skimmer potential 75 V, protein concentration 10 pmol/ μ L. Molar ratio of protein to biotin: (a) 1:2, (b) 1:5, (c) 1:8.

was 1:2. This increased to 4.2 and 7.0 for the ratios of 1:5 and 1:8, respectively. It is interesting to note that even at a F_{ab} to biotin ratio of 1:2, the native protein is no longer evident. This suggests a 100% molar incorporation of biotin since there is no purification stage between chemical modification and analysis. The data from ESI-MS of the B72.3 monoclonal antibody and fragments are summarized in Table 2.

DISCUSSION

HEL was chosen as the initial model protein for this study as there is substantial literature available concerning the accessibility of the amino acid residues in this protein (33). (*N*-Hydroxysuccinimido)biotin (NHS-biotin) reacts specifically with primary amines (generally lysine ϵ groups) on proteins to form stable amide bonds. HEL has six lysine residues available for conjugation to a biotin molecule, and as seen in Figure 2c all six residues appear to have been conjugated when a 8 mol excess of biotin was used. The possibility of reaction of NHS-biotin with the other amino groups on the protein (e.g., *N*-terminal) cannot be excluded on the basis of this data; however, there is no spectral evidence for the incorporation of a seventh biotin molecule.

The available methods for attachment of iodine to proteins employ the use of oxidative agents for producing an electrophilic iodine species (I^+), thereby enabling iodine to react with aromatic groups, with tyrosine residues the major site of iodination (34). As with the biotinylated species, small changes in the molar ratio of protein to conjugate resulted in a change in the distribution of molecular species as well as a change in their proportions. HEL has three tyrosine residues, and since each tyrosine has the potential to conjugate two iodine atoms, incorporation of up to six iodine atoms might be expected. However, it is well documented that the two most reactive tyrosine residues in HEL are Tyr-20 and Tyr-23. The third residue, Tyr-53, generally only reacts under extreme conditions (33). As indicated by the data in Table 1, excessive iodination of HEL only results in the incorporation of a maximum of four iodine atoms which is consistent with an unreactive third tyrosine

Table 2. Expected and Observed Masses for B72.3 Monoclonal Antibody and Fragments

	expected mass (Da)	obsd mass (Da)
F _{ab} fragment (light chain: Asp-1 → Cys-214 plus heavy chain fragment: Gln-1 → Gly-217)	46 832 ^{28,29}	46 525 ± 8
reduced F _c fragment (heavy chain fragment: Val-225 → Lys-438) + core carbohydrate ^a + 2 <i>N</i> -acetylglucosamine	25 739	25 733 ± 7
reduced F _c fragment (heavy chain fragment: Val-225 → Lys-438) + core carbohydrate ^a + 2 <i>N</i> -acetylglucosamine + 1 galactose	25 901	25 895 ± 6
reduced F _c fragment (heavy chain fragment: Val-225 → Lys-438) + core carbohydrate ^a + 2 <i>N</i> -acetylglucosamine + 2 galactose	26 063	26 057 ± 8
B72.3 MAb	143 756 ^{28,29 b}	146 861 ± 52

^a Core carbohydrate = (mannose)₃(*N*-acetylgalactosamine)₄(fucose). ^b Molecular mass of nonglycosylated MAb.

residue. This would not be apparent from other methods used for assessment of iodine incorporation which only yield an average figure. Confirmation of a nonreactive Tyr-53 toward iodination would require further structural analysis by additional experiments involving enzymatic digestion and LC-MS or MS-MS analysis of the resulting peptides.

The spectrum in Figure 4 indicates the addition of one [Co(diAMsar)]³⁺ moiety to HEL, despite the use of a 1000-fold molar excess of ligand. A similar result was obtained by Conrad *et al.* (22) in coupling reactions of [Co(diAMsar)]³⁺ to cytochrome *c*. These workers also used more detailed mass spectrometric studies to demonstrate that there were a number of different attachment sites for this ligand on the protein; however, in all cases, only one [Co(diAMsar)]³⁺ was attached to the protein. In addition, the spectrum also shows that a number of *N*-acylurea EDC-modified glutamic or aspartic acid residues are also present but have not covalently bound the [Co(diAMsar)]³⁺ moiety. This would clearly be undesirable from the point of view of obtaining maximum incorporation of [Co(diAMsar)]³⁺. This type of structural detail could in turn be used in the optimization of the chemistry involved in conjugation of such ligands to the protein. ESI-MS can also provide an estimate of the degree of purity of the required compound. For example, if the desired product is the protein plus one ligand without EDC coupling groups and the ESI spectrum indicates that this is not the predominant component, the chemistry can be modified until this is shown to be the major component by ESI-MS. Alternatively, once it has been established by ESI-MS that the required species is present, ESI-MS can be used to monitor chromatographic separations to ensure maximum purity of the desired product.

The peaks from the intact MAb (in Figure 5) are approximately 1300 Da (~30 *m/z* units) wide due to both glycosylation, salt adducts, and the large number of isotopic states for a molecule of this size. Thus, it would not be possible to resolve a difference of say 226 Da from one biotin molecule which corresponds to ~4 *m/z* units for the 50⁺ charge state and one galactose residue (162 Da, ~3 *m/z* units). However by "oversmoothing" so that an average mass is determined it is likely that average mass shifts would be observed, as has been demonstrated with MALDI-MS (8). Alternatively, MAbs could be modified with the ligand of choice, enzymatically digested to F_{ab} and F_c fragments, and the individual fragments assessed by ESI-MS to determine the loading values of the ligand on the fragments. We have already shown that the detection of a small ligand on a 50 kDa F_{ab} fragment can readily be observed (Figure 8). Experiments are currently under way to remove the carbohydrate side chain from the C_H2 region of B72.3. This will not only allow direct comparison of the molecular weight obtained by ESI-MS with the known DNA sequence of the antibody but it may enable chemical modifications

on a considerably larger protein to be observed. It should also be possible to use ESI-MS for assessing the purity of the MAb, *i.e.*, to determine if one or more MAbs are present.

The carbohydrate structure of murine IgG is an asparagine linked oligosaccharide of the biantennary complex type (35). However, instead of a single oligosaccharide, an array of structurally related yet distinct oligosaccharides is expressed. It has been reported that 94% of murine oligosaccharides have a fucosylated core and that 80% bear either no or a single galactose residue. Solely on the basis of mass, the proposed carbohydrate composition for the B72.3 MAb appears to have the same structure as that elucidated by Mizuochi *et al.* (35).

The molecular mass of the B72.3 F_{ab} fragment as determined from DNA sequencing is 46 832 Da assuming cleavage at the C-terminus of Gly-217 of the MAb heavy chain. This is 307 Da greater than the mass of the papain generated F_{ab} fragment as determined by ESI-MS. There are two plausible explanations for this observed discrepancy. Firstly, it is possible that there may be an error in the DNA sequence which has been translated to errors in the amino acid sequence, or secondly, it is feasible that the papain has overdigested the B72.3 MAb and removed amino acids from the F_{ab} fragment. However, it is not obvious from the sequence and mass differences where this may have occurred. Such a difference may not be readily detected by conventional techniques. SDS-PAGE, for example, would be unable to discriminate between a mass of 46 832 and 46 525 Da. This clearly illustrates the power of ESI-MS for determining small changes in mass on large proteins.

CONCLUSION

The work described here demonstrates the potential of electrospray mass spectrometry for the characterization of chemically-modified proteins including MAb fragments. ESI-MS offers the capability for determining both the numbers of ligands bound to the protein and the actual distributions of individual species, thus providing a degree of structural information not accessible by other methods. Thus, ESI-MS should prove to be a powerful aid in the design and development of improved radioimmunoconjugates for future clinical applications. Finally, we and others have demonstrated ESI-MS can be used for the measurement of intact MAbs thus providing a means of establishing the purity of antibody-based pharmaceuticals.

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LITERATURE CITED

- (1) Riethmüller, G., Schneider-Gädick, E., and Johnson, J. P. (1993) Monoclonal antibodies in cancer therapy. *Curr. Opin. Immunol.* 5, 732–739.
- (2) James, K. (1993) Human monoclonal antibodies and engineered antibodies in the management of cancer. *Cancer Biol.* 1, 243–253.
- (3) Hiltunen, J. V. (1993) Search for new and improved radiolabelling methods for monoclonal antibodies. *Acta Oncol.* 32, 831–839.
- (4) Griffiths, G. L., Goldenberg, D. M., Jones, A. L., and Hansen, H. J. (1992) Radiolabelling of monoclonal antibodies and fragments with technetium and rhenium. *Bioconjugate Chem.* 3, 91–99.
- (5) Khazaeli, M. B., Conry, R. M., and LoBuglio, A. F. (1994) Human immune response to monoclonal antibodies. *J. Immunother.* 15, 42–52.
- (6) Pietersz, G. A., Cunningham, Z., and McKenzie, I. F. C. (1988) Specific *in vitro* anti-tumour activity of methotrexate-monomonal antibody conjugates prepared using human serum albumin as an intermediary. *Immunol. Cell Biol.* 66(1), 43–49.
- (7) Meares, C. F., McCall, M. J., Reardan, D. T., Goodwin, D. A., Diamanti, C. I., and McTigue, M. (1984) Conjugation of antibodies with bifunctional chelating agents: isothiocyanate and bromoacetamide reagents, methods of analysis and subsequent addition of metal ions. *Anal. Biochem.* 142, 68–78.
- (8) Siegel, M. M., Hollander, I. J., Hamann, P. R., James, J. P., Hinman, L., Smith, B. J., Farnsworth, A. P. H., Phipps, A., King, D. J., Karas, M., Ingendoh, A., and Hillenkamp, F. (1991) Matrix-assisted UV-laser desorption/ionisation mass spectrometric analysis of monoclonal antibodies for the determination of carbohydrate, conjugated chelator and conjugated drug content. *Anal. Chem.* 63, 2470–2481.
- (9) Fenn, J. B., Mann, M., Meng, C. K., Wong, S. F., and Whitehouse, C. M. (1989) Electrospray ionisation for mass spectrometry of large biomolecules. *Science* 246, 64–71.
- (10) Smith, R. D., Loo, J. A., Edmonds, C. G., Barinaga, C. J., and Udseth, H. R. (1990) New developments in biochemical mass spectrometry: electrospray ionisation. *Anal. Chem.* 62, 882–899.
- (11) Feng, R., and Konishi, Y. (1992) Analysis of antibodies and other large glycoproteins in the mass range of 150 000–200 000 Da by electrospray ionisation mass spectrometry. *Anal. Chem.* 64, 2090–2095.
- (12) Feng, R., Konishi, Y., and Bell, A. W. (1991) High accuracy molecular weight determination and variation characterisation of proteins up to 80 ku by ionspray mass spectrometry. *J. Am. Soc. Mass Spectrom.* 2, 387–401.
- (13) Jiskoot, W., van de Werken, G., Coco Martin, J. M., Green, B. N., Beuvery, E. C., and Crommelin, D. J. A. (1992) Application of electrospray mass spectrometry (ES-MS) for the analysis of monoclonal antibody F_c subunits. *Pharm. Res.* 9, 945–951.
- (14) Feng, R., and Konishi, Y. (1993) Collisionally-activated dissociation of multiply charged 150-kDa antibody ions. *Anal. Chem.* 65, 645–649.
- (15) Lewis, D. A., Guzzetta, A. W., Hancock, W. S., and Costello, M. (1994) Characterization of humanised anti-TAC, an antibody directed against the interleukin 2 receptor, using electrospray ionisation mass spectrometry by direct infusion, LC/MS, and MS/MS. *Anal. Chem.* 66, 585–595.
- (16) Verentchikov, A. N., Ens, W., and Standing, K. G. (1994) Reflecting time-of-flight mass spectrometer with an electrospray ion source and orthogonal extraction. *Anal. Chem.* 66, 126–133.
- (17) Bourell, J. H., Clauser, K. P., Kelley, R., Carter, P., and Stults, J. T. (1994) Electrospray ionisation mass spectrometry of recombinantly engineered antibody fragments. *Anal. Chem.* 66, 2088–2095.
- (18) Ashton, D. S., Beddell, C. R., Cooper, D. J., Craig, S. J., Lines, A. C., Oliver, R. W. A., and Smith M. A. (1995) Mass spectrometry of the humanised monoclonal antibody CAM-PATH 1H. *Anal. Chem.* 67, 835–842.
- (19) Colcher, D., Esteban, J. M., Carrasquillo, J. A., Sugarbaker, P., Reynolds, J. C., Bryant, G., Larson, S. M., and Schlom, J. (1987) Quantitative analyses of selective radiolabelled monoclonal antibody localisation in metastatic lesions of colorectal cancer patients. *Cancer Res.* 47, 1185–1189.
- (20) Bayer, E. A., and Wilchek, M. (1990) Protein Biotinylation. *Methods in Enzymology* Vol. 184, pp 138–157, Academic Press, San Diego, CA.
- (21) Fraker, P. J., and Speck, J. C., Jr. (1978) Protein and cell membrane iodination with a sparingly soluble chloroamide, 1,3,4,6-tetrachloro-3 α ,6 α -diphenylglycoluril. *Biochem. Biophys. Res. Commun.* 80, 849–857.
- (22) Conrad, D. W., Zhang, H., Stewart, D. E., and Scott, R. A. (1992) Distance dependence of long-range electron transfer in cytochrome c derivatives containing covalently attached cobalt cage complexes. *J. Am. Chem. Soc.* 114, 9909–9915.
- (23) Geue, R. J., Hambley, T. W., Harrowfield, J. M., Sargeson, A. M., and Snow, M. R. (1984) Metal ion encapsulation: cobalt cages derived from polyamines, formaldehyde and nitromethane. *J. Am. Chem. Soc.* 106, 5478–5488.
- (24) Ferrige, A. G., Seddon, M. J., Green, B. N., Jarvis, S. A., and Skilling, J. (1992) Disentangling electrospray spectra with maximum entropy. *Rapid Commun. Mass Spectrom.* 6, 707–711.
- (25) Kilby, G. W., Truscott, R. J. W., and Sheil, M. M. Unpublished observations.
- (26) Mather, S. J. (1986) Radioiodinated monoclonal antibodies: a critical review. *Appl. Radiat. Isot.* 37, 727–733.
- (27) Parker, D. (1990) Tumour targeting with radiolabelled macrocycle-antibody conjugates. *Chem. Soc. Rev.* 19, 271–291.
- (28) Kabat, E. A., Wu, T. T., Reid-Miller, M., Pery, H. M., and Gottesman, K. S. (1987) *Sequences of Proteins of Immunological Interest*, 4th ed., U.S. Department of Health and Human Services, National Institute of Health, Bethesda, MD.
- (29) Whittle, N., Adair, J., Lloyd, C., Jenkins, L., Devine, J., Schlom, J., Raubitschek, A., Colcher, D., and Bodmer, M. (1987) Expression in COS cells of a mouse-human chimaeric B72.3 antibody. *Protein Eng.* 1, 499–505.
- (30) Goding, J. W. (1986) *Monoclonal Antibodies: Principles and Practice*, 2nd ed., Academic Press, London, UK.
- (31) Bennett, K. L., Hick, L. A., Truscott, R. J. W., Sheil, M. M., and Smith, S. V. (1995) Optimum conditions for electrospray mass spectrometry of a monoclonal antibody. *J. Mass Spectrom.* 30, 769–771.
- (32) Rothman, R. J., Warren, L., Vliegthart, J. F. G., and Härd, K. J. (1989) Clonal analysis of the glycosylation of immunoglobulin G secreted by murine hybridomas. *Biochemistry* 28, 1377–1384.
- (33) Imoto, T., Johnson, L. N., North, A. C. T., Phillips, D. C., and Rupley, J. A. (1972) Vertebrate Lysozymes. *The Enzymes*, 3rd ed., (P. D. Boyer, Ed.) Vol. 7 pp 665–868, Academic Press, New York.
- (34) Seevers, R. H., and Counsell, R. E. (1982) Radioiodination techniques for small organic molecules. *Chem. Rev.* 82, 575–590.
- (35) Mizuochi, T., Hamako, J., and Titani, K. (1987) Structures of the sugar chains of mouse immunoglobulin. *Arch. Biochem. Biophys.* 257, 387–394.

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Studies on the Activity of Barnase Toxins *in Vitro* and *in Vivo*[†]

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Pseudomonas exotoxin A (PE) is a protein toxin composed of three structural domains which are responsible for cell binding (domain Ia, amino acids 1–252), translocation into the cytosol (domain II, amino acids 253–364) and ADP-ribosylation activity (domain III, amino acids 405–613). We have previously described (Prior, T. I., FitzGerald, D. J., and Pastan, I. (1992) *Biochem.* 31, 3555–3559) a molecule composed of amino acids 1–412 of PE and the extracellular ribonuclease of *Bacillus amyloliquefaciens*, barnase (Bar), and shown that this protein (PE^{1–412}-Bar) is toxic to cells due to its ribonuclease activity, which had been delivered to the cytosol. We have now used this model to evaluate the role played by the carboxyl end of domain II (amino acids 347–364), domain Ib, and the amino end of domain III (amino acids 405–412) in the translocation event. Toxins completely lacking domain III, termed PE^{1–380}-Bar, or both domains Ib and III, termed PE^{1–364}-Bar, were equally cytotoxic to a murine fibroblast cell line (L929) as was PE^{1–412}-Bar. Extending the deletion to include part of the E-helix and all of the F-helix of domain II (amino acids 347–364) resulted in a toxin (PE^{1–346}-Bar) that was 10-fold less toxic. Previously tested on only murine cell lines, we demonstrate that barnase toxins are cytotoxic also to a variety of human cell lines. Cytotoxicity was assessed by measuring inhibition of DNA synthesis. Surprisingly, PE^{1–380}-Bar is not lethal when injected into mice, either intraperitoneally or intravenously, at 9 nmol, which is 2200-fold more than the amount required for killing by PE (4 pmol). In cell culture these barnase-containing toxins are 100-fold less toxic to murine fibroblast cells than PE. Barnase toxin has a greater survival time in the blood of mice than PE, with a half-life of 102 min. We conclude that domain II is sufficient to transport proteins into the cytosol. Further, since domain Ia can be replaced with other cell targeting moieties, we propose that barnase-toxins should be evaluated for utility in targeted cancer therapy.

INTRODUCTION

Pseudomonas exotoxin A (PE) is a 66 000 Da (Da) (1) protein toxin which kills eukaryotic cells that have specific cell-surface receptors for the toxin (2, 3). The toxin enters the cell *via* receptor-mediated endocytosis (4), and a 37 000 Da carboxyl-terminal fragment is translocated into the cytosol (5), where it catalyzes the irreversible ADP-ribosylation of elongation factor 2 (EF-2) (6), leading to cessation of protein synthesis and cell death. The three-dimensional structure of PE has been solved (7), and one or more functions have been assigned to each of the structural domains (8). Domain Ia (amino acids 1–252) is responsible for cell recognition and binding, and domain III (amino acids 405–613) contains the ADP-ribosylation activity. No role for domain Ib (amino acids 365–404) has been determined (9), although its presence may facilitate protein folding during synthesis (10). Mutational analysis of the carboxyl terminus of PE has shown that the last few amino acids of the toxin (...REDLK) are critical for cytotoxic activity (11) but have a role independent of ADP-ribosylation activity (11) or endosome–membrane binding (12). Further investigation showed that the sequence REDLK could be replaced (13) by the endoplasmic retention signal sequence KDEL (14). Toxins with this modified carboxyl end were more

cytotoxic than their native counterparts (13), suggesting that the carboxyl terminus of PE has a role in the translocation process.

It was originally proposed that domain II (amino acids 253–364) was responsible for translocation of domain III across the cell membrane (8) since a construct in which the first half of this domain was deleted had both cell binding and ADP-ribosylation activity, but was not cytotoxic. Since these initial experiments, several studies confirmed the role of domain II in the translocation process and defined the regions of this domain necessary for cytotoxicity. The amino terminus of domain II appears to be necessary since deletion of amino acids 254–263 inactivates full-length PE (8). Using a toxin targeted to EGF-receptor bearing cells, TGF α -PE40, in which domain Ia had been replaced with transforming growth factor α (TGF α), Siegfal *et al.* (9, 15) demonstrated that amino acids 346–364 could be deleted without loss of toxin activity. Further deletion of amino acids 344 and 345 resulted in complete inhibition of cytotoxic activity (15). In addition, arginine residues at positions 276, 279, 330, and 337 are important for full toxicity (16), with proteolytic cleavage occurring between arginine 279 and glycine 280 (17). This processing generates a 37 000 Da fragment derived from the carboxyl end of the toxin consisting of a portion of domain II and all of domain III; this is the fragment that is translocated to the cytosol. The carboxyl-terminal fragment can self-catalyze its translocation to the cytosol since a molecule which begins at residue 280 and ends at amino acid 613, and which contains TGF α inserted at position 606, immediately prior to the carboxyl end of PE, is toxic to EGF-receptor bearing cells (18). Specifically, the amino-terminal region (amino acids 280–313) of this 37 000 kDa carboxyl-terminal fragment appears to carry a membrane translocation signal sequence (19, 20). A

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role for domain Ia in translocation can be discounted since this domain can be replaced with many other targeting moieties of diverse structure (21, 22).

In addition to transporting domain III, PE also can transport foreign proteins into the cytosol of target cells. We have described a molecule in which the extracellular ribonuclease of *Bacillus amyloliquefaciens*, barnase (reviewed in 23), was inserted between domain III and the carboxyl end of PE (termed PE-Bar) and demonstrated that PE could deliver barnase to the cytosol (24). We subsequently described a molecule in which 96% of domain III had been removed by deletion (25). This toxin, termed PE¹⁻⁴¹²-Bar, was cytotoxic to cells in culture indicating that domain II of PE is sufficient to promote the translocation of domain III and of proteins inserted in place of domain III. Guidi-Rontani (26) has demonstrated that the ADP-ribosylation domain of PE (domain III) could be replaced with the A fragment of diphtheria toxin (DTA), which possesses the ADP-ribosylation activity of this toxin. In addition to being able to transport foreign proteins, PE is also able to carry nonpolypeptides, such as poly(ethylene glycol) (27) into the cytosol.

Ribonucleases such as barnase (24, 25), ribonuclease A (28, 29), and angiogenin (30, 31) have been shown to possess enzymatic activities which may be exploited for the development of novel cytotoxic agents (reviewed in ref 32). In order to evaluate further the potential of using barnase toxins for the treatment of human disease, we identified three areas for further study. The first was to determine more precisely the functional boundary of the translocation activity encoded by domain II. Secondly, we needed to investigate the cytotoxic activity of barnase toxins on human cell lines. And finally, we needed to evaluate the toxicity, and the pharmacokinetics of the toxins, in mice.

MATERIALS AND METHODS

Reagents. Chemicals and enzymes were purchased from standard suppliers. [³H]Thymidine (6.7 Ci/mmol) was purchased from NEN/Dupont (Boston, MA). Purified barnase was a gift from Dr. R. W. Hartley, National Institutes of Health.

Plasmids, Bacterial Strains, and Cell Lines. Plasmid pTP32 f(+)T carries the DNA coding for the chimeric toxin PE-Bar (24). This plasmid was designed such that the DNA encoding barnase (amino acids 1–110) was preceded by amino acids 1–607 and followed by amino acids 604–613 of PE. DNA encoding the barnase-specific inhibitor, barstar (23), was placed between the stop codon of the toxin and the transcription terminator. Plasmid pTP50 f(+)T (25) encodes PE¹⁻⁴¹²-Bar, which is identical to plasmid pTP32 f(+)T except that barnase is preceded by amino acids 1–412 of PE. *Escherichia coli* (*E. coli*) strain BL21(λDE3) has been described previously (33). L929 is a murine fibroblast cell line. The following human cell lines were obtained from the American Type Culture Collection: epidermoid carcinoma line A431, breast carcinoma line MCF-7, and hepatoma line HepG2. Glioblastoma line U251 was obtained from NCI-Frederick Cancer Research Facility.

Plasmid Construction. Plasmid DNA was propagated in *E. coli* strain DH5α and prepared using QIAGEN columns (QIAGEN, Studio City, CA). Plasmid pTP32 f(+)T contains a *Stu*I recognition site immediately prior to the DNA encoding barnase. This plasmid also has two *Sfi*I recognition sites, both located close to the junction of domain II and domain Ib. Plasmid pTP32 f(+)T was digested with *Stu*I and *Sfi*I, and the 5'-phosphates were removed using calf-intestinal alkaline phosphatase (CIP). The 4766 bp fragment was purified on a 1% (w/v) low-

Table 1. Oligonucleotides Used in This Study

Oligo 1:	5'- TGG CCC TGA CCC TGG CCG CCG CCG AG-3'
Oligo 2:	5'- CTC GGC GGC GGC CAG GGT CAG GGC CAG ACG-3'
Oligo 3:	5'- TGG CCC TGA CCC TGG CCG CCG CCG AGA GCG AGC GCT TCG TCC GCG AGG GCA CCG GCA ACG ACG AGG CCG GCG CGG CCA AC-3'
Oligo 4:	5'- GTT GGC CGC GCC GGC CTC GTC GTT GCC GGT GCC CTG CCG GAC GAA GCG CTC GCT CTC GGC GGC GGC CAG GGT CAG GGC CAG ACG

melting point agarose gel (SeaPlaque GTG agarose, FMC Bioproducts, Rockland, ME). Meanwhile, the oligonucleotides, oligo 1 and oligo 2 (Table 1), were annealed and 5'-phosphate groups added using T4 polynucleotide kinase. Oligo 1 was designed from the coding strand and extends from the upstream *Sfi*I cleavage site to the codon equivalent to amino acid 346; oligo 2 is derived from the complementary strand. When annealed, the duplex has a 3'-extension equivalent to the *Sfi*I cleavage site at one end and is blunt-ended at the other. The purified DNA fragment derived from plasmid pTP32 f(+)T and the oligonucleotide duplex were ligated, and the recombinants were screened by restriction digestion analysis. Several positive clones were checked for protein expression. Plasmid pTP77 f(+)T was identified as carrying the gene for the chimeric toxin PE¹⁻³⁴⁶-Bar.

Plasmid pTP32 f(+)T has an *Apa*I recognition site within the DNA encoding domain Ib. Plasmid pTP32 f(+)T was digested with *Stu*I and *Apa*I and the 3'-protruding end of the *Apa*I site removed using Klenow. After purification, the 4899 bp fragment was self-ligated, and the resulting recombinants were analyzed as discussed earlier. Plasmid pTP87 f(+)T was identified as carrying the gene for the chimeric toxin PE¹⁻³⁸⁰-Bar.

Plasmid pTP109 f(+)T, encoding PE¹⁻³⁶⁴-Bar, was constructed in a manner similar to that for plasmid pTP77 f(+)T, except that oligo 3 and oligo 4 were used. Oligo 3 was designed from the coding strand and extends from the *Sfi*I cleavage site to the codon equivalent to amino acid 364; oligo 4 is derived from the complementary strand.

Protein Expression and Purification. The chimeric toxins were prepared using *E. coli* strain BL21(λDE3). All of the constructions used in this study contained the ompA signal peptide; consequently, the expressed proteins were secreted into the periplasm. After induction with isopropyl β-D-thiogalactopyranoside (IPTG), the chimeric toxins PE¹⁻³⁸⁰-Bar and PE¹⁻³⁶⁴-Bar were purified essentially as described earlier (24, 25). Briefly, the proteins were purified using Q-Sepharose Fast-Flow and Mono Q (Pharmacia/LKB Biotechnology Inc., Piscataway, NJ) anion-exchange chromatography and gel-filtration chromatography (TSK G3000SW column), with removal of the tightly bound barstar from the barnase chimeras accomplished by denaturation in 7 M guanidine hydrochloride, 5 mM EDTA, 100 mM Tris-HCl (pH 8.0), and chromatography over a TSK G3000SW column equilibrated with this same buffer. The larger PE-Bar toxins are separated from the smaller barstar by this procedure. The chimeric toxin was renatured by dialysis versus 20 mM Tris-HCl (pH 7.4) and 1 mM EDTA.

The toxin PE¹⁻³⁴⁶-Bar did not bind to the anion exchange columns under the conditions used to purify the other barnase-containing proteins (20 mM Tris-HCl, [pH 7.4], 1 mM EDTA). Consequently, periplasm from cells expressing PE¹⁻³⁴⁶-Bar was prepared in 20 mM Tris-HCl (pH 8.0), 1 mM EDTA, and anion exchange chromatography performed at this pH. The protein was eluted using a linear gradient of 0–500 mM NaCl in 20

mM Tris-HCl (pH 8.0), 1 mM EDTA. Purification of PE¹⁻³⁴⁶-Bar was continued using the protocol described above.

Gel Electrophoresis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Laemmli (34).

DNA Synthesis Inhibition Assay. The cytotoxicity of the chimeric toxins was measured using a [³H]-thymidine incorporation assay as described (24). Briefly, the cells were placed in 96-well tissue culture dishes at 5×10^3 cells per well for 24 h before assaying. The toxins were diluted in phosphate-buffered saline containing 0.2% (w/v) human serum albumin and added at various concentrations to the cells. After incubation for 48 h at 37 °C in 5% CO₂, the cells were cultured with [³H]-thymidine (0.2 μ Ci) for 3 h. The cells were harvested by filtration onto glass fiber membranes (TomTec 96-well harvester), and the amount of [³H]thymidine incorporation was measured (Beta-Plate Scintillation Counter, Pharmacia/Wallac, Ltd.).

Animal Toxicity. To test animal toxicity, the purified toxins were diluted into phosphate-buffered saline containing 0.2% (w/v) human serum albumin and injected either intraperitoneally (ip) or intravenously (iv) into 8-week-old Balb/c mice. The animals were observed for 48 h and the number of dead mice counted.

Serum Levels of PE¹⁻³⁸⁰-Bar in Mice. Mice were given a single dose of PE¹⁻³⁸⁰-Bar iv (60 μ g) diluted in 200 μ L of PBS containing 0.2% (w/v) HSA into the tail. Blood (70 μ L) was drawn at various times postinjection from the retro-orbital plexus. Each mouse was bled no more than three times. The samples were allowed to clot on ice and centrifuged to obtain serum. The level of PE¹⁻³⁸⁰-Bar was determined by incubating serum with L929 cells and measuring its effects on DNA synthesis. A standard DNA synthesis inhibition curve was made with PE¹⁻³⁸⁰-Bar diluted in control mouse serum. The α and β half-lives were calculated by the R-strip program (Salt Lake City, UT).

Survival of PE¹⁻³⁸⁰-Bar in Serum. PE¹⁻³⁸⁰-Bar was diluted to a final concentration of 1 μ g/mL in mouse serum and incubated for 8 h at 37 °C. The survival of PE¹⁻³⁸⁰-Bar in serum was determined by comparing the cytotoxic activity of material incubated in serum with toxin freshly diluted into serum.

RESULTS

Construction of Expression Plasmids Encoding PE¹⁻³⁸⁰-Bar, PE¹⁻³⁶⁴-Bar and PE¹⁻³⁴⁶-Bar. The plasmid pTP87 f(+)T encoding PE¹⁻³⁸⁰-Bar was constructed as described in the Materials and Methods. This chimeric toxin is composed of the ompA signal peptide, amino acids 1-380 of PE fused to amino acids 1-110 of mature barnase, and finally, amino acids 604-613 of PE. There is a single amino acid residue, proline, between amino acid 380 of PE and the first amino acid of barnase, which was introduced by the cloning procedure and corresponds to Pro-607 of PE.

Plasmids pTP77 f(+)T and pTP109 f(+)T, encoding PE¹⁻³⁴⁶-Bar and PE¹⁻³⁶⁴-Bar, respectively, were constructed in similar fashion, as described in the Materials and Methods. The toxins they encode are identical to that described for PE¹⁻³⁸⁰-Bar, except that in PE¹⁻³⁴⁶-Bar the barnase moiety is preceded by amino acids 1-346 of PE and by amino acids 1-364 of PE in PE¹⁻³⁶⁴-Bar. A schematic representation of the proteins encoded by these genes is shown in Figure 1; sequences were confirmed by DNA sequencing (data not shown).

Expression and Purification of the Chimeric Toxins. The chimeric toxins were expressed by cultures

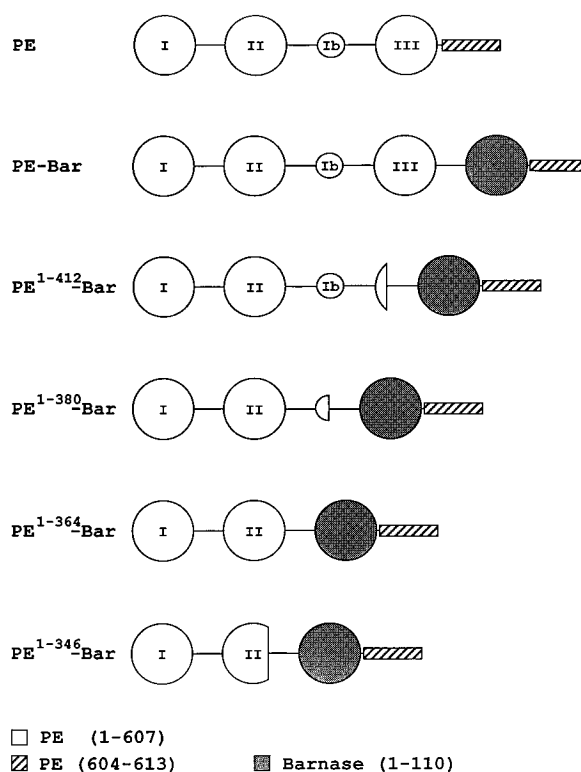


Figure 1. Schematic representation of the domain structure of PE and PE-barnase toxins. Roman numerals refer to the domains of PE. The schematic presentation of native PE represents PE 1-613.

of *E. coli* BL21(λ DE3). Since the toxin genes contained the *ompA* signal sequence, induction with IPTG resulted in the appearance of the expressed protein in the periplasm. The barnase toxins were purified from this source. We observed that deletion of amino acids 365-380, in PE¹⁻³⁶⁴-Bar, caused a significant decrease in the amount of material that could be recovered from the periplasm (data not shown). A similar low-level expression was obtained with PE¹⁻³⁴⁶-Bar. The toxins PE¹⁻³⁸⁰-Bar and PE¹⁻³⁶⁴-Bar were purified essentially as described earlier (24, 25). Initial purification was achieved by Q-Sepharose Fast-Flow and Mono Q anion-exchange chromatography. Both proteins eluted from the anion-exchange columns at salt concentrations (130 mM) similar to those observed when purifying PE¹⁻⁴¹²-Bar (25); this concentration is significantly lower than that observed with PE or PE¹⁻⁶⁰⁷-Bar (220 mM) (24) (data not shown).

The chimeric toxin PE¹⁻³⁴⁶-Bar did not bind to the anion exchange columns under the same conditions (20 mM Tris-HCl (pH 7.4), 1 mM EDTA) used to purify the other proteins. Consequently, periplasm from bacterial cultures expressing this toxin were prepared in 20 mM Tris-HCl (pH 8.0), 1 mM EDTA. At this pH, the protein bound to the resins. PE¹⁻³⁴⁶-Bar was eluted from the Q-Sepharose column, pooled, diluted 10-fold with 20 mM Tris-HCl (pH 8.0), 1 mM EDTA, and loaded onto a Mono Q anion-exchange column. The protein eluted at a salt concentration of 100 mM (data not shown).

Further purification of all the toxins was achieved by gel filtration over a TSK G3000SW sizing column. Since barstar binds tightly to, and copurifies with, barnase (23) and PE-Bar chimeric proteins (24, 25), it is necessary to chaotropically separate the proteins. Consequently, the purified preparations were treated with 7 M guanidine hydrochloride and loaded onto a TSK G3000SW equilibrated with buffer containing guanidine hydrochloride.

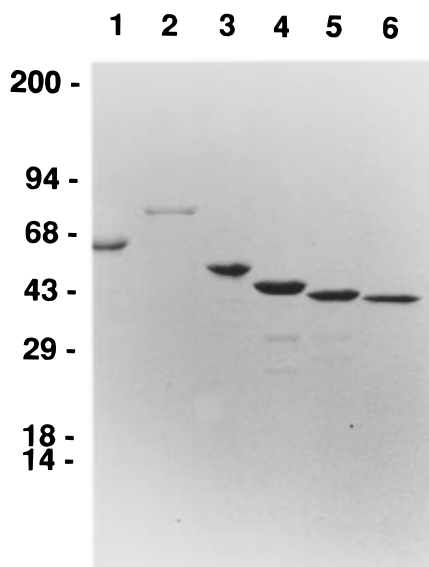


Figure 2. SDS-PAGE of PE and PE-barnase toxins. Purified proteins were analyzed using 4–20% (w/v) SDS-PAGE and stained with Coomassie blue: lane 1, PE; lane 2, PE¹⁻⁶⁰⁷-Bar; lane 3, PE¹⁻⁴¹²-Bar; lane 4, PE¹⁻³⁸⁰-Bar; lane 5, PE¹⁻³⁶⁴-Bar; lane 6, PE¹⁻³⁴⁶-Bar.

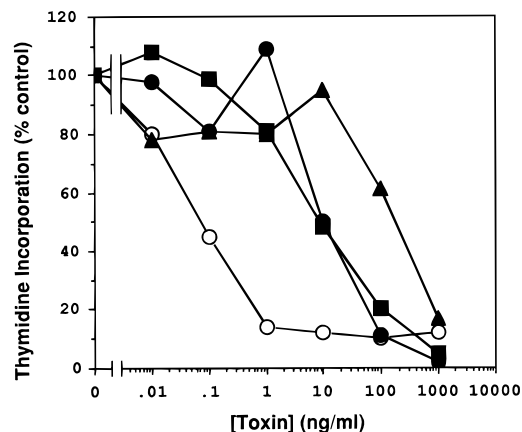


Figure 3. DNA synthesis inhibition by PE, PE¹⁻³⁸⁰-Bar, PE¹⁻³⁶⁴-Bar, and PE¹⁻³⁴⁶-Bar. L929 cells (5×10^3 cells in 0.2 mL) were incubated overnight. Various amounts of PE (○), PE¹⁻³⁸⁰-Bar (●), PE¹⁻³⁶⁴-Bar (■), or PE¹⁻³⁴⁶-Bar (▲) were added to the cultures. After 48 h of incubation, DNA synthesis was measured. Results are expressed as the percentage of DNA synthesis activity of cells incubated without toxin.

The first peak, corresponding to the PE-Bar toxins, was renatured by removing the guanidine hydrochloride by dialysis. The recovered protein was substantially pure, as determined by SDS-PAGE (Figure 2).

Cytotoxic Activity of the Chimeric Toxins. The cytotoxic activity of the various PE-Bar chimeric toxins was first evaluated on the murine fibroblast cell line L929. The cells were incubated for 48 h with varying amounts of toxin, and the rate of DNA synthesis was measured to assess the cytotoxic activity of the proteins. As shown in Figure 3, PE and the chimeric toxins all inhibited DNA synthesis on L929 cells. The concentration of toxin giving a 50% inhibition of DNA synthesis (ID_{50}) was 0.1 ng/mL for PE, 10 ng/mL for PE¹⁻³⁸⁰-Bar and PE¹⁻³⁶⁴-Bar, and 180 ng/mL for PE¹⁻³⁴⁶-Bar. Thus, maximal cytotoxic activity of the chimeric toxins was maintained when all of domain III and domain Ib were removed by deletion. Subsequent removal of 18 amino acids from the carboxyl end of domain II resulted in a decrease in cytotoxic activity.

Table 2. Cytotoxic Activity of PE and PE¹⁻³⁸⁰-Bar on Various Cell Lines^a

cell line	cell type	ID_{50} (ng/mL)		ratio
		PE	PE ¹⁻³⁸⁰ -Bar	
L929	murine fibroblast	0.1	10	100
A431	epidermoid carcinoma	7	60	9
MCF7	breast carcinoma	2	40	20
HepG2	hepatoma	5	>1000	>200
U251	glioblastoma	20	60	3

^a DNA synthesis was measured after 48 h of incubation with toxin as described in the Materials and Methods. Unconjugated barnase had no cytotoxic activity when added to the medium of L929 cells (24).

Table 3. Toxicity of PE, Barnase, and Chimeric Toxins on Mice

protein	route of administrn	LD_{50}		ref
		μ g	nmol	
PE	ip	0.3	4×10^{-3}	8
PE40 ^a	ip	50	1.25	8
Barnase	ip	>200	>16.7	this study
PE ¹⁻³⁸⁰ -Bar	ip	>500	>9.1	this study
	iv	>200	>3.6	this study

^a PE40 is a mutant form of PE(8) lacking the cell binding domain (domain Ia).

Since PE¹⁻³⁸⁰-Bar could be obtained in greater yield than PE¹⁻³⁶⁴-Bar, we used this toxin to evaluate further barnase toxins. We tested the cytotoxic activity of PE and PE¹⁻³⁸⁰-Bar on several human cell lines (Table 2). On all cell lines tested, PE¹⁻³⁸⁰-Bar was less toxic than PE. However, there was no discernable pattern regarding the relative sensitivities of the cell lines to the toxins. For example, on U251 cells, PE was approximately 3-fold more toxic (20 ng/mL) than PE¹⁻³⁸⁰-Bar (60 ng/mL). By contrast, PE (5 ng/mL) was at least 200-fold more toxic to HepG2 cells than PE¹⁻³⁸⁰-Bar, for which the ID_{50} was greater than 1000 ng/mL. On L929 cells, the most active barnase-containing toxins, PE¹⁻³⁸⁰-Bar and PE¹⁻³⁶⁴-Bar, were approximately 100-fold less toxic than PE (Figure 3). Nonetheless, the data indicated that PE¹⁻³⁸⁰-Bar is toxic to a variety of cell lines.

Toxicity of Barnase and PE-Barnase on Mice. To compare the animal toxicity of barnase and PE¹⁻³⁸⁰-Bar with PE and a mutant form of PE lacking the cell binding domain, termed PE40 (8), we injected barnase ip, and PE¹⁻³⁸⁰-Bar ip and iv, into Balb/c mice. The results (Table 3) indicate that barnase was nontoxic to mice up to 16.7 nmol (200 μ g). Surprisingly, PE¹⁻³⁸⁰-Bar was also nontoxic to mice when injected either ip (up to 9.1 nmol (500 μ g)) or iv (up to 3.6 nmol (200 μ g)). Thus, PE¹⁻³⁸⁰-Bar is at least 2200-fold less toxic than PE (8) when injected ip into mice, although it is only 100-fold less toxic when tested on mouse L929 fibroblasts.

Serum Level of PE¹⁻³⁸⁰-Bar after ip and iv Injection. The low animal toxicity of PE¹⁻³⁸⁰-Bar could have been due to rapid inactivation or clearance from the blood of mice. To determine the blood level of PE¹⁻³⁸⁰-Bar achieved after a single dose injection, 60 μ g of toxin was injected iv by tail vein into a group of mice. Blood was collected at various times postinjection and assayed for its content of PE¹⁻³⁸⁰-Bar using L929 cells. One minute after iv injection, 30 μ g/mL of PE¹⁻³⁸⁰-Bar was detected in the blood, and a level of 2.5 μ g/mL was detected 2 h after injection (Figure 4). The survival curve shows a biphasic decay; the α decay is rapid, with a half-life ($t_{1/2}$) survival of about 1.2 min. However, the β -decay curve is much more prolonged with a $t_{1/2}$ of 102 min.

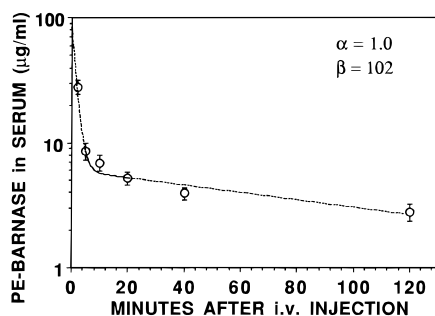


Figure 4. Serum levels of PE¹⁻³⁸⁰-Bar following iv injection. Mice were injected via the tail vein with 3 mg/kg of PE¹⁻³⁸⁰-Bar and blood drawn at 2, 5, 10, 20, 30, 40, and 120 min. Serum levels were quantified by cytotoxicity assay and are expressed as means with the standard deviations shown by error bars. The $t_{1/2\alpha}$ and $t_{1/2\beta}$ were 1 and 102 min ($r^2 = 0.99$), calculated using a weighting factor of 2.0 by the program RSTRIP (MicroMath, Inc., Salt Lake City, UT).

Incubation of PE¹⁻³⁸⁰-Bar in Serum. In order to determine the sensitivity of PE¹⁻³⁸⁰-Bar to serum proteases, we incubated toxin at 1 µg/mL in mouse serum for 8 h at 37 °C and measured cytotoxicity on L929 cells. There was no significant loss in the toxicity of the toxin after incubation in serum (data not shown).

DISCUSSION

In this paper, we describe the activities of chimeric toxins in which the extracellular ribonuclease of *B. amyloliquefaciens*, barnase, is inserted into PE in place of all of domains Ib and III and part of domain II. We also describe the cytotoxic activity of these proteins on a variety of cell lines *in vitro* and their toxicity *in vivo*.

We have described a series of new chimeric toxins (24, 25) in which barnase was inserted into PE. We showed that the cytotoxic action of these molecules, termed PE-Bar and PE¹⁻⁴¹²-Bar, was due to their ribonuclease activity which had been translocated into the cytosol because the degradation of radiolabeled cytosolic RNA was increased (24, 25). We further concluded from this evidence that domain II was essential and sufficient to transport a soluble enzyme into the cytosol, essentially independent of the other domains of PE. Since removal of 96% of domain III from PE-Bar resulted in more efficient transfer of barnase to the cytosol (25), we wished to determine if further deletions would cause increased activity. Siegall *et al.* (9, 15) reported that domain Ib (amino acids 365–404) could be deleted without affecting the cytotoxicity of a mutant form of PE in which domain Ia had been replaced with transforming growth factor α (TGF α), termed TGF α -PE40. However, deletion of amino acids 365–399 caused a 10-fold reduction in cytotoxicity when compared with toxin lacking only amino acids 365–380 (15); this additional deletion caused only a 2-fold reduction of ADP-ribosylation activity, raising the possibility that amino acids 381–399 may be required for efficient translocation. Our results indicate that this is not the case since a toxin (PE¹⁻³⁸⁰-Bar) lacking, in part, amino acids 381–399 was equally cytotoxic as a PE¹⁻⁴¹²-Bar (25), which contain these amino acids. Indeed, further deletion of amino acids 365–380, in PE¹⁻³⁶⁴-Bar, resulted in a molecule which was equally as cytotoxic as PE¹⁻³⁸⁰-Bar and PE¹⁻⁴¹²-Bar.

However, further deletion of amino acids 347–364 resulted in a significant (approximately 10-fold) loss of cytotoxicity. By contrast, Siegall *et al.* (15) reported that amino acids 346–380 could be deleted from TGF α -PE40 without affecting either cytotoxicity or ADP-ribosylation activity. Examination of the three-dimensional structure

of PE (7) reveals that this region consists of part of the E-helix (amino acids 333–350) and all of the F-helix (amino acids 360–363) of domain II. It may be that in the absence of these helices, or in the absence of the intervening amino acids (351–359), barnase causes a perturbation of the structure of domain II. For example, the barnase moiety may interfere with the correct folding of domain II, thus resulting in inefficient processing of the toxin within the endocytic vesicle. Alternatively, barnase may interact nonspecifically with the remainder of the PE molecule, preventing efficient unfolding, and interfering with transport of the carboxyl terminal fragment into the cytosol.

Nonetheless, the data indicate that all of domains Ib and III can be deleted without affecting translocation of barnase by domain II and, further, that transport can still occur, at a reduced level, with a deletion of the carboxyl end of domain II. Since only the 37-kDa carboxyl terminus of PE is translocated into the cytosol (5), it seems logical that this fragment contains all the information necessary for the transport event to occur.

Recently, we have shown that a protein composed of only domain III and TGF α (inserted after amino acid 607 and prior to the carboxyl amino acids 604–613) was toxic to EGF-receptor-bearing cells (12). Since the cytotoxic activity of chimeric toxins comprising only domain III appears limited to TGF α -constructs (A. Kihara and I. Pastan, unpublished observations), the delivery of ADP-ribosylation activity to the cytosol in these constructs may be due to the specifics of internalization and processing of EGF-receptors, rather than domain II catalyzed translocation. Nonetheless, since proteolytic cleavage within the endosome occurs between arginine-279 and glycine-280 (17), the ability to translocate the carboxyl end of PE into the cytosol is located within a relatively small (84 amino acids) region (amino acids 280–364). Indeed, translocation can still occur, albeit less efficiently, with only 66 amino acids (amino acids 280–346). It seems unlikely that this region is large enough to carry out protein transport by itself. Rather, it seems likely that this region contains information necessary for its interaction with cellular components that help catalyze translocation. Indeed, we have ascertained recently that amino acids 280–313 may comprise such a translocation signal sequence (19, 20).

Although very cytotoxic to a variety of cell lines *in vitro*, PE¹⁻³⁸⁰-Bar was nontoxic (Table 3) when injected into mice at levels significantly greater (approximately 2200-fold, ip) than those required to kill with PE. Indeed, PE¹⁻³⁸⁰-Bar was less toxic than PE40, a mutant form of PE that lacks the cell-binding domain (domain Ia) but retains translocation and ADP-ribosylation activity. The lack of toxicity *in vivo* is not due to an inability of PE¹⁻³⁸⁰-Bar to cross from the peritoneal cavity into the blood stream since toxin can be detected in the circulation after ip injection (data not shown). Furthermore, there was no apparent toxicity when the toxin (200 µg, 3.6 nmol) was injected intravenously. Further, PE¹⁻³⁸⁰-Bar is apparently resistant to circulating proteases since incubation of toxin in serum at 37 °C for 8 h did not result in a significant loss of activity. The difference in toxicity *in vivo* between PE and PE¹⁻³⁸⁰-Bar is not due to differences in targeting since both molecules bind through domain Ia.

PE¹⁻³⁸⁰-Bar was cleared from the serum of mice with an $t_{1/2\alpha}$ of 1 min and a $t_{1/2\beta}$ of 102 min (Figure 4). These values were very close to what was observed when PE was administered to mice (35), where the $t_{1/2\alpha}$ and $t_{1/2\beta}$ calculated in the same way were 5.7 and 97 min, respectively. The rapid α phase, which is likely due to

redistribution to liver and other organs which contain the PE receptor, is highly dependent on the exact time of the first blood draw, and hence, it could not be concluded that the $t_{1/2\alpha}$ of PE¹⁻³⁸⁰-Bar and PE differ significantly. The area under the curve (AUC) of percent of peak serum level versus minutes was 3600 and 5700 for PE¹⁻³⁸⁰-Bar and PE, respectively, the difference likely due to the difference in $t_{1/2\alpha}$. Certainly, the pharmacokinetic comparison PE¹⁻³⁸⁰-Bar and PE could not explain the marked difference in their toxicity of mice.

The lack of toxicity with PE¹⁻³⁸⁰-Bar may reflect the mechanism by which this molecule kills cells. PE kills cells by the irreversible inactivation of protein synthesis (6); PE¹⁻³⁸⁰-Bar kills cells by the destruction of RNA (24). This latter mechanism is not irreversible, and cell death will depend upon the equilibrium between *de novo* RNA synthesis and destruction by barnase. Thus, cells which are especially dependent upon a rapid turnover of protein, such as rapidly dividing cells, may be more sensitive to decreased levels of RNA. Indeed, the results presented here (Table 2) support this conclusion since relative sensitivity of cells to PE does not predict their sensitivity to PE¹⁻³⁸⁰-Bar. Had cytotoxicity to PE¹⁻³⁸⁰-Bar been dependent solely upon the number of PE receptors per cell, then the ratio of cytotoxicity between PE and PE¹⁻³⁸⁰-Bar should have remained constant; this was not observed.

Beyond their potential role as therapeutic agents, chimeric proteins such as PE-Bar, which possess activities derived from a number of different sources, should be useful for the study of biological processes. For example, we have recently isolated a number of cDNA clones that encode proteins which can render transfected cells partially resistant to PE and DT (36), both of which kill cells via inhibition of protein synthesis by ADP-ribosylation of EF-2 (21, 22), but not to ricin or cycloheximide (36). The mechanism of this resistance activity is currently unknown. If cells transfected with these cDNA clones were resistant also to barnase toxins, then the expressed protein's mechanism of action must occur at a point in the cell-killing pathway prior to ADP-ribosylation, since a cell line resistant to ADP-ribosylation was sensitive to barnase toxins (24, 25).

The results presented here show that all of domains Ib and III of PE can be deleted without affecting transport of a soluble enzyme into the cytosol. Translocation can still occur, although at reduced levels, even if a small deletion is made in domain II. Since domain Ia of PE, which binds to cells as a targeting domain, can be replaced with growth factors, lymphokines or single-chain antibodies for targeting to specific cells *in vitro* or in animals (21, 22), the results suggest that barnase toxins may be useful for the treatment of cancer. The differential of sensitivity of cells to barnase toxins independent of the number of cell-surface binding sites may permit selective targeting to rapidly growing tumors.

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LITERATURE CITED

- (1) Leppla, S. H. (1976) Large scale purification and characterization of the exotoxin of *Pseudomonas aeruginosa*. *Infect. Immunol.* 14, 1077-1086.
- (2) FitzGerald, D. J., Padmanabhan, R., Pastan, I., and Willingham, M. C. (1983) Adenovirus-induced release of epidermal growth factor and *Pseudomonas* toxin into the cytosol of KB cells during receptor-mediated endocytosis. *Cell* 32, 607-617.
- (3) Pastan, I., Willingham, M. C., and FitzGerald, D. (1986) Immunotoxins. *Cell* 47, 641-648.
- (4) FitzGerald, D., Morris, R. E., and Saelinger, C. B. (1980) Receptor-mediated internalization of *Pseudomonas* toxin by mouse fibroblasts. *Cell* 21, 867-873.
- (5) Ogata, M., Chaudhary, V. K., Pastan, I., and FitzGerald, D. J. (1990) Processing of *Pseudomonas* exotoxin by a cellular protease results in the generation of a 37,000-toxin fragment that is translocated to the cytosol. *J. Biol. Chem.* 265, 20678-20685.
- (6) Iglewski, B. H., and Kabat, D. (1975) NAD-dependent inhibition of protein synthesis by *Pseudomonas aeruginosa* toxin. *Proc. Natl. Acad. Sci. U.S.A.* 72, 2284-2288.
- (7) Allured, V. S., Collier, R. J., Carroll, S. F., and McKay, D. B. (1987) Structure of exotoxin A of *Pseudomonas aeruginosa* at 3.0-Angstrom resolution. *Proc. Natl. Acad. Sci. U.S.A.* 83, 1320-1324.
- (8) Hwang, J., FitzGerald, D. J., Adhya, S., and Pastan, I. (1989) Functional domains of *Pseudomonas* exotoxin identified by deletion analysis of the gene expressed in *E. coli*. *Cell* 48, 129-136.
- (9) Siegall, C. B., Chaudhary, V. K., FitzGerald, D. J., and Pastan, I. (1989) Functional analysis of domains II, Ib and III of *Pseudomonas* exotoxin. *J. Biol. Chem.* 264, 14256-14261.
- (10) Kihara, A., and Pastan, I. (1994) Analysis of sequences required for the cytotoxic action of a chimeric toxin composed of *Pseudomonas* exotoxin and transforming growth factor α . *Bioconjugate Chem.* 5, 532-538.
- (11) Chaudhary, V. K., Jinno, Y., FitzGerald, D. J., and Pastan, I. (1990) *Pseudomonas* exotoxin contains a specific sequence at the carboxyl terminus that is required for cytotoxicity. *Proc. Natl. Acad. Sci. U.S.A.* 87, 308-312.
- (12) Kihara, A., and Pastan, I. (1994) Small chimeric toxins containing only transforming growth factor α and domain III of *Pseudomonas* exotoxin with good antitumor activity in mice. *Cancer Res.* 54, 5154-5159.
- (13) Seetharam, S., Chaudhary, V. K., FitzGerald, D. J., and Pastan, I. (1991) Increased cytotoxic activity of *Pseudomonas* exotoxin and two chimeric toxins ending in KDEL. *J. Biol. Chem.* 266, 17376-17381.
- (14) Monroe, S., and Pelham, M. R. B. (1987) A C-terminal signal prevents secretion of luminal ER proteins. *Cell* 48, 899-907.
- (15) Siegall, C. B., Ogata, M., Pastan, I., and FitzGerald, D. J. (1991) Analysis of sequences in domain II of *Pseudomonas* exotoxin A which mediate translocation. *Biochemistry* 30, 7154-7159.
- (16) Jinno, Y., Ogata, M., Chaudhary, V. K., Willingham, M. C., Adhya, S., FitzGerald, D. J., and Pastan, I. (1989) Domain II mutants of *Pseudomonas* exotoxin deficient in translocation. *J. Biol. Chem.* 264, 15953-15959.
- (17) Ogata, M., Fryling, C. M., Pastan, I., and FitzGerald, D. J. (1992) Cell-mediated cleavage of *Pseudomonas* exotoxin between Arg²⁷⁹ and Gly²⁸⁰ generates the enzymatically active fragment which translocates to the cytosol. *J. Biol. Chem.* 267, 25396-25401.
- (18) Theuer, C. P., FitzGerald, D. J., and Pastan, I. (1992) A recombinant form of *Pseudomonas* exotoxin directed at the epidermal growth factor receptor that is cytotoxic without requiring proteolytic processing. *J. Biol. Chem.* 267, 16872-16877.
- (19) Theuer, C. P., Buchner, J., FitzGerald, D., and Pastan, I. (1993) The N-terminal region of the 37-kDa translocated fragment of *Pseudomonas* exotoxin A aborts translocation by promoting its own export after microsomal membrane insertion. *Proc. Natl. Acad. Sci. U.S.A.* 90, 7774-7778.
- (20) Theuer, C. P., Kasturi, S., and Pastan, I. (1994) Domain II of *Pseudomonas* exotoxin A arrests the transfer of translocating nascent chains into mammalian microsomes. *Biochemistry* 33, 5894-5900.
- (21) Pastan, I., Chaudhary, V. K., and FitzGerald, D. (1992) Recombinant toxins as novel therapeutic agents. *Annu. Rev. Biochem.* 61, 331-354.

- (22) Brinkmann, U., and Pastan, I. (1994) Immunotoxins against cancer. *Biochem. Biophys. Acta* 1198, 27–45.
- (23) Hartley, R. W. (1989) Barnase and barstar: two small proteins to fold and fit together. *Trends Biochem. Sci.* 14, 450–454.
- (24) Prior, T. I., FitzGerald, D. J., and Pastan, I. (1991) Barnase toxin: a new chimeric toxin composed of *Pseudomonas* exotoxin A and barnase. *Cell* 64, 1017–1023.
- (25) Prior, T. I., FitzGerald, D. J., and Pastan, I. (1992) Translocation mediated by domain II of *Pseudomonas* exotoxin A: transport of barnase into the cytosol. *Biochemistry* 31, 3555–3559.
- (26) Guidi-Rontani, C. (1992) Cytotoxic activity of a recombinant chimeric protein between *Pseudomonas aeruginosa* exotoxin A and *Corynebacterium diphtheriae* diphtheria toxin. *Mol. Microbiol.* 6, 1281–1287.
- (27) Kuan, C. -T., Wang, Q. -C., and Pastan, I. (1994) *Pseudomonas* exotoxin A mutants. Replacement of surface exposed residues in domain II with cysteine residues that can be modified with polyethylene glycol in a site-specific manner. *J. Biol. Chem.* 269, 7610–7616.
- (28) Rybak, S. M., Hoogenboom, H. R., Newton, D. L., Raus, J. C., and Youle, R. J. (1992) Rational immunotherapy with ribonuclease chimeras. An approach toward humanizing immunotoxins. *Cell Biophys.* 21, 121–138.
- (29) Newton, D. L., Ilercil, O., Laske, D. W., Oldfield, E., Rybak, S. M., and Youle, R. J. (1992) Cytotoxic ribonuclease chimeras. Targeted tumoricidal activity *in vitro* and *in vivo*. *J. Biol. Chem.* 267, 19572–19578.
- (30) Saxena, S. K., Rybak, S. M., Davey, R. T., Jr., Youle, R. J., and Ackerman, E. J. (1992) Angiogenin is a cytotoxic, tRNA-specific ribonuclease in the RNase A superfamily. *J. Biol. Chem.* 267, 21982–21986.
- (31) Rybak, S. M., Hoogenboom, H. R., Meade, H. M., Rous, J. C., Schwartz, D., and Youle, R. J. (1992) Humanization of immunotoxins. *Proc. Natl. Acad. Sci. U.S.A.* 89, 3165–3169.
- (32) Youle, R. J., Newton, D., Wu, Y. N., Gadina, S. N., and Rybak, S. M. (1993) Cytotoxic ribonucleases and chimeras in cancer therapy. *Crit. Rev. Ther. Drug Carrier Syst.* 10, 1–28.
- (33) Studier, F. W., and Moffat, B. A. (1986) Use of bacteriophage T7 polymerase to direct selective expression of cloned genes. *J. Mol. Biol.* 189, 113–130.
- (34) Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227, 680–685.
- (35) Brinkmann, U., Pai, L. H., FitzGerald, D. J., and Pastan, I. (1992) Alteration of a protease-sensitive region of *Pseudomonas* exotoxin prolongs its survival in the circulation of mice. *Proc. Natl. Acad. Sci. U.S.A.* 89, 3065–3069.
- (36) Brinkmann, U., Brinkmann, E., and Pastan, I. (1995) Expression cloning of cDNAs that render cancer cells resistant to *Pseudomonas* and Diphtheria toxin and immunotoxins. *Mol. Med.* 1, 206–216.

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Characterization of Single Site Ricin Toxin B Chain Mutants

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DNA encoding ricin B chain was modified by site-directed mutagenesis, and eight separate mutant RTB cDNAs including four novel mutants were ligated into the baculovirus transfer vector, pAcGP67A. Cotransfection of *S. frugiperda* Sf9 cells with BaculoGold DNA was followed by limiting dilution isolation of recombinant baculoviruses. Infection of Sf9 cells at a multiplicity of infection of 5 in the presence of 25 mM lactose produced 0.05–1 mg/L of soluble, glycosylated 34 kDa proteins immunoreactive with monoclonal and polyclonal antibodies to ricin B chain. Mutant ricin B chains were partially purified by monoclonal antibody immunoaffinity chromatography to 10–50% purity in near milligram quantities. The mutant ricin B chains had decreased lectin binding relative to plant ricin B chain as measured by binding to immobilized lactose and asialofetuin and cell binding immunofluorescence. The mutant ricin B chains reassociated with plant RTA similarly to plant RTB, and the recombinant heterodimers had slightly reduced cell cytotoxicity relative to ricin.

INTRODUCTION

Ricin toxin is a 65 kDa glycoprotein isolated from the seeds of the castor bean plant, *Ricinus communis* (1). Ricin consists of an RNA *N*-glycosidase A chain (RTA)¹ disulfide linked to a galactose-specific lectin B chain (RTB). RTB binds the toxin to mammalian cell surface glycoproteins. Ricin is internalized by receptor-mediated endocytosis (2). Internalized toxin is transported to the TR (trans-reticular) Golgi, where further galactose-specific binding facilitates ricin routing to a specific organelle from which RTA translocates to the cytosol and enzymatically inactivates protein synthesis (3, 4). Thus, the lectin function of RTB may be critical for three steps in ricin intoxication (binding, internalization, and intracellular routing). Because of the extreme potency of this toxin, the available extensive biochemical and structural knowledge, and its importance as a component in a number of immunotoxins currently in clinical trials for leukemias and lymphomas, we have been interested in better characterizing its protein–carbohydrate interactions.

RTB binds β -D-galactopyranose moieties on cell surface glycolipids and glycoproteins. The hydroxyl groups at positions 3, 4, and 6 of the terminal galactose interact with the protein (5). Affinities for galactose and lactose are approximately 10^4 M⁻¹, while association constants for complex oligosaccharides and cell surfaces are 3 and 4 logs higher, respectively (6, 7). These results suggest that multiple low affinity sugar binding sites on ricin interact with complex oligosaccharides and cells to yield high affinity binding.

Equilibrium dialysis studies with plant ricin and plant RTB identified two galactose binding sites with either similar or disparate affinities (K_A 's = 10^3 – 10^4 M⁻¹) (8, 9). X-ray diffraction analysis of ricin crystals soaked with low concentrations of α -lactose (5 mM) revealed two lectin sites in the 1 α and 2 γ subdomains (10). Amino acid residues interacting with the sugars included Asp-22, Trp-37, Gln-35, and Asn-46 in the 1 α subdomain and Asp-234, Tyr-248, and Asn-255 in the 2 γ subdomain. Only a limited number of mutant RTBs have been reported. In one study, modification of an amino acid residue (Asn-255) in one subdomain (2 γ) produced complete loss of sugar binding (11). In another study, mutations of residues in two subdomains (1 α and 2 γ) were required to abolish sugar binding (12). Thus, results of mutational analysis to date are conflicting.

Further, several lines of evidence support three lectin binding sites in ricin. Three distinct sites on ricin were cross-linked by radiolabeled fetuin glycopeptides containing a dichlorotriazine-activated 6-(*N*-methylamino)-6-deoxy-D-galactose moiety (13). The X-ray crystal structure of RTB shows six homologous subdomains with similar folding and primary amino acid sequence that resemble the primitive galactose binding fold in discoidin I from the slime mold *Dictyostelium discoideum* (14). *N*-Bromosuccinimide modification of Trp-37 reduced sugar binding, demonstrating a sugar binding site in subdomain 1 α (15). *N*-Acetylimidazole *O*-acetylation of two tyrosines reduced sugar binding, implicating two additional binding sites in subdomains 1 β (Tyr-78) and 2 γ (Tyr-248) (16).

The disparity between the biochemical/biophysical observations and mutational analyses can be attributed to the previous low level expression of the mutants. Decreased sugar binding due to misfolding or aggregation of recombinant RTBs leads to overestimation of the effect of individual modifications. A major limitation of these previous studies was that they did not report purification or quantitative immunological characterization of the mutants prior to tests of sugar binding.

We have previously reported the expression and partial purification of milligram quantities of wild-type RTB from insect cells using recombinant baculovirus (17). The

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¹ Abbreviations: RTA, ricin toxin A chain; RTB, ricin toxin B chain; SPR, surface plasmon resonance.

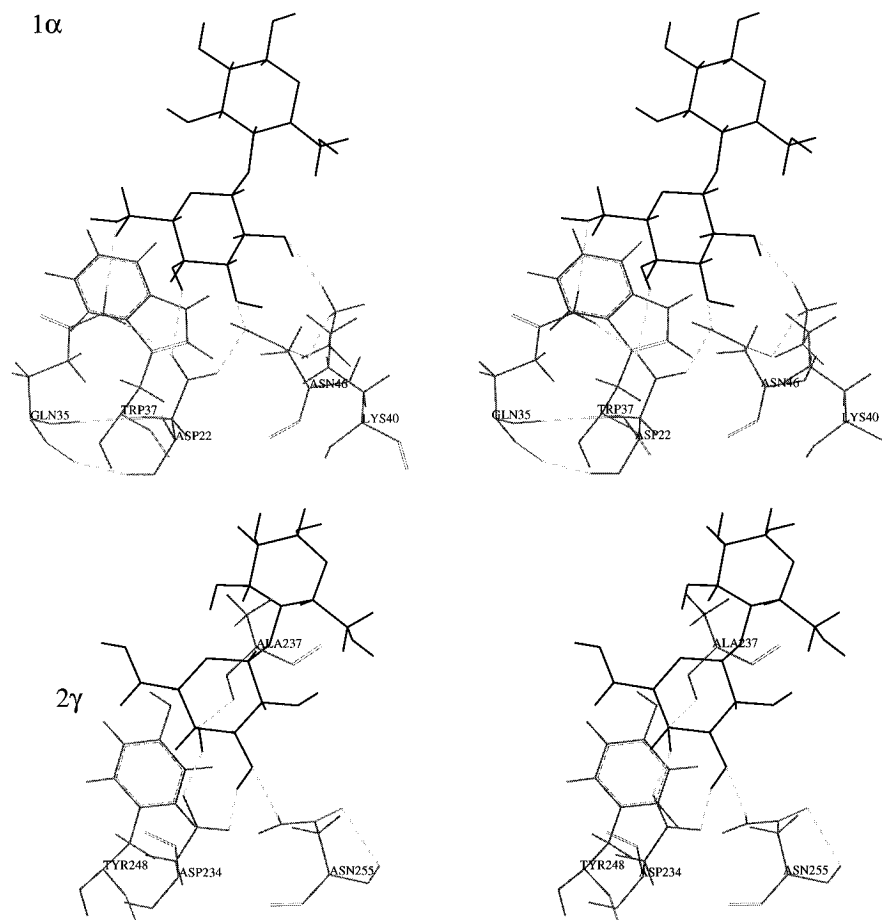


Figure 1. Model of lectin binding sites of RTB. Diagram of association of lactose with amino acid side chains of RTB in subdomains 1 α and 2 γ . Aromatic residues Trp-37 and Tyr-248 interact with the apolar face of terminal galactose. Polar residues Asp-22, Gln-35, Lys-40, Asn-46, Asp-234, and Asn-255 provide hydrogen bonds to hydroxyls of the sugar. Derived from Rutenber and Robertus (10). Wire diagram display of Brookhaven coordinates on SYBYL software on Silicon Graphics Iris Indigo workstation. Stereoviews.

insect RTB had immunologic and biologic properties similar to plant RTB. We have combined this methodology of large scale baculovirus expression of RTB with site-specific mutagenesis to create a more extensive panel of mutants than previously evaluated. Unique modifications at both the 1 α subdomain (W37S and Q35N) and the 2 γ subdomain (Y248S and D234E/A237R) were made based on the X-ray crystallographic models of the lectin binding sites (Figure 1). Other modifications were made to test previously constructed mutants in this system (K40M, K40M/N46G, N255A, and N255G). The modifications either changed the aromatic ring residues which provide van der Waals interactions between the protein and sugar (Trp-37 and Tyr-248) or key polar residues which provide hydrogen bonds to sugar hydroxyls (Gln-35, Lys-40, Asn-46, Asp-234, and Asn-255). We have added both a purification step and a more detailed analysis of the affinity, cell binding, and intoxication properties of mutant proteins to provide an in-depth quantitative study of the ricin lectin binding sites.

MATERIAL AND METHODS

Materials. Restriction endonucleases and T4 ligase were obtained from Promega (Madison, WI). Isotopes and the Sculptor *in vitro* mutagenesis system were obtained from Amersham (Arlington Heights, IL). Polyclonal antibodies and chemicals were from Sigma (St. Louis, MO). EX-CELL400 medium was obtained from JRH Scientific (Lexena, KS). Sf9 insect cells, TMNFH medium, BaculoGold DNA, and pAcGP67A transfer vector were from PharMingen (San Diego, CA). DNA and

plasmid prep kits were obtained from BioRad (Hercules, CA). The Sequenase kit for dideoxy sequencing was obtained from USB (Cleveland, OH). The Random Primer labeling kit and M13K07 phage were obtained from Stratagene (La Jolla, CA). Purified P2, P8, and P10 murine monoclonal antibodies to RTB and purified α BR12 murine monoclonal antibody to RTA were gifts of Dr. Walter Blattler, ImmunoGen (Cambridge, MA). Sera and media were obtained from GIBCO BRL (Grand Island, NY). 3M Emphaze Biosupport medium AB1 azlactone functionality bis-acrylamide and lactosyl acrylamide were obtained from Pierce (Rockford, IL). Triethylamine was obtained from Aldrich (Milwaukee, WI). The alkaline phosphatase Vectastain kit for Western blots was obtained from Vector Laboratories (Burlingame, CA). Ninety-six-well plates were from Costar (Cambridge, MA). Plant RTB, ricin, and RTA were obtained from Inland Laboratories (Austin, TX). Rhodamine conjugated goat anti-(rabbit Ig) was from ImmunoResearch (Bangor, ME).

Construction of Transfer Vectors Encoding Mutant RTBs. pAcGP67A-RTB DNA was prepared using the alkaline lysis method and silica-based matrix purification and then restricted with BamHI and EcoRI. The RTB encoding DNA fragment was ligated to BamHI and EcoRI restricted pUC119 plasmid and used to transform INV α F' *E. coli* cells. Single-stranded DNA was produced by infection of transformants with M13K07 phage as previously described. Oligonucleotides were synthesized on an Applied Biosystems 380B DNA synthesizer and desalted with butan-1-ol. 39-mers were prepared with

the modified codon flanked by 18 bases on each side matching RTB sequence. Site-specific mutagenesis was performed by the Eckstein method using the Amersham *in vitro* mutagenesis kit and manufacturer's instructions (18). Sequences of mutant RTB DNAs were confirmed by double-stranded dideoxy sequencing by the Sanger method using the Sequenase kit (19). Mutant RTB encoding pUC119 DNAs were then restricted with BamHI, and EcoRI and the RTB encoding fragments were subcloned into pAcGP67A plasmid and used to transform INVαF' cells. Transfer vectors with mutant RTBs were then purified by cesium chloride density-gradient centrifugation.

Isolation of Recombinant Baculoviruses. The Sf9 *S. frugiperda* ovarian cell line was maintained on TM-NFH medium supplemented with 10% fetal calf serum. pAcGP67A-mutant RTB DNAs (4 ug) were cotransfected with 0.5 ug of BaculoGold AcNPV DNA into 2×10^6 Sf9 insect cells following the recommendations of the supplier. On day 5 posttransfection, media were centrifuged and the supernatants tested in limiting dilution assays with Sf9 cells. A total of 2×10^4 Sf9 cells were incubated with 10-fold dilutions of supernatants in 96-well plates. Eight days postinfection, supernatants were saved, cells in each assay well were lysed with NaOH, and the lysates were transferred to nitrocellulose. The nitrocellulose was then blocked with Blotto and reacted with random primer ^{32} P-dCTP-labeled RTB DNA. After hybridization for 16 h at 67 °C, the dot blot membranes were washed with 0.1 x (150 mM NaCl/15 mM sodium citrate)/1% SDS, dried, and exposed to X-ray film. Positive wells were identified and supernatants reassayed by limiting dilution until all wells up to 10^{-7} dilution were positive. Two rounds of selection were required for each mutant. Recombinant viruses in the supernatants were then amplified by infecting Sf9 cells at a multiplicity of infection (moi) of 0.1, followed by collection of day 7 supernatants.

Expression of Mutant B Chains in Sf9 Cells. Recombinant baculoviruses were used to infect 2×10^8 Sf9 cells at a moi of 5 in EX-CELL400 media with 50 mM α-lactose in spinner flasks. Media supernatants containing mutant RTBs were collected day 6 postinfection.

Purification of Mutant RTBs. Media supernatants were adjusted to 0.01% sodium azide and maintained through all purification steps at 4 °C. The supernatants were ultracentrifuged at 100 000g for 1 h. The final supernatants were then loaded onto a P2 monoclonal antibody-acrylamide column previously described (17). The affinity column was then washed sequentially with NTEAL and 500 mM NaCl, 25 mM Tris pH 9, 1 mM EDTA, 0.01% sodium azide, 25 mM α-lactose, and 0.1% Tween-20 (NTEALT), and mutant RTBs were eluted with 0.1 M triethylamine pH 11. The alkaline eluants were immediately neutralized with 1 M sodium phosphate pH 4.8 and stored at -20 °C until assayed. Optical densities at 280 nm were determined and aliquots mixed with reducing 2 x SDS sample buffer, boiled 4 min, electrophoresed on a 15% SDS-PAGE, stained with Coomassie Blue R-250, and destained with acetic acid/methanol. Gels were scanned on an IBAS automatic image analysis system to estimate the fraction of protein of molecular weight 30 kDa.

Immunological Properties of Mutant RTBs. Aliquots of mutant RTB, plant RTB, wild-type recombinant RTB and prestained low molecular weight standards were mixed with reducing 2 x SDS sample buffer, boiled for 4 min loaded on a 15% SDS-PAGE, and electrophoresed for 90 min. Gels, Whatman 3M #1 paper, and

nitrocellulose were equilibrated for 15 min in Towbin buffer (20 mM Tris/0.1 M glycine/20% methanol) and placed in a Semi-dry Trans-blot cell (BioRad). After electrophoresis at 15 V for 20 min, the nitrocellulose was blocked with 10% Carnation's nonfat dry milk/0.1% BSA/0.1% Tween 20/0.02% sodium azide. The blots were then washed with PBS plus 0.05% Tween 20 and PBS, incubated with rabbit anti-ricin antibody at 1:400 in PBS plus 0.5% BSA plus 0.01% sodium azide for 1 h, washed again, incubated with alkaline phosphatase conjugated goat anti-(rabbit IgG) at 1:1000 in PBS plus 0.5% BSA plus 0.01% sodium azide for 1 h, washed again, and developed with the Vectastain alkaline phosphatase kit, following the manufacturer's recommendations. Blots were scanned as above to compare 30 kDa Mr band intensities.

Monoclonal antibody P2, P8, or P10 (100 ul) at 5 μg/mL in PBS was incubated in Costar EIA microtiter wells overnight at 4 °C. Samples of plant RTB, wild-type recombinant RTB, and mutant RTBs were treated for 20 min at room temperature with 5% β-mercaptoethanol and then dilutions made in 50 mM NaCl, 25 mM Tris pH 8, 1 mM EDTA. The antibody-coated microtiter wells were then washed with PBS plus 0.1% Tween 20, blocked with 3% BSA/PBS/0.01% sodium azide, rewashed and incubated with dilutions of the reduced RTB samples, rewashed and incubated with rabbit anti-ricin antibody 1:400 in PBS + 0.5% BSA + 0.01% sodium azide, washed again, incubated with alkaline phosphatase conjugated goat anti-(rabbit IgG) at 1:1000 in PBS + 0.5% BSA + 0.01% sodium azide, washed and developed with *p*-nitrophenyl phosphate at 1 mg/mL in 50 mM diethanolamine buffer pH 9.8, and read on a BioRad 450 Microplate reader at 405 nm. For each experiment 12 different concentrations of plant RTB and recombinant RTBs were tested. A plot of absorbance versus dilution was made for plant RTB and recombinant proteins. Dilutions yielding half-maximal binding were used to calculate concentrations.

Lectin Activity of Mutant RTBs. 100 μL volumes of 1 μg/mL asialofetuin in PBS were added to wells of a Costar EIA plate and incubated overnight at 4 °C. Samples of plant RTB, wild-type recombinant RTB, or mutant RTBs in EX-CELL 400 were exposed to 5% β-mercaptoethanol for 20 min at room temperature to remove homodimers and dilutions made in EX-CELL 400 medium with or without 20 μg/mL asialofetuin or 100 mM α-lactose. The asialofetuin coated microtiter wells were then washed with PBS/0.1% Tween-20, blocked with 3% BSA/PBS/0.01% sodium azide, and rewashed. The dilutions of various reduced RTBs were added to wells for 1 h and then removed and the wells washed again. Rabbit anti-ricin antibody was added (1:400 dilution in 0.5% BSA/PBS/0.01% sodium azide) for 1 h; the wells were washed again; alkaline phosphatase conjugated goat anti-(rabbit IgG) (1:5000 in 0.5% BSA/PBS/0.01% sodium azide) incubated in the wells; finally, the wells were washed, reacted with 1 mg/mL of *p*-nitrophenyl phosphate in 50 mM diethanolamine buffer pH 9.6, and measured in a microtiter plate reader at 405 nm.

In each experiment, 12 different concentrations of plant RTB and recombinant protein were tested. As in the antibody elisa, relative reactivity to plant RTB was calculated from concentrations giving half-maximal binding. The effects of 20 μg/mL asialofetuin or 100 mM α-lactose on half-maximal binding were calculated for plant RTB, wild-type recombinant RTB, and mutant RTBs.

Samples of freshly reduced plant RTB, recombinant RTB, or mutant RTB were diluted in 50 mM NaCl/25 mM

Tris pH 8/1 mM EDTA/0.01% sodium azide (NTEA), loaded on a lactosyl acrylamide matrix, and washed with NTEA, and lactose binding protein was eluted with NTEA plus 50 mM lactose. Fractions were assayed for absorbance at 280 nm and asialofetuin binding as described above.

KB cells were washed with PBS and attached to polylysine-coated tissue-culture dishes and centrifuged at 2000g for 10 min. The cells were then incubated live at 4 °C. The cells were washed with 2 mg/mL of BSA in PBS and incubated in PBS plus BSA with or without 100 µg/mL of asialofetuin and with 1 µg/mL of freshly reduced plant RTB, recombinant wild-type RTB or mutant RTB. The incubation was done at 4 °C. The cells were then washed with PBS and incubated with rabbit anti-ricin antibody at 1:400 in PBS plus BSA for 30 min at 4 °C. The cells were then washed with PBS and reacted with goat anti-(rabbit Ig) conjugated to rhodamine at 25 µg/mL for 30 min at 4 °C. The cells were washed again in PBS and fixed in 3.7% formaldehyde in PBS, mounted under a #1 coverslip in glycerol–PBS (90:10), and examined under a Zeiss Axioplan epifluorescence microscope.

Reassociation of Mutant RTB with Plant RTA To Form Heterodimers. Thirty µg portions of plant RTB, wild-type recombinant RTB, and mutant RTBs were mixed with 90 µg of plant RTA in a total volume of 1 mL of PBS, shaking overnight at room temperature. The reaction mixture was then analyzed by a ricin elisa Wells of an EIA plate were coated with 100 µg/mL of asialofetuin in a volume of 100 µL overnight at 4 °C. The wells were washed with PBS plus 0.1% Tween-20, blocked with 3% BSA in PBS plus 0.02% sodium azide, rewashed, and incubated with dilutions of ricin or reassociated heterodimers. The wells were again washed, incubated with αBR12 monoclonal antibody at 1 µg/mL in PBS/0.5% BSA/0.01% sodium azide, rewashed, and incubated with alkaline phosphatase-conjugated goat anti-(mouse Ig) at 1:500 in PBS/0.5% BSA/0.01% sodium azide, washed again, and developed with *p*-nitrophenyl phosphate at 1 mg/mL in 50 mM diethanolamine pH 9.8. Absorbance at 405 nm was read on a microtiter plate reader. Reassociated mixtures were also analyzed by nonreducing SDS/PAGE followed by immunoblots with αBR12 anti-RTA monoclonal antibody and P10 anti-RTB monoclonal antibody. Densitometric scanning using the IBAS 2000 automatic image analysis system (Kontron, Germany) was done to quantify shift of immunoreactive material from 30 to 60 kDa.

Cytotoxicity of Recombinant Mutant Heterodimers. A total of 1.5×10^4 HUT102 human T leukaemia cells in 100 µL were placed in 96-well flat-bottomed plates in leucine-poor RPMI1640 containing 10% dialysed fetal bovine serum. Fifty µL of ricin, recombinant wild-type RTB–plant RTA heterodimer, and mutant RTB–plant RTA heterodimers at varying concentrations were added in the same medium and the cells incubated at 37 °C in 5% CO₂ for 24 h. [³H]leucine, 0.5 µCi per well (120 mCi/mmol), in 50 µL of the same medium was added and incubated for 4 h. Cells were then harvested with a PhD cell harvester onto glass-fiber filter mats. The filters were dried, mixed with 3 mL of liquid scintillation fluid, and counted in an LKB-Wallac liquid scintillation counter gated for ³H. Cells cultured with medium alone served as controls. All assays were performed in triplicate. In some experiments, duplicate samples were incubated in the presence of 50 mM α-lactose. The ID₅₀ was the concentration of protein which inhibited protein synthesis by 50% compared with control.

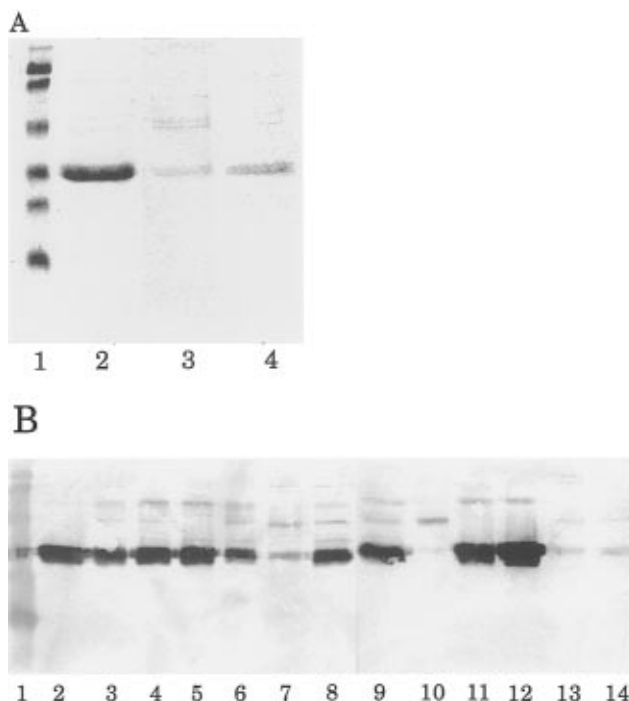


Figure 2. Insect-derived wild-type and mutant RTBs. (A) Coomassie stained 15% reducing SDS/PAGE of wild-type and mutant RTBs: lane 1, low molecular weight prestained BioRad protein standards; lane 2, wild-type; lane 3, N255A; lane 4, Y248S. (B) Immunoblots using rabbit anti-ricin antibody of 15% reducing SDS/PAGE of wild-type and mutant RTBs: lane 1, low molecular weight prestained BioRad protein standards; lane 2, wild-type; lane 3, N255A; lane 4, D234E/A237R; lane 5, N255G; lane 6, K40M; lane 7, N46G/K40M; lane 8, N46G/K40M; lane 9, Q35N; lane 10, Δ32; lane 11, W37S; lane 12, Y248S; lane 13, AcNPV polyhedrin; lane 14, DRA protein.

RESULTS AND DISCUSSION

Yields and Immunoreactivity of Mutant RTBs.

Mutant RTBs were produced with modifications in single putative lectin sites in subdomain 2γ (N255A, N255G, D234E/A237R, and Y248S) and subdomain 1α (W37S, K40M, Q35N, and N46G/K40M). An additional mutant had a nonsense mutation (an additional adenine) at codon 32. The changes in amino acid residues were selected based on their presumed role in lectin function based on X-ray crystallography and, in some cases, their previous analysis in other expression systems (10–12). Four of the mutants have not been previously described (D234E/A237R, Y248S, W37S, and Q35N).

The yields were estimated from the optical density at 280 nm of neutralized alkaline eluants' postaffinity chromatography (plant RTB OD = 1.44 for 1 mg/mL) and densitometry of Coomassie-stained reducing SDS/PAGE (10–50% of the protein migrated at 33 kDa, Figure 2A). These results were confirmed by densitometry of immunoblots with polyclonal rabbit anti-ricin antibody. As shown in Figure 2B, all of the mutants except the nonsense mutant were reactive with the polyclonal antibody. Finally, a monoclonal antibody anti-RTB elisa was used to verify concentrations of each mutant. All three assays gave similar values. Purified wild-type RTB was obtained at 300–500 µg/L culture fluid, while the mutants varied in concentration about 10-fold. The yield of Y248S was 640 µg/L, and the yields of W37S, D234E/A237R, and N255G were 264 µg/L, 240 µg/L, and 160 µg/L, respectively. Lower yields were obtained with Q35N, N255A, K40M/N46G, and K40M. These mutants were produced at 80 µg/L, 40 µg/L, 32 µg/L and 24 µg/L, respectively. The nonsense mutant control did not yield

levels above background ($<0.1 \mu\text{g/L}$). The lower yields of some of the mutants may be due to improper folding. Other investigators have reported bioactivity of mutants without purification or characterization (11, 12). Some of these mutants (N255A, N255G, and K40M/N46G) were identical to those studied in this report. Different expression systems were used (Cos cells and *Xenopus* oocytes), which may have accounted for their differing results. Their assumption that modification of lectin site residues would not affect secondary structure is perhaps inaccurate and overemphasizes the role of individual residues and subdomains in sugar binding and cytotoxicity.

The reactivity of mutant RTBs with different monoclonal antibodies to RTB (P2, P8, and P10) were tested by substituting different monoclonal antibodies as capture reagents in the antibody elisa. Equivalent results were observed for each antibody, suggesting proper folding of all the mutants except the nonsense mutant. The concentration of wild-type recombinant RTB was estimated at $40 \mu\text{g/mL}$ for P2 antibody capture and $75 \mu\text{g/mL}$ for P8 antibody capture. Y248S concentrations were $100 \mu\text{g/mL}$ with P2, $150 \mu\text{g/mL}$ with P8, and $60 \mu\text{g/mL}$ with P10 antibody. W37S concentrations were $20 \mu\text{g/mL}$ with P2, $40 \mu\text{g/mL}$ with P8, and $20 \mu\text{g/mL}$ with P10. D234E/A237R was $25 \mu\text{g/mL}$ with P2, $40 \mu\text{g/mL}$ with P8, and $20 \mu\text{g/mL}$ with P10. N255G gave $20 \mu\text{g/mL}$ with P2, $35 \mu\text{g/mL}$ with P8, and $20 \mu\text{g/mL}$ with P10. Q35N was $10 \mu\text{g/mL}$ with P2, $10 \mu\text{g/mL}$ with P8, and $8 \mu\text{g/mL}$ with P10. N255A was $10 \mu\text{g/mL}$ with P2, $13 \mu\text{g/mL}$ with P8, and $50 \mu\text{g/mL}$ with P10. N46G/K40M was $6 \mu\text{g/mL}$ with P2, $10 \mu\text{g/mL}$ with P8, and $6 \mu\text{g/mL}$ with P10. K40M showed $5 \mu\text{g/mL}$ with P2, $10 \mu\text{g/mL}$ with P8, and $5 \mu\text{g/mL}$ with P10. $\Delta 32$, AnNPV polyhedrin protein and DRA protein gave values of $0.01 \mu\text{g/mL}$ for P2, $0.02 \mu\text{g/mL}$ for P8, and $0.01 \mu\text{g/mL}$ for P10.

Sugar Binding of Mutant RTBs. Single-site mutant RTBs bound immobilized lactose on a molar basis 10–69% as well as plant RTB. Wild-type bound 55% as well as plant RTB. Y248S bound 47% relative to plant RTB; W37S had 48% binding; D234E/A237R had 22% binding; N255G showed 52%; and N255A had 60% binding. Q35N showed 69% binding. In contrast, N46G/K40M had 10% binding and K40M showed 15% binding. Each molecule retained lectin activity. Since multiple modifications were tested for each subdomain, RTB must have at least two sugar-combining sites. Single-site mutants made in *Xenopus* oocytes also retained sugar binding. These observations support a model for RTB lectin containing two or more lectin sites (12) and are incompatible with a model for RTB containing a single dominant sugar-combining site (11).

Binding of partially purified mutant RTBs to immobilized asialofetuin was quantitated by elisa, and the results are shown in Table 1. The results are similar to the immobilized lactose binding and confirm the mutants retain reactivity and, presumably, affinity within 1 log of wild-type.

An independent measure of mutant RTB binding to glycoproteins was made by detecting mutant RTB bound to cell surfaces. All of the single-site mutants bound KB cells at 4°C (Figure 3). Binding of wild-type recombinant and two mutant RTBs (N255A and Y248S) was blocked by asialofetuin (Figure 4). These results confirm the observations made with immobilized lactose above and suggest the mutant heterodimers retain at least some of the intoxication functions of plant ricin. Since binding was assayed at lectin concentrations of $1 \mu\text{g/mL}$ corresponding to $3 \times 10^{-8} \text{ M}$, high affinity interaction, with cell surfaces were retained in the mutants.

Table 1. Binding of Mutant RTBs to Asialofetuin^a

protein	% relative to plant RTB
wild-type	83
N255A	70
Q35N	70
N255G	33
D234E/A237R	30
Y248S	32
W37S	24
K40M	15
K40M/N46G	12

^a Quantity of RTB based on P2 elisa and asialofetuin binding measured by asialofetuin elisa. Both assays employed 5% β -mercaptoethanol to reduce homodimers. Average of three to four assays run on each mutant. $\Delta 32$ nonsense mutant, DRA, and AcNPV gave values equal to background ($0.01 \mu\text{g/mL}$ versus $2\text{--}70 \mu\text{g/mL}$ for the other proteins). Q35N must be assayed within 1 week of preparation or sugar-binding activity decreases.

All of the missense mutants showed lactose, asialofetuin, and cell binding relative avidity within 1 log of plant RTB and wild-type recombinant RTB. The similar decreases in avidity for RTBs with either modification of the 1α subdomain or 2γ subdomain suggest each site has similar affinity for asialofetuin. These observations correspond with the finding of others (12, 21, 22). The weaker sugar binding using equilibrium dialysis observed by some investigators for the 1α subdomain may be due to denaturation during the prolonged isolation or assay incubation times (8, 11). We were able to block binding of $6.9 \mu\text{g/mL}$ of plant RTB, wild-type recombinant RTB, and mutant RTBs to immobilized asialofetuin 20–27-fold in the presence of $20 \mu\text{g/mL}$ of asialofetuin and 200–250-fold in the presence of 100 mM α -lactose. These observations confirm the specificity of the reaction.

We attribute the higher avidity interaction with complex glycoproteins similar to others and suggest multiple low affinity sugar combining sites on ricin interact with complex oligosaccharides and cells to yield high affinity binding. This cluster glycoside effect has been previously described by Lee for hepatic asialoglycoprotein receptor and by Drickamer for the mannose binding proteins (23, 24). The X-ray crystallographic structure of ricin provides a structural basis for the multiple binding site hypothesis. RTB has six subdomains with similar folding and primary amino acid sequence, and these sequences show homology with a galactose-binding motif (14). If single-site mutants have only a single remaining lectin site, the residual avidity in the nanomolar binding range would suggest a much stronger binding than would be predicted from individual sites on other lectins or from X-ray crystallography (10, 23, 24). Cross-linked ricin data support the hypothesis of greater than two sugar-combining sites (16). Ricin cross-linked by two glycopeptides retained sugar binding and cell binding, and only triply cross-linked ricin had no residual lectin activity. Confirmation will require production and testing of double-site mutants with proper folding. An alternate hypothesis is that ricin may bind to polybranched galactose-terminated sugars through only a single binding site.

Reassociation of Mutant RTBs with Plant RTA.

Incubation of mutant RTBs at 10^{-6} M with plant RTA at $3 \times 10^{-6} \text{ M}$ overnight at room temperature yielded 50% of the RTB reassociated with RTA (Figure 5 and legend Figure 6). Similar levels of reassociation were seen using plant RTB or recombinant wild-type RTB with plant RTA at the same concentrations. The equilibrium constant for association of ricin chains was measured as $1.6 \times 10^6 \text{ M}^{-1}$, which would lead to about 50% association at the

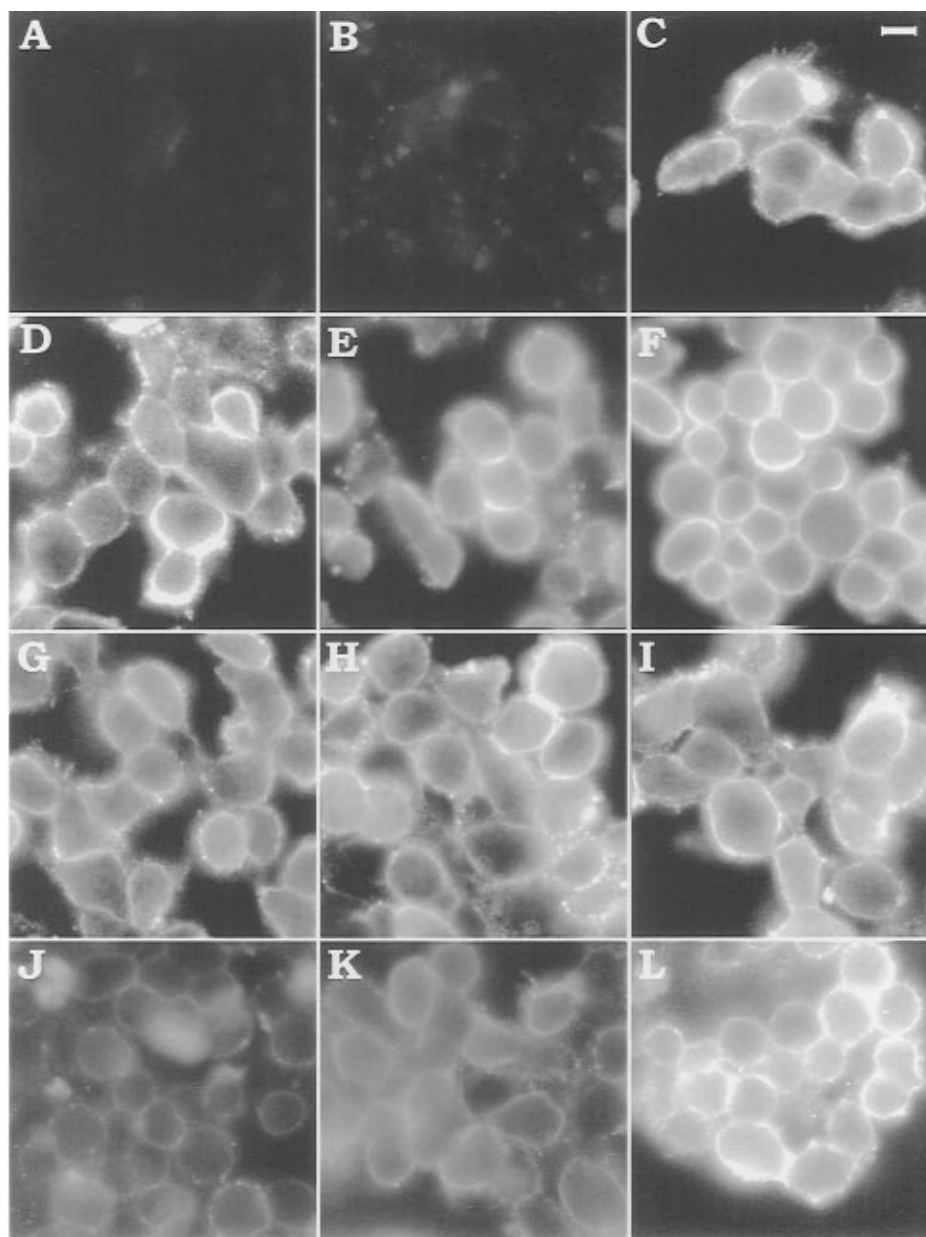


Figure 3. Binding of mutant RTBs to KB cells. Cells were attached to polylysine-coated tissue culture dishes, and all incubations were done at 4 °C. The cells were washed with 2 mg/mL of BSA in PBS and the PBS plus BSA plus 1 μ g/mL of purified mutant RTB or wild-type RTB, rewashed, incubated with 1:200 rabbit antiricin antibody (Sigma) in PBS plus BSA, rewashed, incubated with affinity-purified goat anti-(rabbit Ig) coupled to rhodamine (Jackson ImmunoResearch) at 25 μ g/mL, washed again, and fixed in 3.7% formaldehyde in PBS. Bar represents 20 μ m. Key: A, blank (no RTB); B, DRA control; C, W37S; D, Y248S; E, Q35N; F, wild-type; G, K40M; H, N46G/K40M; I, N255G; J, Δ 32; K, AcNPV polyhedrin; L, D234E/A237R.

concentrations used in our experiments (26). The heterodimer concentrations were quantitated by ricin elisa and by densitometry of 64 kDa bands of immunoblots with anti-RTB or anti-RTA antibodies. Both elisa and immunoblots gave similar values and showed all mutants reassociated well with plant RTA. The amino acid residues of RTB critical for reassociation include Ala-1, Asp-2, Cys-4, Phe-140, Val-141, Phe-218, Lys-219, Asn-220, Pro-260, and Phe-262 and are remote from the amino acid residues of subdomain 1 α and 2 γ changed in this study (10). The stability of the recombinant heterodimers at high dilution implies disulfide bond formation between RTA Cys-259 and mutant RTB Cys-4. Finally, the efficiency of reassociation of the mutant RTBs provides further evidence for the proper folding of these recombinant proteins.

Cytotoxicity of Mutant Heterodimers. The ID₅₀ of ricin on HUT102 cells was 5×10^{-12} M (Figure 6). Previous reports by ourselves and others have shown

ID₅₀'s for ricin in the range of $(1-6) \times 10^{-12}$ M on a variety of mammalian cell lines (11, 12, 17, 27). Again, we found insect RTB-RTA had a comparable ID₅₀ of 5×10^{-12} M. Subdomain 2 γ single-site mutant RTB-plant RTA heterodimers had ID₅₀'s of $(5-8) \times 10^{-11}$ M, representing a drop of about 1 log in potency. This can be accounted for by the reduced cell surface binding avidity and suggests maintenance of intracellular intoxication functions. The subdomain 1 α single-site mutant RTB-plant RTA heterodimers showed a range of ID₅₀'s of 1×10^{-10} to 1×10^{-11} M. The subdomain 1 α mutants showed a broader range of reductions in sugar binding, and the cell cytotoxicity reflects the same heterogeneity. In general, mutants with lower residual sugar binding displayed higher ID₅₀'s. The drop of potency was no more than 1.5 logs for the least potent mutant heterodimer, suggesting most of the alterations in intoxication in subdomain 1 α mutants could also be accounted for by reduced cell surface binding. The residual potent killing

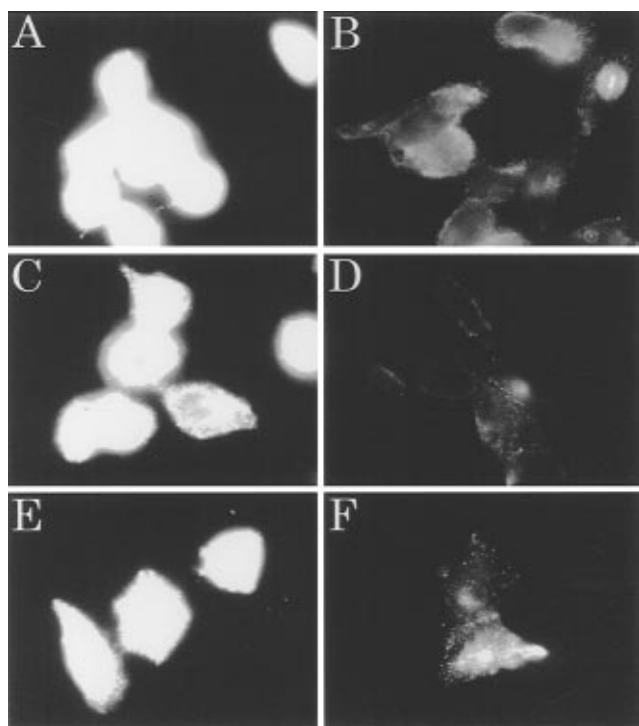


Figure 4. Blocking of RTB binding to KB cells by asialofetuin. Assay was performed as in Figure 3 with 1 μ g/mL of RTB except incubation of toxin with cells performed in the presence of 100 μ g/mL asialofetuin: A, recombinant RTB; B, recombinant RTB with asialofetuin; C, N255A; D, N255A with asialofetuin; E, Y248S; F, Y248S with asialofetuin.

by the mutant heterodimers provides further support that the molecules are properly folded and able to carry out intoxication functions after reassociation.

CONCLUSIONS

This report describes the first partial purification of RTB mutants in usable amounts for immunologic, chemical, and biological characterization. Inhomogeneous reagents can lead to underestimation of sugar binding if the mutant is improperly folded (11) or an overestimation of sugar binding if contaminants have lectin function (12). We have observed both phenomena on some mutants and with incomplete purification (data not shown).

We were able to quantitate relative binding affinities of mutants for asialofetuin. Previous work yielded only qualitative data and used only one or two methods of measurement. Our report documents the high affinity of single-site mutants for complex oligosaccharides using three independent assays. These observations led us to propose a three or more lectin site model for ricin.

The current study for the first time measures cell binding and reassociated heterodimer cytotoxicity quantitatively. The cell binding assay gave analogous results to other tests of sugar binding and suggests *in vitro* reactivity with lactose and asialofetuin models fairly well the chemical interaction of ricin with cell surface oligosaccharides. The correspondence of heterodimer potency with cell binding activity suggests that elimination of single lectin sites does not impair intracellular ricin intoxication functions.

Our study extends mutational analysis to two new residues in the 1 α subdomain and two new residues in the 2 γ subdomain. The X-ray crystallographic structure suggested side groups of each residue interacted with galactose and our results support this hypothesis. Reduction in sugar binding by each mutation yielded similar results for each subdomain and amplified the previous

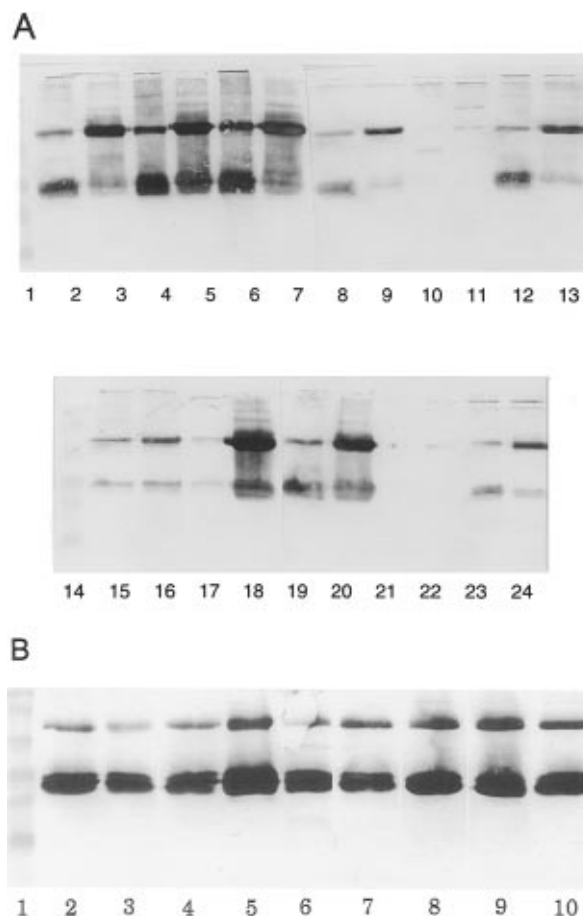


Figure 5. Reassociation of mutant RTBs with plant RTA. (A) Immunoblot using monoclonal antibody P10 anti-RTB of 15% nonreducing SDS/PAGE of plant RTA, mutant RTBs, and reassociated mutant RTB-plant RTA. Arrows indicate low molecular weight BioRad protein standards (106, 80, 49.5, 32.5, 27.5, and 18.5 kDa). RTB immunoreactive material appears at 60 kDa after reassociation with plant RTA: lane 1, low molecular weight prestained BioRad protein standards; lane 2, N255A; lane 3, N255A-plant RTA; lane 4, D234E/A237R; lane 5, D234E/A237R-plant RTA; lane 6, N255G; lane 7, N255G-plant RTA; lane 8, K40M; lane 9, K40M-plant RTA; lane 10, Δ 32; lane 11, Δ 32-plant RTA; lane 12, W37S; lane 13, W37S-plant RTA; lane 14, low molecular weight prestained BioRad protein standards; lane 15, Q35N; lane 16, Q35N-plant RTA; lane 17, Y248S; lane 18, Y248S-plant RTA; lane 19, wild-type RTB; lane 20, wild-type RTB-plant RTA; lane 21, Δ 32; lane 22, Δ 32-plant RTA; lane 23, N46G/K40M; lane 24, N46G/K40M-plant RTA. (B) Immunoblot using α BR12 monoclonal anti-RTA antibody of 15% nonreducing SDS/PAGE of plant RTA and reassociated heterodimers: lane 1, low molecular weight prestained BioRad protein standards; lane 2, N255A-plant RTA; lane 3, D234E/A237R-plant RTA; lane 4, N255G-plant RTA; lane 5, W37S-plant RTA; lane 6, K40M-plant RTA; lane 7, Δ 32-plant RTA; lane 8, Q35N-plant RTA; lane 9, wild-type RTB-plant RTA; lane 10, N46G/K40M-plant RTA.

limited mutational analysis. The properties of Y248S were similar to the properties of ricin agglutinin B chain which has a Y248H substitution (14).

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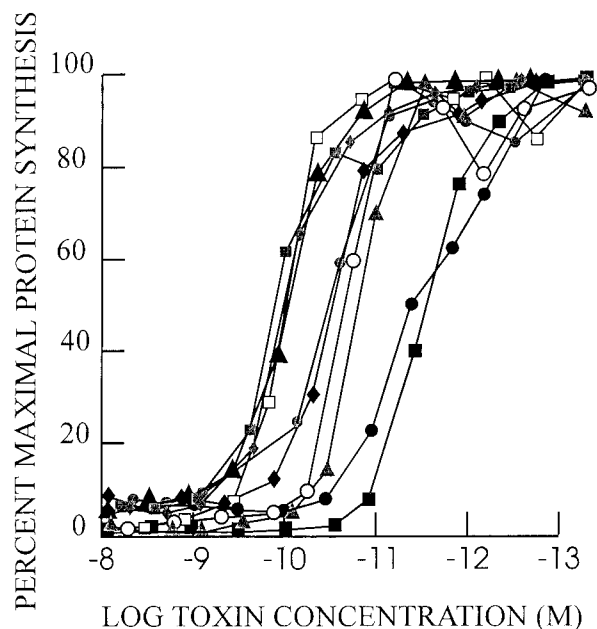


Figure 6. HUT102 cell cytotoxicity. Assay as described in text: solid square, ricin $ID_{50} = 5 \times 10^{-12}$ M; solid circle, wild-type RTB-RTA $ID_{50} = 5 \times 10^{-12}$ M; solid triangle, N255A-RTA $ID_{50} = 8 \times 10^{-11}$ M; lightly shaded square, N255G-RTA $ID_{50} = 8 \times 10^{-11}$ M; solid diamond, Y248S-RTA $ID_{50} = 5 \times 10^{-11}$ M; open square, K40M-RTA $ID_{50} = 1 \times 10^{-10}$ M; shaded circle, K40M/N46G-RTA $ID_{50} = 5 \times 10^{-11}$ M; shaded triangle, Q35N-RTA $ID_{50} = 1 \times 10^{-11}$ M; open circle, W37S-RTA $ID_{50} = 2 \times 10^{-11}$ M. Yield of reassociated heterodimer tested by ricin elisa varied for different RTBs. Fifty percent of plant RTB reassociated; 40% of wild-type recombinant RTB reassociated. Thirty-five percent of Y248S and 11% of W37S reassociated. Fifty percent of D234E/A237R, 100% of N255G, and 34% of N255A reassociated with plant RTA. Sixty-five percent of Q35N, 70% of K40M, and 22% of N46G/K40M reassociated.

LITERATURE CITED

- (1) Olsnes, S., and Pihl, A. (1973) *Biochemistry* **12**, 3121–3126.
- (2) Sandvig, K., and Olsnes, S. (1982) *J. Biol. Chem.* **257**, 7504–7513.
- (3) van Deurs, B., Tonnessen, T., Petersen, O., Sandvig, K., and Olsnes, S. (1986) *J. Cell Biol.* **102**, 37–47.
- (4) Endo, Y., and Tsurugi, K. (1987) *J. Biol. Chem.* **262**, 8128–8130.
- (5) Rivera-Sagredo, A., Solis, D., Diaz-Maurino, T., Jimenez-Barbero, J., and Martin-Lomas, M. (1991) *Eur. J. Biochem.* **197**, 217–228.

- (6) Baenziger, J., and Fiete, D. (1979) *J. Biol. Chem.* **254**, 9795–9799.
- (7) Sandvig, K., Olsnes, S., and Pihl, A. (1976) *J. Biol. Chem.* **251**, 3977–3984.
- (8) Zentz, C., Frenoy, J., and Bourrillon, R. (1978) *Biochim. Biophys. Acta* **536**, 18–26.
- (9) Houston, L., and Dooley, T. (1982) *J. Biol. Chem.* **257**, 4147–4151.
- (10) Rutenber, E., and Robertus, J. (1991) *Proteins* **10**, 260–269.
- (11) Vitetta, E., and Yen, N. (1990) *Biochim. Biophys. Acta* **1049**, 151–157.
- (12) Wales, R., Richardson, P., Roberts, L., Woodland, H., and Lord, J. (1991) *J. Biol. Chem.* **266**, 19172–19179.
- (13) Lambert, J., McIntyre, G., Gauthier, M., Zullo, D., Rao, V., Steeves, R., Goldmacher, V., and Blattler, W. (1991) *Biochemistry* **30**, 3234–3247.
- (14) Rutenber, E., Ready, M., and Robertus, J. (1987) *Nature* **326**, 624–626.
- (15) Hatakeyama, T., Yamasaki, N., and Funatsu, G. (1986) *J. Biochem.* **100**, 781–788.
- (16) Youle, R., Murray, J., and Neville, D. (1981) *Cell* **23**, 551–559.
- (17) Frankel, A., Roberts, H., Afrin, L., Vesely, J., and Willingham, M. (1994) *Biochem. J.* **303**, 787–794.
- (18) Sayers, J., Schmidt, W., Eckstein, F. (1988) *Nucl. Acids Res.* **16**, 791–802.
- (19) Sanger, F., Nicklen, S., and Coulson, A. (1977) *Proc. Natl. Acad. Sci. U.S.A.* **74**, 5463–5467.
- (20) Corr, M., Slanetz, A., Boyd, L., Jelonek, M., Khilko, S., Al-Ramadi, B., Kim, Y., Maher, S., Bothwell, A., and Margulies, D. (1994) *Science* **265**, 946–949.
- (21) Swimmer, C., Lehar, S., McCafferty, J., Chiswell, D., Blattler, D., and Guild, B. (1992) *Proc. Natl. Acad. Sci. U.S.A.* **89**, 3756–3760.
- (22) Lehar, S., Pedersen, J., Kamath, R., Swimmer, C., Goldmacher, V., Lambert, J., Blattler, W., and Guild, B. (1995) *Protein Eng.*, in press.
- (23) Lee, Y. (1992) *FASEB J.* **6**, 3193–3200.
- (24) Taylor, M., Conary, J., Lennartz, M., Stahl, P., and Drickamer, K. (1990) *J. Biol. Chem.* **265**, 12156–12162.
- (25) Goldmacher, V., Lambert, J., and Blattler, W. (1992) *Biochem. Biophys. Res. Commun.* **183**, 758–766.
- (26) Lewis, M., and Youle, R. (1986) *J. Biol. Chem.* **261**, 11571–11577.
- (27) Schlossman, D., Withers, D., Welsh, P., Alexander, A., Robertus, J., and Frankel, A. (1989) *Mol. Cell. Biol.* **9**, 5012–5021.

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Site-Specific Attachment of Functionalized Poly(ethylene glycol) to the Amino Terminus of Proteins

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A convenient method for the construction of site-specifically modified poly(ethylene glycol)–protein conjugates is described. This method relies on the ability to generate a reactive carbonyl group in place of the terminal amino group. If the protein has N-terminal serine or threonine, this can be done by very mild periodate oxidation and generates a glyoxylyl group. A method less restricted by the nature of the N-terminal residue, but which requires somewhat harsher conditions, is metal-catalyzed transamination, which gives a keto group. The N-terminal-introduced reactive carbonyl group specifically reacts, under mild acidic conditions, with an aminooxy-functionalized poly(ethylene glycol) to form a stable oxime bond. Using polymers of different size and shape (linear or multibranched), various conjugates of IL-8, G-CSF, and IL-1ra were constructed and further characterized with respect to their biological activity and pharmacokinetic behavior in rats. Unlike most previous methods, this approach places a single PEG chain at a defined site on the protein. It should therefore be more likely to conserve biological activity when the latter depends on interaction with another macromolecule (unlike enzymic activity which often survives multiple PEGylation).

INTRODUCTION

Covalent attachment of monomethoxypoly(ethylene glycol) to therapeutic proteins prolongs their circulatory life time *in vivo*, reduces their antigenicity and immunogenicity, and improves their resistance to proteolysis (Davis et al., 1980; Abuchowski et al., 1984; Katre et al., 1987). These properties are of great clinical interest, especially in the case of relatively small proteins, where it is believed that an increase of the Stoke's radius is consistent with a reduced renal clearance. PEG functionalization methods have been the subject of a recent review (Zalipsky, 1995). When applied in protein modification, a reactive functional group such as trichloro-s-triazine (Abuchowski et al., 1977), carbonylimidazole (Beauchamp et al., 1983), and succinimidyl succinate (Abuchowski et al., 1984) is introduced to link the polymer to the ϵ -amino group of lysine residues. All involve the preparation of an activated PEG with a reactive functional group which can be coupled to the lysine groups on proteins. Problems in controlling the conjugation chemistry frequently arise because the number and location of lysine residues vary greatly in native proteins and because the location of reactive groups on protein is random. Thus, the stoichiometry of the protein–polymer conjugate and the attachment sites of the polymer to the protein cannot be precisely controlled, factors which might have important implications for protein stability and function. These limitations can be circumvented by appropriate design of both the protein

and the activated polymer. Indeed, there are some examples of site-specific PEGylation, when activated mPEG was linked to a free thiol which has been engineered into the protein (Goodson and Katre, 1990; Benhar et al., 1994).

Another approach for a site-specific conjugation is to take advantage of the presence of a N-terminal serine or threonine which can be converted by very mild periodate oxidation to a glyoxylyl derivative (Fields and Dixon, 1968; Gaertner et al., 1992; Geoghegan and Stroh, 1992) and to functionalize the polymer with a complementary reactive function, the aminooxy group. An analogous method was shown to be straightforward for incorporating synthetic peptides into a protein backbone by introducing at the C-terminus of one fragment or peptide to be recoupled a hydrazide function (Gaertner et al., 1994) and a glyoxylyl group at the N-terminus of the other. In the same way, the aminooxy function will specifically react with the generated aldehyde group at the N-terminus of the polypeptide to form an oxime bond, which is more stable than the hydrazone bond. This approach has already been exploited for the site specific labeling of IL-8 at the N-terminus with an aminooxy-functionalized fluorescent probe (Alouani et al., 1995).

We report here the stoichiometrically precise, site-specific attachment of several functionalized polymers varying in size and shape, via an oxime linkage, to three different proteins: IL-8, G-CSF, and IL-1ra. Indeed, IL-8, as well as G-CSF after aminopeptidase digestion, present a N-terminal serine and threonine, respectively, which can be readily converted to an aldehydic group. A comparably reactive group was introduced at the N-terminus of IL-1ra by metal-catalyzed transamination (Dixon and Fields, 1972), although the conditions for this reaction are known to be potentially much more harmful to the protein. In all cases, the specifically introduced reactive carbonyl group reacts with the complementary aminooxy group of functionalized PEG (Scheme 1). The high homogeneity of PEG conjugates constructed in this way allowed us to explore the influence of the size and shape of the PEG polymer (linear or branched) on the

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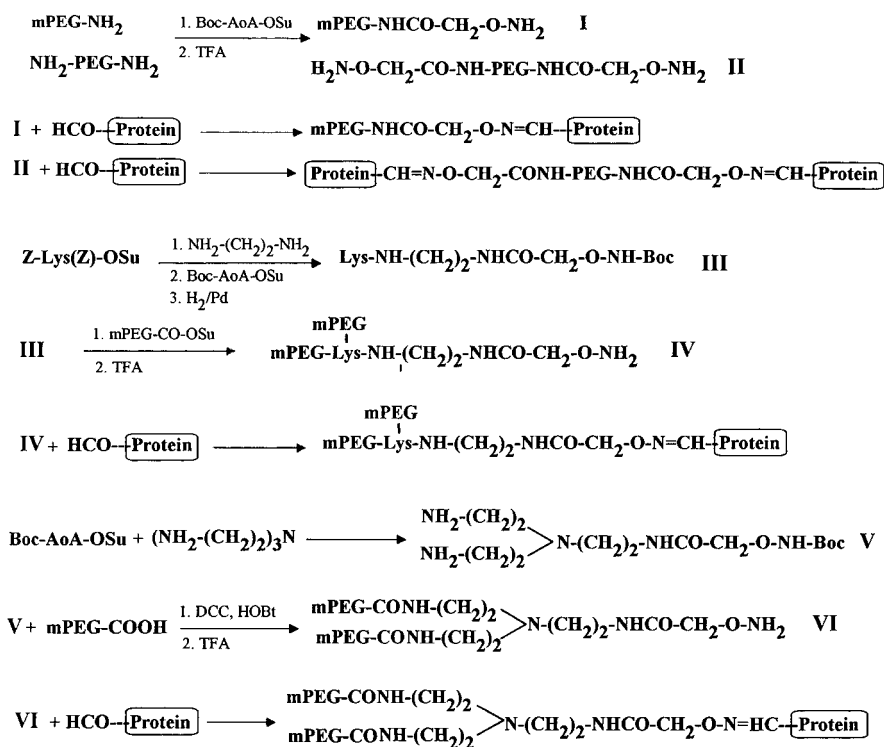
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¹ Abbreviations: mPEG, monomethoxypoly(ethylene glycol); IL-8, interleukin-8; IL-1ra, interleukin-1 receptor antagonist; G-CSF, granulocyte-colony stimulating factor; DCC, *N,N'*-dicyclohexylcarbodiimide; –OSu, *N*-hydroxysuccinimide ester.

Scheme 1. Preparation of the Different Aminoxy Derivatives of PEG and Conjugation to the Reactive Carbonyl Group Generated at the N-Terminus of the Protein.



apparent Stoke's radius of the conjugates, on their *in vitro* activity, and on the blood circulation time in rats.

MATERIALS AND METHODS

Aminomethoxypoly(ethylene glycol) (NH₂-mPEG) of *M_r* 5000 was obtained from Shearwater Polymers (Huntsville, LA) and NH₂-mPEG and mPEG-COOH of *M_r* 10 000 and *M_r* 20 000 as well as NH₂-PEG_{20kD}-NH₂ from Rapp Polymere (Tübingen, Germany).

IL-8 was a gift from Glaxo IMB (Geneva, Switzerland). A mutant analogue of G-CSF was obtained from Zeneca Pharmaceuticals (Macclesfield, U.K.). This analogue known as TG119 is methionyl-[C17S; K16,23,34,40R; A111K; D112S]G-CSF. IL-1ra was a gift from Synergen, Inc. (Boulder, CO). Kidney microsomal aminopeptidase was from Sigma. Na¹²⁵I was from Amersham, and female Wistar rats were from Iffa-Credo (L'Arbresle, France).

Synthesis of Linear (Aminoxy)acetyl-Function-alized Poly(ethylene glycol)(AoA-mPEG). ((Boc-amino)oxy)acetyl *N*-hydroxysuccinimide ester was prepared from aminoxyacetate (Fluka) (Vilaseca et al., 1993) and 144 mg (0.5 mmol) added to 1 g of NH₂-mPEG (0.2 mmol) dissolved in 4 mL of DMSO. The apparent pH was adjusted to 8.0 with *N*-methylmorpholine. After overnight stirring at room temperature, acylation was controlled by the quantitative ninhydrin procedure (Sarin et al., 1981) on a 10 μL aliquot of the reaction mixture. The acylated mPEG was then recovered by dilution with 5 volumes of water, followed by extensive dialysis against water and lyophilization. The Boc group was removed by dissolving the product in 10 mL of anhydrous TFA for 1 h at room temperature. After removal of the acid by rotary evaporation, the material was taken up in water, extensively dialyzed against water, and finally lyophilized yield 0.93 g (93%). The same procedure was used for the synthesis of AoA-NH-mPEG_{10kD} and AoA-NH-mPEG_{20kD} from the corresponding NH₂-mPEG, as well as for AoA-NH-PEG_{20kD}-NH-AoA from NH₂-PEG_{20kD}-NH₂.

AoA-mPEG_{10kD} was analyzed by ¹H-NMR (200 MHz, CDCl₃), but only the PEG polymer signal (δ 3.62 ppm)

was detected. However, the aminoxy end group could be characterized after derivatization with an aromatic ring. For this purpose, the polymer was reacted overnight with a 20-fold excess of benzaldehyde in a 0.1 M acetate buffer, counterion sodium, pH 3.6 containing 20% CH₃CN and the conjugation product isolated by semi-preparative HPLC, using a 35–75% B gradient over 20 min. The two signals at δ 7.4–7.6 ppm with intensities in the ratio 3:2, corresponding to aromatic protons, are consistent with the introduction of a benzaldehyde group through an oxime bond.

Branched Polymer (mPEG)₂Lys-NH-(CH₂)₂-NHCO-CH₂-O-NH₂, (mPEG)₂Lys-AoA. To 2.0 g (3.9 mmol) of Z-Lys(Z)-OSu were added 4 mL of ethylenediamine (60 mmol) and the mixture stirred for 2 h at room temperature. The coupling reaction was quantitative as checked by analytical HPLC on a C8 column using a linear gradient of 0–100% B over 50 min (*t_R* = 41.5 min instead of 48.5 for Z-Lys(Z)-OSu). The coupling product was purified by flash chromatography on a silica column equilibrated in CHCl₃/MeOH (9/1, v:v) and dried by rotary evaporation to yield 1.4 g of material. The product had the expected molecular weight, as determined by ESI-MS (calcd *M* + *H*, *m/z* 457.2; found *m/z* 458.0).

Z-Lys(Z)-NH-(CH₂)₂-NHCO-CH₂-O-NH-Boc was synthesized by adding 1.1 g of Boc-NH-O-CH₂-COOSu (3.8 mmol) to 0.9 g of Z-Lys(Z)-NH-(CH₂)₂-NH₂ (1.9 mmol) dissolved in 3 mL of dry DMSO and the apparent pH adjusted to 8–9 with *N*-methylmorpholine. The solution was stirred during 5 h at room temperature and diluted with 10 volumes of 0.1% TFA and the product purified on a preparative HPLC column 250 × 25 mm i.d. (Nucleosil 300A, 7 μm C8, Macherey Nagel, Oensingen, Switzerland) using a linear gradient from 40 to 80% B over 30 min with a flow rate of 8 mL/min. After lyophilization, the product (weight, 1.1 g; yield 90%) was characterized by ESI-MS (calcd *M* + *H*, *m/z* 630.2, found *m/z* 630.9).

The Z group was cleaved by catalytic hydrogenation. For this purpose, the material was dissolved in absolute

ethanol (50 mL) and acidified with 100 μ L of CH_3COOH , 10% Pd/C (0.1 g) was added, and the mixture was hydrogenated in a hydrogenation apparatus overnight. The catalyst was filtered and the solvent removed by rotary evaporation. The product was purified on the same column that was previously used with a linear gradient from 0 to 50% B over 25 min. After lyophilization (weight, 600 mg; yield, 95%), the product was characterized by ESI-MS (calcd $M + H$, m/z 360.2; found m/z 361.8). The 2HCl salt was obtained by solubilizing the material in 1 mM HCl, freeze drying, and repeating the operation a second time.

Lys-NH-(CH_2)₂-NHCO-CH₂-O-NH-Boc-2HCl was then acylated with mPEG_{5kD}-COOH, mPEG_{10kD}-COOH and mPEG_{20kD}-COOH.

The PEG_{20kD}-COOH was preactivated by reacting 0.4 g (20 μ mol) with *N*-hydroxysuccinimide (23 mg, 200 μ mol) and DCC (21 mg, 100 μ mol) in 3 mL of EtOAc. After overnight incubation at 37 °C, the precipitated dicyclohexylurea was removed by centrifugation and the supernatant treated with active carbon. After centrifugation, the PEG derivative was precipitated by cooling the solution to 4 °C. This precipitation was repeated three times to remove the excess of HOSu and DCC, and finally the material was washed with cold EtOAc and ether and dried in a vacuum desiccator. Two mg of the linker was then reacted with 210 mg of mPEG-COOSu (approximately a 1.5-fold excess over amino groups of the lysine derivative) in 200 μ L of DMF. Acylation was complete after overnight stirring at room temperature. The solution was then diluted with 5 volumes of water, extensively dialyzed against distilled water, and finally lyophilized (180 mg).

The Boc group was then cleaved by dissolving the product in 2 mL of TFA for 1 h at room temperature. TFA was removed under vacuum, and the material was taken up in water, extensively dialyzed against water, and finally lyophilized to give the following derivative: mPEG_{20kD}-Lys(mPEG_{20kD})-NH-(CH_2)₂-NHCO-CH₂-O-NH₂.

A multivalent linker comparable to that described above was obtained by acylation of Tris-(2-aminoethyl)amine with 1 equiv of Boc-AoAOSu in DMSO. The monosubstituted derivative was isolated by HPLC and characterized by ESI-MS (calcd $M + H$, m/z 319.4, found m/z 320.6). The material was solubilized in 10 mM HCl, lyophilized, and further acylated with mPEG_{5kD}-COOH (1.2 molar excess over remaining amino groups) in DMF, in the presence of equimolar amounts of hydroxybenzotriazole and DCC. The solution was stirred overnight at room temperature and the fully acylated derivative purified after extensive dialysis against distilled water by ion exchange chromatography on DEAE-A25 and CMC-25 column and finally deprotected by TFA treatment to give the following derivative: (mPEG_{5kD}-CONH-CH₂-CH₂)₂-N-(CH₂)₂-NHCO-CH₂-O-NH₂.

Functionalization of the N-Terminus of the Protein and Conjugation with the Polymer. 1. *IL-8*. The N-terminal serine of IL-8 (2–5 mg/mL) was oxidized with a 10-fold excess of sodium periodate in a 1% NH_4HCO_3 buffer, pH 8.3, in the presence of a 50 molar excess of methionine (a scavenger) during 10 min at room temperature. After 10 min, the reaction was stopped by the addition of a 2000-fold molar excess of ethylene glycol over periodate and the solution further incubated for 15 min at room temperature. The solution was then dialyzed against a 0.1 M AcONa buffer, pH 4.6. The oxidized protein solution was stored frozen if not used immediately. A 10-fold molar excess of a 10 mM aqueous solution of polymer was added to the oxidized protein,

the pH adjusted to 3.6 with glacial acetic acid, and the mixture incubated for 20 h at room temperature. The conjugation product was purified from reagents by ion exchange chromatography on a Pharmacia Mono S column equilibrated in 25 mM AcONa buffer, pH 4.7 using a linear gradient from 0 to 2 M NaCl over 20 min (to eliminate the excess of polymer), followed by reversed phase HPLC on an analytical C8 column using a linear gradient from 30 to 65% B over 35 min (to separate unreacted IL-8).

2. *G-CSF*. G-CSF was concentrated to 4–8 mg/mL in a Tris·HCl 50 mM, 0.1% sodium lauroyl sarcosinate, pH 7.5 buffer and incubated with aminopeptidase (enzyme/substrate ratio 1/20, w/w) in the presence of MgCl_2 for 20 h at 37 °C. The commercial enzyme suspension was previously, after centrifugation, resolubilized in a 50 mM Tris·HCl buffer, pH 7.5, containing 10 mM MgCl_2 , 2 mM PMSF, benzamidine and 2 mg/mL of aprotinin to prevent any cleavage of the protein by any contaminating proteolytic enzyme present in the commercial enzyme preparation. Aminopeptidase digestion was stopped by adding ethylenediamine tetraacetate (EDTA) at a 10 mM concentration, the solution dialyzed, at 4 °C, against a 20 mM Tris·HCl buffer, pH 8.0, and then against water, and the material finally purified by reversed phase HPLC on a 250 $\times$ 10 mm i.d. Nucleosil C8 column, using a flow rate of 3 mL/min and a linear gradient of 45–70% B over 15 min.

The material was then dissolved in 1% NH_4HCO_3 , pH 8.3, containing 6 M guanidinium hydrochloride at 5 mg/mL and oxidation performed at room temperature with a 5-fold excess of periodate over 10 min. The reaction was stopped by the addition of a 1000-fold excess of ethylene glycol over periodate, and a 5 molar excess of AoA-mPEG_{20kD} was added to the reaction medium. The solution was then diluted with 5 volumes of 0.1 M AcONa, pH 4.6 containing 6 M guanidinium chloride and concentrated on a Centricon to the initial volume. The pH was then adjusted to 3.6 with acetic acid, and a further 5-fold excess of AoA-mPEG_{20kD} was added to the reaction medium, which was then stirred overnight at room temperature. The conjugation product was isolated by reversed phase HPLC on an analytical C8 column using a 50–90% B linear gradient over 40 min with a flow rate of 0.6 mL/min.

3. *IL-1ra*. The concentrated protein solution (40 mg/mL) was diluted 10 times in a 2.5 M sodium acetate buffer, pH 5.5; CuSO_4 and glyoxylic acid were added to a final concentration of 2 mM and 0.1 M, respectively. The solution (final pH, 5.3) was then incubated for 30 min at room temperature and the reaction stopped by the addition of solid EDTA (50 mM, final concentration). The mixture was then extensively dialyzed against a 0.1 M AcONa buffer at pH 4.6 and the soluble fraction used for the conjugation reaction. The transaminated protein was incubated 40 h with a 10-fold excess of polymer in a 0.1 M AcONa buffer, pH 3.6 and conjugation followed by SDS-PAGE. The conjugation product was purified by hydrophobic interaction chromatography using a polypropyl aspartamide column (200 $\times$ 4 mm i.d., 1000A, PolyLC Inc., Columbia, MA), followed by gel filtration on a 600 $\times$ 7.8 mm i.d. BioSep-SecS 2000 Phenomenex column and ion exchange chromatography on a MonoQ column equilibrated in a 25 mM Tris·HCl, pH 7.6 buffer with a linear gradient to 25% of a second buffer containing 1 M NaCl with a flow rate of 1 mL/min.

In Vitro Biological Assay. IL-8 activity was evaluated in a chemotaxis assay involving human neutrophils from healthy donors as already described (Alouani et al., 1995).

G-CSF Biological Activity. Granulocyte colony formation by peripheral blood progenitors isolated by cytopheresis was assayed in standard methylcellulose culture as described (Bender et al., 1994). The colony assay medium containing the cells, 10 ng/mL of IL-3 and IL-6, 100 ng/mL of SCF, 3 U/mL of EPO, and increasing concentrations of G-CSF was plated in duplicate in 35 mm Nunc suspension dishes and incubated at 37 °C in 5% CO₂, 5% O₂, and 95% relative humidity. Plates were scored after 14 days for colonies containing more than 50 cells.

The ability of modified IL-1ra to inhibit, in a dose responsive fashion, the PGE₂ production by dermal fibroblasts induced by IL-1 β was measured after 72 h culture in the absence or presence of IL-1ra, as already described (Balavoine et al., 1986).

Pharmacokinetics of Native and Modified Proteins in Rats. IL-8 and its derivatives were iodinated under conditions, which have already been shown to retain full biological activity as determined by neutrophil chemotaxis (Grob et al., 1990). The specific activity of the radioiodinated IL-8 was 5–10 mCi/mg protein. ¹²⁵I-labeled IL-8 or its derivative was injected as a bolus (10 μ g/kg, an amount limited by the potential toxicity of IL-8) in the tail vein of female Wistar rats weighing between 150 and 200 g. Blood samples were collected from the tail at 3, 7, 15, 30 min, 1, 3, 7, and 24 h. The samples were weighed to determine their exact volume, and their radioactivity was measured. Since a 2–3 min period is necessary to mix the injected material in the blood compartment, the sample collected at 3 min was considered as the initial concentration.

IL-1ra was also iodinated by the chloramine T methodology. Briefly, the protein was dialyzed against a 0.1 M Tris·HCl, 0.15 M NaCl, pH 7.0 buffer and concentrated to about 300 μ L. This solution was then mixed with 500 μ Ci Na¹²⁵I and 30 μ L of a 1% (w/v) chloramine T solution prepared in the same buffer as that used for the protein. The iodination reaction was terminated after 90 s by the addition of 30 μ L of a 5% (w/v) sodium metabisulfite solution and 25 μ L of a 50% (w/v) KI solution. The entire reaction mixture was transferred on a GF5 column (Pierce) equilibrated in PBS and previously washed with the same buffer containing 0.1% BSA to separate the radioiodinated protein from free ¹²⁵I. The specific radioactivity thus obtained was in the range of 100–300 μ Ci/mg. The pharmacokinetics of IL-1ra and its derivatives was studied at a dose of 300 μ g protein/kg under the same conditions as previously described.

Analytical Methods. SDS–PAGE was carried out on 20% Phast homogenous gels (Pharmacia) according to the manufacturer's instructions and the protein visualized by silver staining. IL-8, G-CSF, and IL-1ra were quantified by extinction coefficient $A_{1\text{cm}}^{0.1\%} = 0.89, 0.86, \text{ and } 0.93$, respectively.

Analytical HPLC (250 mm $\times$ 4 mm i.d. Nucleosil 300A 5 μ m C8 column) operated at 0.6 mL/min; 100% solvent A, 5 min then 2% B/min to 100% B; solvent A (1 g of TFA/1 L of HPLC grade water), solvent B (1 g of TFA/100 mL of HPLC water, made up to 1 L with HPLC grade acetonitrile). Electrospray ionization mass spectrometry (ESI-MS) was performed as already described (Gaertner et al., 1994).

RESULTS AND DISCUSSION

Coupling of Functionalized Polymers to the Proteins. 1. *IL-8.* Oxidation of the amino terminal serine under conditions described was considered to be almost quantitative since two components could be identified ESI-MS corresponding to the glyoxylyl derivative derived

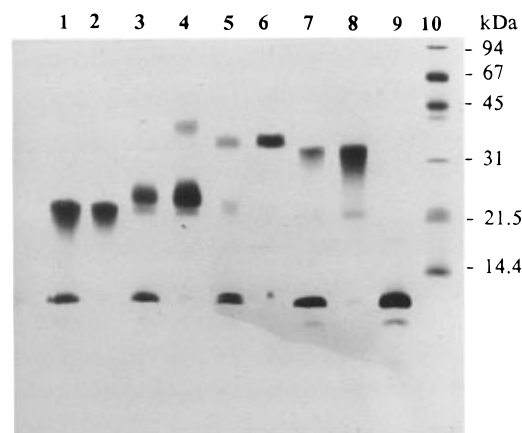


Figure 1. SDS–polyacrylamide gel electrophoresis of coupling between oxidized IL-8 and different functionalized mPEG polymers. Lanes 1, 3, 5, 7: result of 20 h of incubation of oxidized IL-8 with functionalized AoA-mPEG_{5kD}, AoA-mPEG_{10kD}, AoA-mPEG_{20kD}, and AoA-N(mPEG_{5kD})₂, respectively. Lanes 2, 4, 6, 8: purified mPEG_{5kD}–IL-8, mPEG_{10kD}–IL-8, and mPEG_{20kD}–IL-8 and (mPEG_{5kD})₂–IL-8. Lane 9: oxidized IL-8. Lane 10: protein markers from top to bottom, phosphorylase B (94 kD), bovine serum albumin (67 kD), ovalbumin (45 kD), carbonic anhydrase (31 kD), soybean tyrosin inhibitor (21.5 kD), lysozyme (14.4 kD). Unreacted PEG polymers were not visualized by silver staining.

from the methanol/water/acetic acid solvent used to inject samples (calcd, m/z 8350.7; found m/z 8350.8 $\pm$ 1.0) and especially the methyl hemiacetal derivative (calcd, m/z 8382.7; found m/z 8382.2 $\pm$ 0.6) while a unique mass spectrum was obtained for IL-8 (calcd, m/z 8381.7; found m/z 8380.9 $\pm$ 0.7). This result was further confirmed by the high coupling yield of the aminoxy functionalized polymers to the oxidized IL-8 (Figure 1). The SDS–PAGE characterization of mPEG–IL-8 samples shows also that a single homogeneous conjugate with increased molecular weight as compared to the parent protein is obtained, where the size is only dependent on the length of the polymer used and not on the degree of modification, as it occurred for attachment involving amino groups. The staining intensity of IL-8 and mPEG–IL-8 bands of analyzed reaction mixtures (Figure 1, lanes 1 and 3) and HPLC profiles (results not shown) allowed us to estimate the coupling yield to be about 80% in the case of AoA-mPEG_{5kD} and AoA-mPEG_{10kD}. The lower coupling yields observed with AoA-mPEG_{20kD} and AoA-N(mPEG_{5kD})₂ after 20 h of incubation (approximately 30%), could be significantly increased by prolonging the reaction period to 2–3 days.

The chemoselectivity of the ligation reaction allowed us to investigate the attachment of non standard PEG polymers, such as branched or bifunctional ones (Figures 1 and 2). The bifunctional derivative resulted in the formation of a protein dimer linked by a PEG chain (“dumbbell”). Although SDS–PAGE is not the most appropriate technique to characterize the effective size of each species, it can be clearly shown that constructs differing in topology but not in average molecular weight exhibit different apparent hydrodynamic radii. For example, in Figure 1, the behavior of (mPEG_{5kD})₂–IL-8 is much near to that of mPEG_{20kD}–IL-8 than to that of mPEG_{10kD}–IL-8. It is also worth stressing that the dimer construct was obtained in a single coupling step, even with a 5- or 10-fold excess of the bifunctional polymer over the oxidized protein.

2. *G-CSF.* The same approach was used in the conjugation of aminoxy-functionalized mPEG to G-CSF. But in this case, the N-terminal methionine had to be removed in a first step to reveal the threonine which will

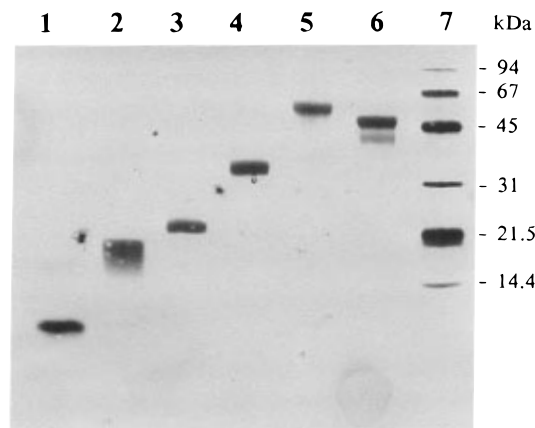


Figure 2. Characterization of IL-8 conjugates obtained with the different functionalized polymers after purification: lane 1; IL-8; lanes 2, 3, 4, mPEG_{5kD}-IL-8, mPEG_{10kD}-IL-8, and mPEG_{20kD}-IL-8; lane 5, (mPEG_{20kD})₂Lys-IL-8; lane 6, IL-8-PEG_{20kD}-IL-8; lane 7, protein markers.

be the target of periodate oxidation. This could be readily achieved by enzymatic digestion with microsomal leucine aminopeptidase. This specific cleavage takes advantage of the presence of vicinal proline residue, which avoids any overdigestion of the protein substrate. This specific cleavage was confirmed by ESI-MS analysis of the digestion product, where only two compounds could be identified, the most important corresponding to the *des*-Met-G-CSF (calcd, m/z 18792.9; found 18792.1 ± 1.8) and the other to the native protein (calcd, m/z 18923.9; found m/z 18925.0 $\pm$ 2.8).

The oxidation could be performed under the same conditions as described for IL-8 and confirmed by mass spectrometry, but no coupling occurred without the addition of guanidinium chloride to the medium. This suggests that oxidation leads either to an unreactive species with no detectable mass change relative to the initial oxidized protein (by formation, for example, of an hemiacetal with a serine side chain) or to a conformational change leading to the steric inaccessibility of the carbonyl function. Under these conditions, the coupling yield did not exceed 20–30% and only the mPEG_{20kD}-G-CSF conjugate was synthesized at a larger scale for biological studies.

3. IL-1ra. The N-terminal Met-Arg-Pro- sequence of IL-1ra does not allow the use of the previously described method so that, in this case, selective modification of α -amino group was achieved by metal-catalyzed transamination. This method is likely to be much more general with regard to the N-terminal residue, but has the drawback of exposing the protein to a metal. Indeed, disappearance of the N-terminal amino group could not be assessed either by mass spectrometry, where several copper adducts only could be identified, or by electrophoresis, and so conditions chosen were close to those already described (Dixon and Fields, 1972). The appearance of the reactive keto group was detected by its ability to react with AoA-NH-mPEG. The transaminated protein was incubated for 48h with a 10-fold excess of polymer in a 0.1 M acetate (sodium), pH 3.9 at room temperature. mPEG_{5kD}-IL-1ra, mPEG_{10kD}-IL-1ra, and mPEG_{20kD}-IL-1ra were thus constructed and purified as shown in Figure 3. Owing to the fact that the coupling yield is lower than in the case of IL-8 and that some side reactions occur, isolation of the conjugation product required three purification steps and significantly decreased the overall yield of conjugate.

Biological Activities in Vitro and Pharmacokinetics in Rats. **1. PEG Conjugate of IL-8.** While IL-8

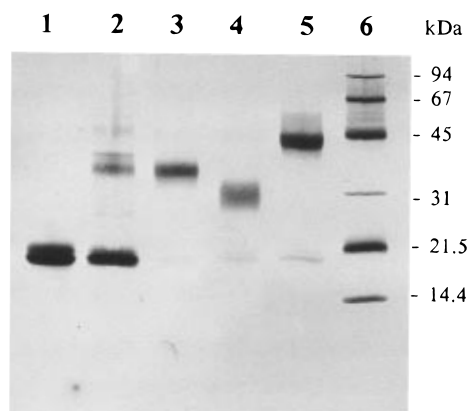


Figure 3. Conjugation of functionalized AoA-mPEG_{5kD}, AoA-mPEG_{10kD}, and AoA-mPEG_{20kD} to IL-1ra: lane 1, IL-1ra; lane 2, coupling of AoA-mPEG_{10kD} to transaminated IL-1ra after 48 h of incubation at room temperature; lanes 3, 4, 5, purified conjugates mPEG_{10kD}-IL-1ra, mPEG_{5kD}-IL-1ra, and mPEG_{20kD}-IL-1ra; lane 6, protein markers.

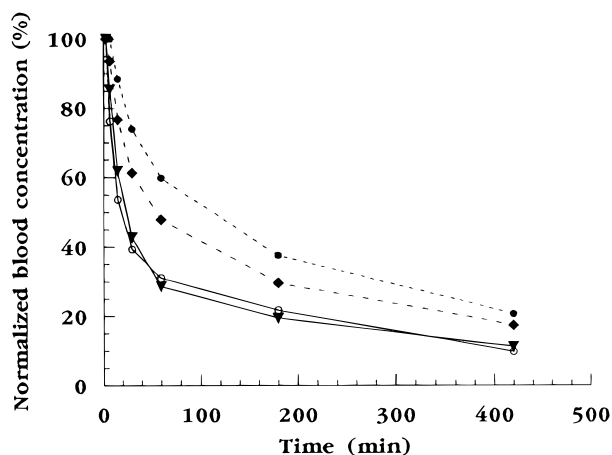


Figure 4. Pharmacokinetics of IL-8, mPEG_{20kD}-IL-8, (mPEG_{20kD})₂Lys-IL-8, and IL-8-PEG_{20kD}-IL-8. Rats were given a single injection of 10 μ g/kg, i.e., IL-8 ($\circ$), mPEG_{20kD}-IL-8 ($\blacktriangledown$), (mPEG_{20kD})₂Lys-IL-8 ($\blacklozenge$), and IL-8-PEG_{20kD}-IL-8 ($\bullet$). Each determination is the mean value of four determinations with four animals.

had an EC₅₀ of 2 nM in the neutrophil chemotaxis assay (concentration corresponding to 50% of the maximal chemoattractant activity), mPEG_{5kD} and mPEG_{10kD} derivatives were shown to have an EC₅₀ of 10 nM and mPEG_{20kD}, (mPEG_{20kD})₂Lys-IL-8, and IL-8-PEG_{20kD}-IL-8 an EC₅₀ ranging between 20 and 40 nM.

The curves of relative blood levels for IL-8, PEG_{20kD}-IL-8, (mPEG_{20kD})₂Lys-IL-8, and IL-8-PEG_{20kD}-IL-8 given intravenously to the rats are compared in Figure 4. Conjugation of a single 20 kD chain to IL-8 has almost no influence on the pharmacokinetic curve, but the dumbbell and the (mPEG_{20kD})₂Lys derivatives have considerably increased area under the pharmacokinetic curve. A manual fit to these curves gives apparent first $T_{1/2}$ values of approximately 10 min for IL-8, 17 min for mPEG_{20kD}-IL-8, 30 min for IL-8-PEG_{20kD}-IL-8, and 60 min for (mPEG_{20kD})₂Lys-IL-8. No quantifiable differences were observed in the apparent second $T_{1/2}$ among all protein derivatives, but while less than 1% of the initially observed radioactivity remained in circulation after 24 h for IL-8, 6% of the initial amount was still present in case of (mPEG_{20kD})₂Lys-IL-8.

2. mPEG Conjugate of G-CSF. As shown in Figure 5, a dose-response curve was obtained for both *des*-Met-G-CSF and mPEG_{20kD}-*des*-Met-G-CSF. The biological activity of the *des*-Met-G-CSF derivative was slightly lower than that obtained with Neupogen which was used

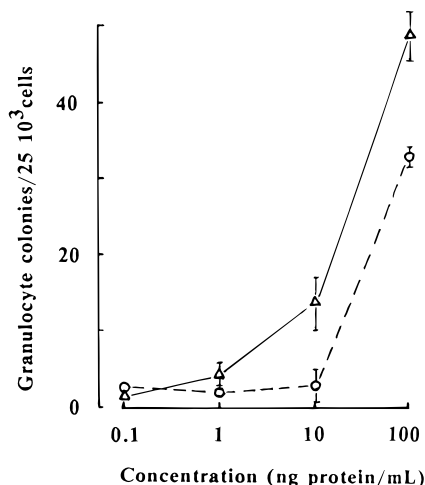


Figure 5. Comparison of *in vitro* biological activities of des-Met-G-CSF (Δ) and mPEG_{20kD}-des-MetG-CSF ($\circ$) using a methylcellulose culture medium to which were added IL-3 and IL-6 at 10 ng/mL, SCF at 100 ng/mL, and EPO at 3 units/mL using progenitor cells of human blood. 25×10^3 cells were deposited in each well, and G-CSF was added at increasing concentrations. Granulocyte colonies were scored after 14 days of incubation at 37 °C.

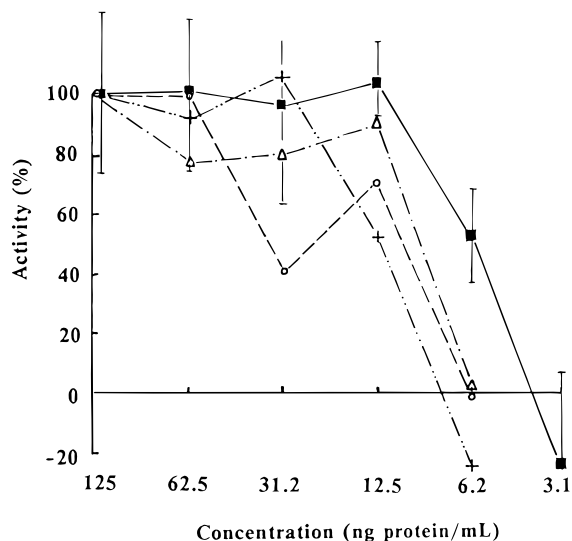


Figure 6. Inhibition of IL-1 β -induced PGE₂ production by dermal fibroblasts in the presence of decreasing concentrations of IL-1ra derivatives. Each point is derived from a mean of three PGE₂ determinations, from which is subtracted the mean of three determinations obtained when buffer only was used in place of IL-1ra. For clarity, error bars which are of the same order of magnitude as for IL-1ra ($\blacksquare$) were not plotted for mPEG_{5kD}-IL-1ra (+), mPEG_{10kD}-IL-1ra ($\circ$), and mPEG_{20kD}-IL-1ra (Δ) derivatives.

as control (22 colonies/ 25×10^3 cells at 10 ng/mL), but the attachment of a mPEG_{20kD} chain to the N-terminus resulted in a 2–3-fold decrease of the activity.

3. mPEG Conjugate of IL-1ra. As shown in Figure 6, the IL-1 β effect is inhibited in a dose-responsive manner by all conjugates, and an IC₅₀ value (concentration necessary to block 50% of IL-1 β response) can be approximately determined: the values are approximately 13 and 9 ng/mL for mPEG_{5kD}- and mPEG_{20kD}-IL-1ra, respectively. The IC₅₀ value for authentic IL-1ra was found to be about 6 ng/mL, and a similar profile was obtained for the transaminated derivative without conjugation of the polymer (results not shown).

The curves of relative blood levels for IL-1ra, mPEG_{10kD}-IL-1ra, and mPEG_{20kD}-IL-1ra given intravenously to the rats are compared in Figure 7. An

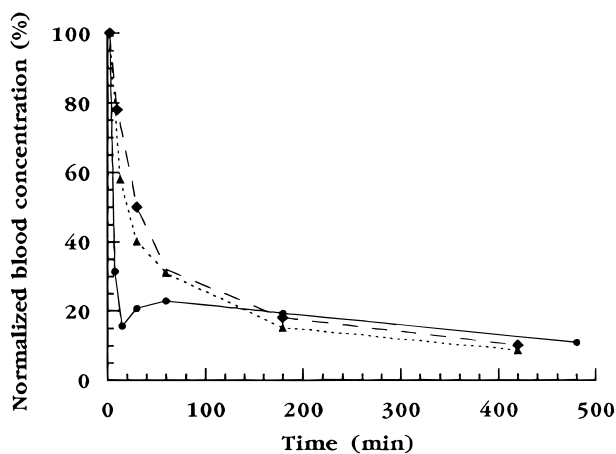


Figure 7. Pharmacokinetics of mPEG_{10kD}-IL-1ra and mPEG_{20kD}-IL-1ra in rats, compared with authentic IL-1ra. Rats were given a single injection of 300 μ g/kg, i.e., IL-1ra ($\bullet$), mPEG_{10kD}-IL-1ra ($\blacktriangle$), and mPEG_{20kD}-IL-1ra ($\blacklozenge$). Samples of blood were collected at 3, 10, 15, 30 min, 1, 3, 7, 12, and 24 h. Each determination represents the mean value of three determinations with three animals.

unexpected shoulder, reproducible from one animal to another, could be observed in the IL-1ra clearance curve for blood samples recovered after 30 min, corresponding to a slight increase of circulating radioactivity which did not occur for other protein derivatives. A manual fit to these curves gives apparent first $T_{1/2}$ values of approximately 3 min for IL-1ra, 14 min for PEG_{10kD}-IL-1ra, and 20 min for PEG_{20kD}-IL-1ra. No detectable differences were observed in the apparent second $T_{1/2}$ amongst all three proteins, and in all animals less than 2% of the initially observed radioactivity remained in circulation after 24 h.

It is clear from the results presented herein that by increasing the polymer mass or its molecular volume, either by enhancing its length or by adding multiple copies of the polymer at one site, one can increase the circulating half-life without marked loss of functionality. For these small proteins, where size is the rate-determining step in the *in vivo* clearance mechanism, only the size of the single polymer attached to the N-terminus of the protein will influence the pharmacokinetics of the conjugate, while with other conventional methods, the extent of modification of the different amino groups, which is less reproducible, has to be taken into account. Moreover, this method has the advantage of neither significantly decreasing the isoelectric point upon modification nor of introducing groups at locations likely to interfere with the binding properties of the target molecule. Pharmacokinetic data obtained with the 8 kD molecular weight protein IL-8 show that attachment of a single PEG_{20kD}-chain is not enough for significantly increasing the effective molecular size of the conjugate to overcome the cut-off point of kidney glomerular filtration (70 kD), whereas a comparable modification of the 14 kD IL-1ra results in at least 6-fold increase of the first half-life which is more consistent with a partial exclusion from kidney filtration. However, attachment of the bi-antennary (mPEG_{20kD})₂Lys-AoA construct markedly increased the circulatory half-life of IL-8, when compared to the single 20 kD polymer-modified protein.

The convenient functionalization of commercial amino-derivatized PEG polymers, or constructed multibranched structures described here, can provide a large panel of PEG derivatives varying in size and shape for a precise tuning of biological activity and plasma clearance of the target protein. Moreover, this approach can be extended

to other polymers such as dextran which can be mono-functionalized at its reducing terminus with a reactive amino group and further derivatized with an aminoxy function (H. Gaertner, unpublished results).

CONCLUSION

Results presented here demonstrate that proteins with an N-terminal serine or threonine are attractive targets for the site-specific attachment of aminoxy functionalized PEG polymers to generate improved biopharmaceutical proteins. This further extends the formerly described applications of this approach in protein backbone engineering (Gaertner et al., 1994) or immunoconjugate preparation (Werlen et al., 1994; Mikolajczyk et al., 1994). IL-8, which is an inflammatory cytokine, was used throughout this study only as a model protein which happens to have the appropriate N-terminal residue. The G-CSF modification demonstrates that even in the presence of a Met-Thr-Pro- N-terminal sequence, the threonine residue can be conveniently revealed by enzymatic digestion. Modification of IL-1ra without marked loss of activity shows that also metal-catalyzed transamination can be used for introducing a comparably reactive keto group at the N-terminus. This method is less restricted in terms of its N-terminal residues that are able to react, and it might therefore be a more general reaction.

Since in our approach the polymer is attached at a single site of the protein, it has to be of sufficient size to increase *in vivo* bioavailability, whereas in other methods such a result relies on the attachment of mPEG chains of relatively small size (5 kD) to several amino groups of the protein. The high coupling efficiency to oxidized IL-8 of PEG polymers, either linear or branched and varying in size from 5 to 40 kD, supports this hypothesis and prompts us to investigate the attachment of even larger multibranched polymers to the single reactive site of the protein via an oxime bond.

ACKNOWLEDGMENT

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LITERATURE CITED

- Abuchowski, A., McCoy, J. R., Palczuk, N. C., van Es, T., and Davis, F. F. (1977) Alteration of immunological properties of bovine serum albumin by covalent attachment of polyethylene glycol. *J. Biol. Chem.* 252, 3582–3586.
- Abuchowski, A., Kazo, G. M., Verhoest, C. R., van Es, T., Kafkewitz, D., Nucci, M. L., Viau, A. T., and Davis, F. F. (1984) Cancer therapy with chemically modified enzymes. I. Antitumor properties of polyethylene glycol asparaginase conjugates. *Cancer Biochem. Biophys.* 7, 175–186.
- Alouani, S., Gaertner, H. F., Mermod, J. J., Power, C. A., Bacon, K. B., Wells, T. N. C., and Proudfoot, A. E. I. (1995) A fluorescent interleukin-8 receptor probe produced by targeted labelling at the amino terminus. *Eur. J. Biochem.* 227, 328–334.
- Balavoine, J. F., de Rochemonteix, B., Williamson, K., Seckinger, P., Cruchaud, A., and Dayer, J. M. (1986) Prostaglandins and collagenase production by fibroblasts and synovial cells is regulated by urine-derived human interleukin 1 and inhibitors. *J. Clin. Invest.* 78, 1120–1124.
- Beauchamp, C. O., Gonias, S. L., Menapace, D. P., and Pizzo, S. V. (1983) A new procedure for the synthesis of polyethylene glycol–protein adducts; effects of function, receptor recognition, clearance of superoxide dismutase, lactoferrin, and α_2 -macroglobulin. *Anal. Biochem.* 131, 25–33.
- Bender, J. G., Lum, L., Unverzagt, K. L., Lee, W., Van Epps, D., George, S., Coon, J., Ghalie, R., McLeod, B., Kaizer, H., and Williams, S. F. (1994) Correlation of colony-forming cells, long term culture initiating cells and CD34+ cells in apheresis products from patients mobilized from peripheral blood progenitors with different regimens. *Bone Marrow Transpl.* 13, 479–485.
- Benhar, I., Wang, Q., Fitzgerald, D., and Pastan, I. (1994) Pseudomonas exotoxin A mutants. Replacement of surface exposed residues in domain III with cysteine residues that can be modified with polyethylene glycol in a site specific manner. *J. Biol. Chem.* 269, 13398–13404.
- Davis, F. F., Abuchowski, A., Van Es, T., Palczuk, N. C., Savoca, K., Chen, R. H.-L., and Pyatak, P. (1980) Soluble, nonantigenic polyethylene glycol-bound enzymes. *Biomedical Polymers*, pp 441–451, Academic Press, New York.
- Dixon, H. B. F., and Fields, R. (1972) Specific modifications of NH₂-terminal residues by transamination. *Methods Enzymol.* 25, 409–419.
- Fields, R., and Dixon, H. B. F. (1968) A spectrometric method for the micro determination of periodate. *Biochem. J.* 108, 883–887.
- Gaertner, H. F., Rose, K., Cotton, R., Timms, D., Camble, R., and Offord, R. E. (1992) Construction of protein analogues by site-specific condensation of unprotected fragments. *Bioconjugate Chem.* 3, 262, 262–268.
- Gaertner, H. F., Offord, R. E., K. Cotton, R., Timms, D., Camble, and Rose, K. (1994) Chemoenzymic backbone engineering of proteins: site specific incorporation of synthetic peptides that mimic the 64–74 disulfide loop of G-CSF. *J. Biol. Chem.* 269, 7224–7230.
- Geoghegan, K. F., and Stroh, J. G. (1992) Site-directed conjugation of nonpeptide groups to peptides and proteins via periodate oxidation of a 2-amino alcohol. Application to modification of a N-terminal serine. *Bioconjugate Chem.* 3, 138–146.
- Goodson, R. J., and Katre, N. V. (1990) Site-directed pegylation of recombinant interleukin-2 at its glycosylation site. *Bio-Technology* 8, 343–346.
- Grob, P. M., David, E., Warren, T. C., DeLeon, R. P., Farina, P. R., and Homon, C. A. (1990) Characterization of a receptor for human monocyte-derived neutrophil chemotactic factor/interleukin-8. *J. Biol. Chem.* 265, 8311–8316.
- Katre, N. D., Knauf, M. J., and Laird, W. J. (1987) Chemical modification of recombinant interleukin 2 by polyethylene glycol increases its potency in the murine Meth A sarcoma model. *Proc. Natl. Acad. Sci. U.S.A.* 84, 1487–1491.
- Knauf, M. J., Bell, D. P., Hirtzer, P., Luo, Z.-P., Young, J. D., and Katre, N. V. (1988) Relationship of effective molecular size to systemic clearance in rats of recombinant interleukin-2 chemically modified with water soluble polymers. *J. Biol. Chem.* 263, 15064–15070.
- Mikolajczyk, S. D., Meyer, D. L., Starling, J. J., Law, K. L., Rose, K., Dufour, B., and Offord, R. E. (1994) High yield, site specific coupling of N-terminally modified β -lactamase to a proteolytically derived single sulfhydryl murine Fab'. *Bioconjugate Chem.* 5, 636–646.
- Sarin, V. K., Kent, S. B. H., Tam, J. P., and Merrifield, R. B. (1981) Quantitative monitoring of solid phase peptide synthesis by the ninhydrin reaction. *Anal. Biochem.* 117, 147–157.
- Vilaseca, L. A., Rose, K., Werlen, R., Meunier, A., Offord, R. E., Nichols, C. L., and Scott, W. L. (1993) Protein conjugates of defined structure: synthesis and use of a new carrier molecule. *Bioconjugate Chem.* 4, 515–520.
- Werlen, R. C., Lankinen, M., Rose, K., Blakey, D., Shuttleworth, H., Melton, R., and Offord, R. E. (1994) Site-specific conjugation of an enzyme to an antibody fragment. *Bioconjugate Chem.* 5, 411–417.
- Zalipsky, S. (1995) Functionalized poly(ethylene glycol) for preparation of biologically relevant conjugates. *Bioconjugate Chem.* 6, 150–165.

Purification of Subunit B of Shiga Toxin Using a Synthetic Trisaccharide-Based Affinity Matrix

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The blood group P₁ antigenic trisaccharide (**3**), which is the receptor-binding ligand of Shiga-like toxins, is synthesized in a spacer-equipped form (**32**) from 2-(trimethylsilyl)ethyl glucoside **5** and the 1-thiogalactoside building blocks **10** and **22** in a stereocontrolled, stepwise fashion. Covalent attachment of **32** to hydrazine group-containing agarose gel by reductive amination provided the P₁ trisaccharide-containing affinity sorbent which was used for preparative scale isolation of subunit B of Shiga toxin.

INTRODUCTION

The Shiga and Shiga-like families of secreted toxins play an important role in the pathogenesis of infections caused by *Shigella dysenteriae* type 1 and various enterotoxigenic strains of *Escherichia coli* and are associated with a number of clinical manifestations of infections such as hemorrhagic colitis and hemolytic uremic syndrome (1, 2). These toxins are multimeric proteins consisting of a single enzymatic subunit (subunit A, MW 32 kDa) and a symmetrically arranged, pentameric subunit B (MW 7.7 kDa) (1, 2). The latter was shown to mediate the binding of the holotoxin to cell-surface glycolipids as the first step of its endocytosis. Galabiose (**1**) was identified as the minimum binding receptor that recognizes the toxin, placed either terminally or internally in glycolipids (3). The binding was stronger with P blood group (4) determinant trisaccharides, such as the P_k antigen (globotriose, **2**) and the P₁ antigen (**3**) (Chart 1). More recent reports confirmed these findings and showed that the α -(1→4) linkage between the two galactose residues is essential for the recognition (5, 6). It has been suggested (5) that specific adsorbents of Shiga (Shiga-like) toxins may aid the development of serologic tests for rapid diagnosis of *E. coli* infections, and receptor-analog oligosaccharides that inhibit the binding of these toxins to their cell-surface receptors can be candidates for therapeutics against such diseases (6). Three methods were used for the isolation and purification of the toxin components. Functional subunits A and B were isolated from the holotoxin in small quantities by molecular sieve chromatography (7). In an elegant method, the P₁ glycoprotein of the hydatid cyst fluid was covalently attached to Sepharose 4B and the receptor-based affinity material was used to isolate subunit B in a single step on the mg scale (8). Unfortunately, the low

capacity of this column precludes scale-up. Recently, large scale purification was also reported (9). Although the latter approach provided pure subunit B in the 100 mg level, it required a two-step chromatographic protocol including ion-exchange chromatography on DEAE-Sephadex followed by chromatofocusing using Polybuffer Exchanger 94 and, finally, by ammonium-sulfate precipitation. We now report a simple and efficient method for the preparative scale isolation of subunit B of Shiga toxin, using a receptor-analog affinity sorbent. Specifically, we describe the chemical synthesis of a glycosyl donor derivative of the P₁ trisaccharide antigen and the covalent attachment of the P₁ trisaccharide (**3**) through a chemical anchor to a solid support, using a general method reported earlier from this laboratory (10). We also demonstrate the utility of the affinity material for the rapid isolation of pure subunit B in a semiautomated fashion.

EXPERIMENTAL PROCEDURES

General. Melting points were taken on a Meltemp capillary melting point apparatus and are uncorrected. All chemicals were of commercial grade and were used without purification. Anhydrous solvents were obtained from Aldrich. Optical rotations were measured at 22 °C with a Perkin-Elmer type 241MC or 341 polarimeter for CHCl₃ solutions, except where indicated otherwise. Column chromatography was performed on silica gel 60 (0.040–0.063 mm). The NMR spectra were obtained at 296 K, on a Varian XL-300 or a Gemini 300 spectrometer operating at 300 MHz for ¹H and at 75.5 MHz for ¹³C. Internal references: TMS (0.000 ppm for ¹H for solutions in organic solvents), acetone (2.225 ppm for ¹H and 31.00 ppm for ¹³C for solutions in D₂O) and CDCl₃ (77.00 ppm for ¹³C for solutions in CDCl₃). The methylene carbon resonances were identified by a DEPT-135 experiment. Subscripts A-C refer to the individual sugar residues, with A standing for the reducing-end unit. Protons linked to the same carbon atom are differentiated by an asterisk. Aglyconic atoms are denoted by a prime for compounds **28–32**. For compound **32**, the assignments for the hydrated form are shown in italics. The fast atom bombardment mass spectra were run on a JEOL SX102 mass spectrometer using 6 keV xenon atoms to ionize

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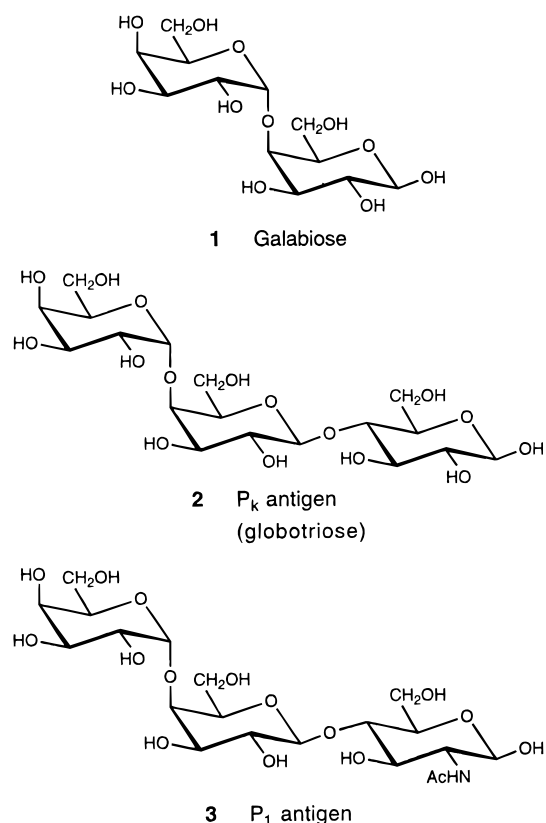
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Chart 1^a

^a Minimum carbohydrate structures recognized by subunit B of Shiga toxin. The reducing end unit is shown as the β anomer.

the samples which were desorbed from a mixture of dithiothreitol and dithioerythritol from glycerol or from 3-nitrobenzyl alcohol as the matrix. Chemical ionization mass spectra (CI-MS) were obtained by using NH_3 as the ionizing gas. Elemental analyses were performed by Atlantic Microlab, Inc., Norcross, GA. Affinity chromatography was performed in a Pharmacia FPLC system.

Vibrio cholerae Strain. *V. cholerae* strain 0395-N1 (pSBC32) containing the gene of subunit B of Shiga toxin but lacking in the gene for subunit A has been described by Acheson *et al.* (9).

Protein Determination. Protein determination was carried out by the bicinchonic acid (BCA) method (27), using the BCA protein assay reagent of Pierce (Rockford, IL). Bovine serum albumin was used as the reference.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE). SDS-PAGE was performed in Novex (16% Tris-tricine) slab gels, 8 $\times$ 8 cm, and stained with Coomassie blue R 350 stain, obtained from Pharmacia Biotechnology (Uppsala, Sweden).

Antigenicity of Subunit B of Shiga Toxin. Purified subunit B of Shiga toxin was reacted with a monoclonal antisubunit B rabbit monoclonal antibody as described earlier (9).

Chemical Synthesis of the Affinity Sorbent. 2-(Trimethylsilyl)ethyl 3-O-benzyl-4,6-O-benzylidene-2-deoxy-2-phthalimido- β -D-glucopyranoside (**4**). Compound **4** prepared essentially as described in ref 11 was obtained in crystalline form: mp 103–105 °C (hexane), $[\alpha]_D^{25} +43^\circ$ (c 0.7) (lit. (11) syrup, $[\alpha]_D^{25} +39^\circ$ (c 0.7, CHCl_3)). Anal. ($\text{C}_{33}\text{H}_{37}\text{NO}_7\text{Si}$) C, H, N.

Methyl 2,3,4,6-Tetra-O-acetyl-1-thio- β -D-galactopyranoside (6**).** To a solution of 1,2,3,4,6-penta-O-acetyl- β -D-galactopyranoside (127 g, 325 mmol) in anhydrous CH_2Cl_2 (350 mL) were added $\text{BF}_3 \cdot \text{Et}_2\text{O}$ (100 mL, 813 mmol) (**12**) and liquid MeSH (25 g, 520 mmol) at 0 °C. The

solution was allowed to reach 22 °C in 1 h. TLC (1:1 EtOAc–hexane) indicated complete conversion of the starting material to a slightly faster-moving compound. The solution was slowly poured into ice-cold, aqueous NaHCO_3 in a 4 L Erlenmeyer flask under stirring. The organic layer was washed with H_2O , dried (Na_2SO_4), and concentrated. The resulting solids were treated with diethyl ether (300 mL). Filtration afforded **6** (109 g, 88.5%), which was used for the next step without further purification. Recrystallization of a sample afforded analytically pure **6** having physical properties identical to those of an independent preparation (13c).

Methyl 4,6-O-Benzylidene-1-thio- β -D-galactopyranoside (8**).** To a stirred mixture of **7** (13) (60 g, 303 mmol) and benzaldehyde dimethyl acetal (200 mL, 800 mmol) in DMF (100 mL) was added a catalytic amount of 10-camphorsulfonic acid (**14**). After 12 h, Et_3N (excess) was added, and approximately half of the volatiles were evaporated under vacuum. The residual solution was stirred with H_2O and hexane at ambient temperature for 1 h to afford a crystalline product which was isolated by filtration followed by washing alternatively with water and hexane and drying to give colorless **8** (69 g, 76%): mp 148–151 °C; $[\alpha]_D^{25} -32^\circ$ (c 0.9) (lit. (15) mp 149–151; $[\alpha]_D^{25} -34^\circ$ (c 0.5, H_2O)); NMR (CDCl_3) ^1H , δ 5.516 (s, 1 H, CHPh), 4.322 (dd, 1 H, $J_{5,6} = 1.5$ Hz, $J_{6,6^*} = 12.2$ Hz, H-6), 4.263 (d, 1 H, $J_{1,2} = 9.3$ Hz, H-1), 4.205 (br d, 1 H, H-4), 4.000 (dd, 1 H, $J_{5,6^*} = 1.7$ Hz, H-6*), 3.814 (t, 1 H, $J_{1,2} = J_{2,3} = 9.3$ Hz, H-2), 3.650 (dd, 1 H, H-3), 3.482 (d, 1 H, H-5), and 2.180 (s, 3 H, CH_3S); ^{13}C , δ 129.3, 128.3, and 126.4 (aromatic), 101.4 (CHPh), 85.0 (C-1), 75.9, 73.8, 69.9, and 68.8 (C-2,3,4,5), 69.2 (C-6), and 10.9 (CH_3S). Anal. ($\text{C}_{14}\text{H}_{18}\text{O}_5\text{S}$) C, H, S.

Methyl 2,3-Di-O-acetyl-4,6-O-benzylidene-1-thio- β -D-galactopyranoside (9**).** To a stirred solution of compound **8** (68 g, 227 mmol) in pyridine (100 mL) was added Ac_2O (100 mL) at 0 °C. After 3 h the volatiles were evaporated under vacuum. Extractive workup of the residue afforded **9** as a syrup (74 g, 85%), which was sufficiently pure for the next step. Column chromatographic purification (1:1 hexane–EtOAc) afforded analytically pure **9** as a syrup: $[\alpha]_D^{25} +44^\circ$ (c 1.2); NMR (CDCl_3) ^1H , δ 5.486 (dd, 1 H, H-2), 5.486 (s, 1 H, CHPh), 4.990 (dd, 1 H, $J_{2,3} = 9.9$ Hz, $J_{3,4} = 3.4$ Hz, H-3), 4.407 (br d, 1 H, H-4), 4.376 (d, 1 H, $J_{1,2} = 9.8$ Hz, H-1), 4.330 (dd, 1 H, $J_{5,6} = 1.5$ Hz, $J_{6,6^*} = 12.4$ Hz, H-6), 3.998 (dd, 1 H, $J_{5,6^*} = 1.6$ Hz, H-6*), 3.570 (m, 1 H, H-5), 2.239, 2.075, and 2.180 (3 s, 9 H, CH_3CO and CH_3S); ^{13}C , δ 170.5 and 169.4 (C=O), 137.5–126.3 (aromatic), 101.1 (CHPh), 81.9 (C-1), 73.5, 72.8, 69.5, and 69.0 (C-2,3,4,5), 65.7 (C-6), 20.80 and 20.75 (CH_3CO), and 10.1 (CH_3S). Anal. ($\text{C}_{18}\text{H}_{22}\text{O}_7\text{S}$) C, H, S.

Methyl 2,3,4-Tri-O-acetyl-6-O-benzyl-1-thio- β -D-galactopyranoside (10**).** To a stirred mixture of **9** (38 g, 99.4 mmol), NaCNBH_3 (30 g, 477 mmol), and 4A molecular sieves in tetrahydrofuran (500 mL) was added dropwise a saturated solution of HCl in diethyl ether (~130 mL) at 0 °C until TLC (2:1 hexane–EtOAc) indicated complete conversion (**16**). EtOAc (150 mL) was added, and the mixture was filtered through a layer of Celite 577. The solution was carefully neutralized with aqueous NaHCO_3 at 0 °C. The mixture was equilibrated between CHCl_3 and H_2O . The CHCl_3 layer was dried (Na_2SO_4) and then concentrated. A solution of the syrupy residue in pyridine (100 mL) was treated with Ac_2O (100 mL) at 25 °C. After 12 h the volatiles were removed under vacuum. Extractive workup of the residue afforded crystalline **10** (31.5 g, 74%): mp 102–104 °C; $[\alpha]_D^{25} +44^\circ$ (c 1.2); NMR (CDCl_3) ^1H , δ 7.32–7.24 (aromatic), 5.516 (dd, 1 H, $J_{4,5} = 1.1$ Hz, H-4), 5.239 (t, 1 H, $J_{1,2} = J_{2,3} = 9.8$ Hz, H-2), 5.061 (dd, 1 H, $J_{3,4} = 3.2$ Hz, H-3), 4.550 and 4.414 (2 d,

2 H, $J \sim 12$ Hz, CH_2 of Bn), 4.395 (d, 1 H, H-1), 3.898 (ddd, 1 H, H-5), 3.547 (dd, 1 H, $J_{5,6} = 6.0$ Hz, $J_{6,6^*} = 9.4$ Hz, H-6), 3.454 (dd, 1 H, $J_{5,6^*} = 7.0$ Hz, H-6*), 2.184, 2.067, 2.053, and 1.982 (4 s, 12 H, CH_3CO and CH_3S); ^{13}C , δ 170.1, 170.0, and 169.7 (C=O), 137.4–127.9 (aromatic), 83.5 (C-1), 75.8, 72.0, 67.7, and 66.7 (C-2,3,4,5), 75.8 (CH_2 of Bn), 67.3 (C-6), 20.7 and 20.6 (CH_3CO), and 11.5 (CH_3S). Anal. ($\text{C}_{19}\text{H}_{26}\text{O}_8\text{S}$) C, H, S.

2,3,4-Tri-O-acetyl-6-O-benzyl- β -D-galactopyranosyl chloride (11). To a solution of compound **10** (13.4 g, 31.4 mmol) in anhydrous CH_2Cl_2 (100 mL) was added a solution of Cl_2 in CCl_4 [containing ~ 2.6 g (37 mmol) of Cl_2] at 0 °C (17). After 20 min, 1-hexene (excess) was added and the solution was concentrated to give **11** as a solid (11.5 g, 88%): $[\alpha]_D^{20} -2^\circ$ (c 1.4); NMR (CDCl_3) ^1H , δ 7.37–7.23 (aromatic), 5.499 (dd, 1 H, H-4), 5.361 (dd, 1 H, $J_{1,2} = 8.7$ Hz, $J_{2,3} = 10.1$ Hz, H-2), 5.232 (d, 1 H, H-1), 5.003 (dd, 1 H, $J_{3,4} = 3.4$ Hz, H-3), 4.561 and 4.413 (2 d, 2 H, $J \sim 12$ Hz, CH_2 of Bn), 3.941 (ddd, 1 H, H-5), 3.594 (dd, 1 H, $J_{5,6} = 6.0$ Hz, $J_{6,6^*} = 9.6$ Hz, H-6), 3.487 (dd, 1 H, $J_{5,6^*} = 6.8$ Hz, H-6*), 2.080, 2.074, and 1.982 (3 s, 9 H, CH_3CO); ^{13}C , δ 170.0, 169.8, and 169.1 (C=O), 137.2, 128.4, and 128.0 (aromatic), 88.3 (C-1), 75.9 (CH_2 of Bn), 73.6, 71.1, 70.9, 67.1, and 67.0 (C-2,3,4,5,6), 20.6 and 20.5 (CH_3CO). Anal. ($\text{C}_{19}\text{H}_{23}\text{ClO}_8$) C, H, Cl.

2-(Trimethylsilyl)ethyl O-(2,3,4-Tri-O-acetyl-6-O-benzyl- β -D-galactopyranosyl)-(1 $\rightarrow$ 4)-3,6-di-O-benzyl-2-deoxy-2-phthalimido- β -D-glucopyranoside (12). To a stirred mixture of **5** (11) (10.5 g, 17.8 mmol), **11** (20.0 g, 48.2 mmol), 2,6-di-*tert*-butyl-4-methylpyridine (8 g, 38.9 mmol), and 4A molecular sieves (15 g) in CH_2Cl_2 (150 mL) was added AgOTf (25 g, 97.3 mmol), at -40 °C (18). The reaction mixture was allowed to reach 25 °C in 1 h and then was treated with aqueous NaHCO_3 . The mixture was filtered, and the solids were washed with CHCl_3 . Concentration of the organic phase afforded a syrup, which was chromatographed (3:1 hexane–EtOAc) to give amorphous **12** (13.3 g, 77%): $[\alpha]_D^{10} +10^\circ$ (c 0.8); NMR (CDCl_3) ^1H , δ 7.55–6.98 (aromatic), 5.542 (br d, 1 H, H-4_B), 5.278 (dd, 1 H, $J_{1,2} = 8.0$ Hz, $J_{2,3} = 10.4$ Hz, H-2_B), 5.268 (d, 1 H, $J_{1,2} = 8.1$ Hz, H-1_A), 5.018 (dd, 1 H, $J_{3,4} = 3.5$ Hz, H-3_B), 4.730 (d, 1 H, H-1_B), 2.154 and 2.104 (2 s, 9 H, CH_3CO), 1.04–0.81 (m, 2 H, H_2CSi), and 0.129 (s, 9 H, SiMe_3); ^{13}C , δ 170.0, 169.9, and 169.2 (C=O), 138.6–123.1 (aromatic), 100.2 (C-1_B), 97.7 (C-1_A), 74.3, 73.5, 73.3, 67.5, 66.8, and 66.7 (C-6_A,6_B, $\text{CH}_2\text{CH}_2\text{Si}$, and CH_2 of Bn), 55.7 (C-2_A), 20.7 (2C) and 20.5 (CH_3CO), 17.6 (CH_2Si), and -1.6 (SiMe_3); FABMS m/z 966 $[(M + H - \text{H}_2)^+]$, 940 $[(M + H - \text{HO}(\text{CH}_2)_2\text{SiMe}_3 + \text{HOSiMe}_3)^+]$, 850 $[(M + H - \text{HO}(\text{CH}_2)_2\text{SiMe}_3)^+]$. Anal. ($\text{C}_{52}\text{H}_{61}\text{NO}_{15}\text{Si}$) C, H, N.

2-(Trimethylsilyl)ethyl O-(6-O-Benzyl-3,4-O-isopropylidene- β -D-galactopyranosyl)-(1 $\rightarrow$ 4)-3,6-di-O-benzyl-2-deoxy-2-phthalimido- β -D-glucopyranoside (13). To a solution of **12** (13 g, 13.4 mmol) in MeOH (100 mL) was added a catalytic amount of NaOMe at 25 °C. The usual workup afforded **13** as a syrup (10.9 g, 96%): $[\alpha]_D^{47} +47^\circ$ (c 1.4); NMR (CDCl_3) ^{13}C , δ 138.5–123.1 (aromatic), 103.4 ($^1J_{\text{C-1,H-1}} = 160$ Hz, C-1_B), 97.7 ($^1J_{\text{C-1,H-1}} = 163$ Hz, C-1_A), 74.3, 73.3 (2C), 69.2, 68.2, and 66.8 (C-6_A,6_B, $\text{CH}_2\text{CH}_2\text{Si}$, and CH_2 of Bn), 55.9 (C-2_A), 17.8 (CH_2Si), and -1.6 (SiMe_3). Anal. ($\text{C}_{46}\text{H}_{55}\text{NO}_{12}\text{Si}$) C, H, N.

2-(Trimethylsilyl)ethyl O-(6-O-benzyl-3,4-O-isopropylidene- β -D-galactopyranosyl)-(1 $\rightarrow$ 4)-3,6-di-O-benzyl-2-deoxy-2-phthalimido- β -D-glucopyranoside (14). To a solution of **13** (10 g, 11.9 mmol) in 2,2-dimethoxypropane (40 mL, 325 mmol) was added a catalytic amount of 10-camphorsulfonic acid at 25 °C (19). After 2 h, the solution was treated with an excess of Et_3N . Extractive workup ($\text{CHCl}_3/\text{H}_2\text{O}$) afforded **14** as a syrup (10.8 g, 94%): $[\alpha]_D^{49} +49^\circ$ (c 1.0); NMR (CDCl_3) ^{13}C , δ 138.5–123.1 (aromatic),

109.9 (CMe₂), 102.6 ($^1J_{\text{C-1,H-1}} = 160$ Hz, C-1_B), 97.9 ($^1J_{\text{C-1,H-1}} = 163$ Hz, C-1_A), 74.6, 73.6, 73.4, 69.2, 68.2, and 66.7 (C-6_A,6_B, $\text{CH}_2\text{CH}_2\text{Si}$, and CH_2 of Bn), 55.8 (C-2_A), 28.2 and 26.3 $[(\text{CH}_3)_2\text{C}]$, 17.7 (CH_2Si), and -1.6 (SiMe_3). Anal. ($\text{C}_{49}\text{H}_{59}\text{NO}_{14}\text{Si}\cdot\text{EtOAc}$) C, H, N.

2-(Trimethylsilyl)ethyl O-(2,6-di-O-benzyl-3,4-O-isopropylidene- β -D-galactopyranosyl)-(1 $\rightarrow$ 4)-3,6-di-O-benzyl-2-deoxy-2-phthalimido- β -D-glucopyranoside (15). To a stirred solution of **14** (23 g, 26.3 mmol) in DMF (140 mL) was added NaH (2.8 g of a 60% suspension in oil, ~ 70 mmol) at 0 °C. After 30 min, the mixture was treated with benzyl bromide (10 mL, 84 mmol). The reaction mixture was stirred for 4 h at 0 °C. The usual workup followed by column chromatographic purification (4:1 hexane–EtOAc) afforded **15** as a syrup (21 g, 82%): $[\alpha]_D^{41} +41^\circ$ (c 1.4); NMR (CDCl_3) ^{13}C , δ 102.2 (C-1_B), 97.5 (C-1_A), 74.3, 73.4 (2C), 73.1, 69.1, 67.9, and 66.6 (C-6_A,6_B, $\text{CH}_2\text{CH}_2\text{Si}$, and CH_2 of Bn), 55.7 (C-2_A), 27.9 and 26.3 $[(\text{CH}_3)_2\text{C}]$, 17.7 (CH_2Si), and -1.6 (SiMe_3). Anal. ($\text{C}_{56}\text{H}_{65}\text{NO}_{12}\text{Si}$) C, H, N.

2-(Trimethylsilyl)ethyl O-(2,6-di-O-benzyl- β -D-galactopyranosyl)-(1 $\rightarrow$ 4)-3,6-di-O-benzyl-2-deoxy-2-phthalimido- β -D-glucopyranoside (16). A solution of **15** (20 g, 20.6 mmol) in 4:1 AcOH–H₂O (100 mL) was kept at 80 °C for 2 h. Evaporation of the volatiles under vacuum followed by column chromatographic purification (2:1 hexane–EtOAc) afforded **16** as a syrup (15 g, 78%): $[\alpha]_D^{33} +33^\circ$ (c 1.5); NMR (CDCl_3) ^{13}C , δ 138.7–123.1 (aromatic), 103.0 (C-1_B), 97.7 (C-1_A), 75.0, 74.3, 73.5, 73.1, 69.1, 68.0, and 66.6 (C-6_A,6_B, $\text{CH}_2\text{CH}_2\text{Si}$, and CH_2 of Bn), 55.7 (C-2_A), 17.7 (CH_2Si), and -1.6 (SiMe_3); CIMS m/z 954 $[(M + \text{Na})^+]$, 930 $[(M + H - \text{H}_2)^+]$, 904 $[(M + H - \text{HO}(\text{CH}_2)_2\text{SiMe}_3 + \text{HOSiMe}_3)^+]$, 814 $[(M + H - \text{HO}(\text{CH}_2)_2\text{SiMe}_3)^+]$. Anal. ($\text{C}_{53}\text{H}_{61}\text{NO}_{12}\text{Si}$) C, H, N.

2-(Trimethylsilyl)ethyl O-(2,3,6-Tri-O-benzyl- β -D-galactopyranosyl)-(1 $\rightarrow$ 4)-3,6-di-O-benzyl-2-deoxy-2-phthalimido- β -D-glucopyranoside (17). A stirred mixture of **16** (7.0 g, 7.5 mmol), Bu_2SnO (2.0 g, 8 mmol), and toluene (150 mL) was refluxed under a Dean-Stark adapter for 2 h, and then approximately half of the volatiles were evaporated under vacuum. Bu_4NBr (2.5 g, 7.8 mmol) and benzyl bromide (1.2 mL, 10.1 mmol) were added, and the mixture was stirred at 40 °C for 3 h. The mixture was concentrated under vacuum. Column chromatography (2:1 hexane–EtOAc) of the residue afforded **17** as a syrup (7.5 g, 98%): $[\alpha]_D^{25} +25^\circ$ (c 1.2); NMR (CDCl_3) ^{13}C , δ 133.7–123.2 (aromatic), 102.9 (C-1_B), 97.8 (C-1_A), 75.4, 74.4, 73.5, 73.0, 72.0, 68.8, 67.9, and 66.6 (C-6_A,6_B, $\text{CH}_2\text{CH}_2\text{Si}$, and CH_2 of Bn), 55.7 (C-2_A), 17.5 (CH_2Si), and -1.7 (SiMe_3); CIMS m/z 1094 $[(M + \text{SiMe}_3)^+]$, 994 $[(M + H - \text{HO}(\text{CH}_2)_2\text{SiMe}_3 + \text{HOSiMe}_3)^+]$, 904 $[(M + H - \text{HO}(\text{CH}_2)_2\text{SiMe}_3)^+]$. Anal. ($\text{C}_{60}\text{H}_{67}\text{NO}_{12}\text{Si}$) C, H, N.

Methyl 2-O-Benzyl-6-O-(tert-butyldiphenylsilyl)-3,4-O-isopropylidene-1-thio- β -D-galactopyranoside (19). To a solution of methyl 6-O-(*tert*-butyldiphenylsilyl)-3,4-O-isopropylidene-1-thio- β -D-galactopyranoside [**18**, (20)] (7.7 g, 15.8 mmol) in DMF (60 mL) was added NaH (0.7 g of a 60% suspension in oil, ~ 17.5 mmol) at 0 °C. After 30 min, the mixture was treated with benzyl bromide (3.5 mL, 29.4 mmol). The reaction mixture was stirred for 2 h at 0 °C. The usual workup followed by column chromatographic purification (8:1 hexane–EtOAc) afforded **19** as a crystalline solid (7.6 g, 83%): mp 108–110 °C; $[\alpha]_D^{-4} -4^\circ$ (c 1.1); NMR (CDCl_3) ^1H , δ 7.72–7.23 (aromatic), 4.860 and 4.747 (2 d, 2 H, $J \sim 12$ Hz, CH_2 of Bn), 4.316 (dd, 1 H, $J_{3,4} = 5.5$ Hz, $J_{4,5} = 2.0$ Hz, H-4), 4.284 (d, 1 H, H-1), 4.224 (dd, 1 H, H-3), 3.925 (m, 1 H, H-6,6*), 3.825 (m, 1 H, H-5), 4.442 (dd, 1 H, $J_{1,2} = 9.6$ Hz, H-2), 2.156 (s, 3 H, CH_3S), 1.436 and 1.358 [2 s, 6 H, $(\text{CH}_3)_2\text{C}]$, and 1.050 (s, 9 H, CMe₃); ^{13}C , δ 135.6–127.7 (aromatic), 109.8 $[(\text{CH}_3)_2\text{C}]$, 84.5 (C-1), 79.7, 78.9, 76.8, and 73.5 (C-

2,3,4,5), 73.5 (CH_2 of Bn), 62.7 (C-6), 27.9 and 26.3 [$(\text{CH}_3)_2\text{C}$], 26.7 [$\text{C}(\text{CH}_3)_3$], and 12.6 (CH_3S). Anal. ($\text{C}_{56}\text{H}_{65}\text{NO}_{12}\text{Si}$) C, H, N.

Methyl 2-O-benzyl-3,4-O-isopropylidene-1-thio- β -D-galactopyranoside (20). To a solution of compound **19** (7.0 g, 12.1 mmol) in tetrahydrofuran (50 mL) was added Bu_4NF (6.0 mL of a 1.4 M solution in tetrahydrofuran, ~ 8.4 mmol) at 25 °C. After 12 h, the solution was concentrated. Column chromatography (3:1 $\rightarrow$ 1:1 hexane–EtOAc) afforded **20** as a syrup (4.0 g, 97%): $[\alpha]_D^{+4^\circ}$ (c 1.0); NMR (CDCl_3) ^1H , δ 7.42–7.24 (aromatic), 4.850 and 4.746 (2 d, 2H, $J \sim 11$ Hz, CH_2 of Bn), 4.322 (d, 1 H, $J_{1,2} = 9.6$ Hz, H-1), 4.250 (t, 1 H, $J_{2,3} = J_{3,4} = 6$ Hz, H-3), 4.182 (dd, 1 H, $J_{4,5} = 1.9$ Hz, H-4), 3.98–3.75 (m, 3 H, H-5, 6, 6*), 3.451 (dd, 1 H, H-2), 2.190 (s, 3 H, CH_3S), 1.442 and 1.351 [2 s, 6 H, $(\text{CH}_3)_2\text{C}$]; ^{13}C , δ 137.7, 128.3, and 127.8 (aromatic), 110.1 [$\text{C}(\text{CH}_3)_2$], 84.3 (C-1), 79.6, 78.5, 76.7, and 73.9 (C-2, 3, 4, 5), 73.3 (CH_2 of Bn), 62.4 (C-6), 27.7 and 26.2 [$(\text{CH}_3)_2\text{C}$], and 12.7 (CH_3S). Anal. ($\text{C}_{17}\text{H}_{24}\text{O}_5\text{S}$) C, H, S.

Methyl 2-O-Benzyl-1-thio- β -D-galactopyranoside (21). A solution of **20** (3.8 g, 11.2 mmol) in 3:1 AcOH– H_2O (40 mL) was kept at 80 °C for 3 h and then concentrated under vacuum. Toluene (3 $\times$ 50 mL) was added to and evaporated from the residue. TLC (1:1 hexane–EtOAc) indicated the disappearance of **20** and the formation of a major and a minor product. A solution of the residue in MeOH was treated with a catalytic amount of NaOMe. After 2 h, the solution was neutralized (Dowex 50×2 , H^+) and concentrated. Trituration of the residue in hexane afforded **21** as a crystalline solid (3.2 g, 95%): mp 157–159 °C; $[\alpha]_D^{+0.4^\circ}$ (c 0.7, MeOH); NMR (CDCl_3) ^1H , δ 7.48–7.24 (aromatic), 4.864 and 4.815 (2 d, 2H, $J = 10.5$ Hz, CH_2 of Bn), 4.316 (d, 1 H, $J_{1,2} = 9.3$ Hz, H-1), 3.920 (dd, 1 H, H-4), 3.797 (dd, 1 H, $J_{5,6} = 6.6$ Hz, $J_{6,6^*} = 11.4$ Hz, H-6), 3.721 (dd, 1 H, $J_{5,6^*} = 5.3$ Hz, H-6*), 3.638 (dd, 1 H, $J_{2,3} = 9.1$ Hz, $J_{3,4} = 3.2$ Hz, H-3), 3.557 (t, 1 H, H-2), 5.51 (m, 1 H, H-5), and 2.237 (s, 3 H, CH_3S); ^{13}C , δ 137.6–127.0 (aromatic), 84.9 (C-1), 78.3, 77.8, 74.3, and 68.8 (C-2, 3, 4, 5), 74.6 (CH_2 of Bn), 60.6 (C-6), and 11.4 (CH_3S). Anal. ($\text{C}_{17}\text{H}_{24}\text{O}_5\text{S}$) C, H, S.

Methyl 3,4,6-Tri-O-acetyl-2-O-benzyl-1-thio- β -D-galactopyranoside (22). To a solution of **21** (2.9 g, 9.6 mmol) in pyridine (20 mL) was added Ac_2O (20 mL) at 0 °C. The reaction mixture was allowed to reach 20 °C in 1 h, and then the volatiles were removed under vacuum. Column chromatography (5:2 hexane–EtOAc) afforded **22** as a syrup (4.0 g, 97%): $[\alpha]_D^{+15^\circ}$ (c 1.0); NMR (CDCl_3) ^1H , δ 7.36–7.25 (aromatic), 5.403 (dd, 1 H, $J_{3,4} = 3.4$ Hz, H-4), 5.017 (dd, 1 H, $J_{2,3} = 9.5$ Hz, H-3), 4.85 and 4.62 (2 d, 2 H, $J \sim 11$ Hz, CH_2 of Bn), 4.463 (d, 1 H, $J_{1,2} = 9.7$ Hz, H-1), 4.160 (dd, 1 H, $J_{5,6} = 6.7$ Hz, $J_{6,6^*} = 11.2$ Hz, H-6), 4.085 (dd, 1 H, $J_{5,6^*} = 6.6$ Hz, H-6*), 3.871 (br t, 1 H, H-5), 3.675 (t, 1 H, H-2), 2.266, 2.131, 2.036, and 1.946 (4 s, 12 H, 3 CH_3CO and CH_3S); ^{13}C , δ 170.5, 170.2, and 170.0 (C=O), 137.7–127.9 (aromatic), 85.8 (C-1), 75.6, 74.0, 73.9, 67.6 (C-2, 3, 4, 5), 75.4 (CH_2 of Bn), 61.4 (C-6), 20.5 (CH_3CO), and 12.9 (CH_3S); CIMS m/z 444 [(M + NH_3) $^+$], 379 [(M + H- CH_3SH) $^+$]. Anal. ($\text{C}_{20}\text{H}_{26}\text{O}_8\text{S}$) C, H, S.

2-(Trimethylsilyl)ethyl O-(3,4,6-Tri-O-acetyl-2-O-benzyl- α -D-galactopyranosyl)-(1 $\rightarrow$ 4)-O-(2,3,6-tri-O-benzyl- β -D-galactopyranosyl)-(1 $\rightarrow$ 4)-3,6-di-O-benzyl-2-deoxy-2-phthalimido- β -D-glucopyranoside (23). To a stirred mixture of disaccharide **17** (7.0 g, 6.85 mmol), thioglycoside **22** (6.1 g, 14.3 mmol), 2,6-di-*tert*-butyl-4-methylpyridine (3 g, 14.6 mmol), and 4A molecular sieves (3 g) in diethyl ether (100 mL) at 25 °C was added MeOTf (4 mL, 35 mmol) over a period of 96 h. The usual workup, followed by column chromatographic purification (3:1 hexane–EtOAc),

afforded **23** as an amorphous solid (6.9 g, 73%): $[\alpha]_D^{+48^\circ}$ (c 0.4); NMR (CDCl_3) ^1H , 7.8–6.9 (aromatic), 5.381 (dd, 1 H, $J_{3,4} = 3.3$ Hz, $J_{4,5} = 1.3$ Hz, H-4 $_C$), 5.258 (dd, 1 H, $J_{1,2} = 3.2$ Hz, $J_{2,3} = 10.1$ Hz, H-3 $_C$), 5.053 (d, 1 H, H-1 $_C$), 3.770 (dd, 1 H, H-2 $_C$), 1.988, 1.848, and 1.851 (3 s, 9 H, 3 CH_3CO), and -0.177 [s, 9 H, (SiMe $_3$)]; ^{13}C , δ 179.9 and 169.6 (C=O), 139.2–127.9 (aromatic), 102.6, 99.3, and 97.8 (C-1 $_A$, 1 $_B$, 1 $_C$), 80.3, 79.4, 78.1, 77.1, 75.1, 74.1, 73.8, 72.3, 70.0, 68.4, and 66.0 (C-2 $_B$, 2 $_C$, 3 $_A$, 3 $_B$, 3 $_C$, 4 $_A$, 4 $_B$, 4 $_C$, 5 $_A$, 5 $_B$, 5 $_C$), 75.2, 74.7, 73.5, 73.0, 72.9, 72.6, 68.0, 66.8, and 66.5 (C-6 $_B$, 6 $_C$, 6 CH_2 of Bn, $\text{CH}_2\text{CH}_2\text{Si}$), 60.9 (C-6 $_A$), 55.8 (C-2 $_A$), 20.6 and 20.5 (CH_3CO), 17.8 (CH_2Si), and -1.6 (SiMe $_3$); FABMS m/z 1398 [(M + H – H $_2$) $^+$]. Anal. ($\text{C}_{79}\text{H}_{89}\text{NO}_{20}\text{Si}$) C, H, N.

2-(Trimethylsilyl)ethyl O-(3,4,6-Tri-O-acetyl- α -D-galactopyranosyl)-(1 $\rightarrow$ 4)-O-(β -D-galactopyranosyl)-(1 $\rightarrow$ 4)-2-deoxy-2-phthalimido- β -D-glucopyranoside (24). A mixture of **23** (6.8 g, 5 mmol), 10% Pd–C (1.5 g), EtOH (50 mL), and AcOH (5 mL) was stirred under hydrogen at 25 °C at 345 kPa for 4 days. Removal of the catalyst by filtration and the volatiles under vacuum afforded amorphous **24** (3.94 g, 92%): $[\alpha]_D^{+47^\circ}$ (c 1); NMR (CDCl_3) ^1H , 7.78–7.70 (aromatic), 5.413 (br d, 1H, H-4 $_C$), 5.175 (dd, 1H, H-3 $_C$), 5.235 (d, 1H, $J_{1,2} = 8$, 7 Hz, H-1 $_A$), 5.068 (d, 1H, $J_{1,2} = 3.7$ Hz, H-1 $_C$), 4.567 (d, 1H, $J_{1,2} = 8.9$ Hz, H-1 $_B$), 2.119, 2.058, and 1.993 (3 s, 9 H, 3 CH_3CO), and -0.150 (s, 9 H, SiMe $_3$); ^{13}C , δ 171.4, 171.2, and 170.4 (C=O of Ac), 168.6 (C=O of Phth), 134.3 and 131.7 (aromatic), 103.9 and 100.8 (C-1 $_B$, 1 $_C$), 97.8 (C-1 $_A$), 80.6, 78.4, 74.9, 74.6, 73.0, 71.6, 70.2 (2 $_C$), 70.1, 68.3, 67.3, 67.2, and 66.9 (C-2 $_B$, 2 $_C$, 3 $_A$, 3 $_B$, 3 $_C$, 4 $_A$, 4 $_B$, 4 $_C$, 5 $_A$, 5 $_B$, 5 $_C$, and $\text{CH}_2\text{CH}_2\text{Si}$), 62.0, 61.2, and 60.7 (C-6 $_A$, 6 $_B$, 6 $_C$), 56.4 (C-2 $_A$), 20.7, 20.6, and 20.5 (CH_3CO), 17.7 (CH_2Si), and -1.7 (SiMe $_3$); FABMS m/z 882 [(M + Na) $^+$], 840 [(M + H – HO(CH $_2$) $_2$ SiMe $_3$ + HOSiMe $_3$) $^+$], 742 [(M + H – HO(CH $_2$) $_2$ SiMe $_3$) $^+$]. Anal. ($\text{C}_{37}\text{H}_{53}\text{NO}_{20}\text{Si}$) C, H, N.

2-(Trimethylsilyl)ethyl O-(2,3,4,6-Tetra-O-acetyl- α -D-galactopyranosyl)-(1 $\rightarrow$ 4)-O-(2,3,6-tri-O-acetyl- β -D-galactopyranosyl)-(1 $\rightarrow$ 4)-3,6-di-O-acetyl-2-deoxy-2-phthalimido- β -D-glucopyranoside (25). A solution of **24** (3.7 g, 4.3 mmol), 4-(dimethylamino)pyridine (100 mg, 0.8 mmol), pyridine (10 mL), and Ac_2O (10 mL) was kept at 25 °C for 12 h. Removal of the volatiles at 30 °C under vacuum followed by column chromatographic purification (2:1 hexane–EtOAc) afforded amorphous **25** (4.5 g, 94%): $[\alpha]_D^{+52^\circ}$ (c 0.6); NMR (CDCl_3) ^1H 7.89–7.73 (aromatic), 5.727 (dd, 1 H, $J_{2,3} = 10$ Hz, $J_{3,4} = 7.8$ Hz, H-3 $_A$), 5.564 (br d, 1 H, H-4 $_C$), 5.398 (d, 1 H, $J_{1,2} = 8.4$ Hz, H-1 $_A$), 5.377 (dd, 1 H, $J_{2,3} = 11$ Hz, $J_{3,4} = 3.2$ Hz, H-3 $_C$), 5.173 (dd, 1 H, $J_{1,2} = 3.5$ Hz, H-2 $_C$), 5.130 (dd, 1 H, $J_{1,2} = 7.7$ Hz, $J_{2,3} = 10.7$ Hz, H-2 $_B$), 4.997 (d, 1 H, $J_{1,2} = 3.5$ Hz, H-1 $_C$), 4.800 (dd, 1 H, $J_{3,4} = 2.4$ Hz, H-3 $_B$), 4.614 (d, 1 H, H-1 $_B$), 4.185 (H-2 $_A$), 2.155, 2.138, 2.083, 2.070, 2.056, 1.964, and 1.949 (7 s, 27 H, 9 CH_3CO), and -0.127 (s, 9 H, SiMe $_3$); ^{13}C , δ 171–169.2 (C=O of Ac), 134.3–123.3 (aromatic), 100.8, 99.3, and 97.2 (C-1 $_A$, 1 $_B$, 1 $_C$), 76.9, 76.7, 72.6, 72.3, 71.5, 71.4, 68.9, 68.6, 67.8, 67.1, 66.9, and 66.8 (C-2 $_B$, 2 $_C$, 3 $_A$, 3 $_B$, 3 $_C$, 4 $_A$, 4 $_B$, 4 $_C$, 5 $_A$, 5 $_B$, 5 $_C$, and $\text{CH}_2\text{CH}_2\text{Si}$), 62.3, 61.1, and 60.1 (C-6 $_A$, 6 $_B$, 6 $_C$), 54.7 (C-2 $_A$), 20.4–20.0 (CH_3CO), 17.3 (CH_2Si), and -2.1 (SiMe $_3$); FABMS m/z 1134 [(M + Na) $^+$], 1084 [(M + H – HO(CH $_2$) $_2$ SiMe $_3$ + HOSiMe $_3$) $^+$], 1052 [(M + H – AcOH) $^+$], 994 [(M + H – HO(CH $_2$) $_2$ SiMe $_3$) $^+$], 934 [(994 – AcOH) $^+$]. Anal. ($\text{C}_{49}\text{H}_{65}\text{NO}_{26}\text{Si}$) C, H, N.

O-(2,3,4,6-Tetra-O-acetyl- α -D-galactopyranosyl)-(1 $\rightarrow$ 4)-O-(2,3,6-tri-O-acetyl- β -D-galactopyranosyl)-(1 $\rightarrow$ 4)-3,6-di-O-acetyl-2-deoxy-2-phthalimido- β -D-glucopyranose (26). A mixture of **25** (5.0 g, 4.5 mmol), CHCl_3 (15 mL), and CF_3COOH (15 mL) was kept at 25 °C for 4 h, and then the volatiles were removed under vacuum at 25 °C. Tritu-

ration of the residue in hexane afforded **26** as an amorphous solid (3.5 g 77%): $[\alpha]_D^{+85^\circ}$ (c 0.63); NMR (CDCl_3) ^1H , 7.85–7.70 (aromatic), 5.757 (m, 1 H, $J_{2,3} = 10.5$ Hz, H-3_A), 5.664 (d, 1 H, $J_{1,2} = 8.4$ Hz, H-1_A), 5.565 (dd, 1 H, H-4_C), 5.367 (dd, 1 H, $J_{2,3} = 11$ Hz, $J_{3,4} = 3.3$ Hz, H-3_C), 5.157 (dd, 1 H, $J_{1,2} = 3.5$ Hz, H-2_C), 5.116 (dd, 1 H, $J_{1,2} = 7.6$ Hz, $J_{2,3} = 10.8$ Hz, H-2_B), 4.975 (d, 1 H, $J_{1,2} = 3.5$ Hz, H-1_C), 4.753 (dd, 1 H, $J_{3,4} = 2.5$ Hz, H-3_B), 4.588 (d, 1 H, H-1_B), 4.16 (H-2_A), 2.129, 2.116, 2.061, 2.060, 2.055, 2.053, 2.031, 1.948, and 1.941 (9 s, 27 H, 9 CH_3CO); ^{13}C , δ 170.65, 170.61, 170.5, 170.4, 170.0, 169.7, 169.5, 168.9, 167.8 (C=O of Ac), 167.8 (C=O of Phth), 134.3–123.5 (aromatic), 100.9 and 99.5 (C-1_B, 1_C), 92.3 (C-1_A), 76.9, 76.7, 72.7 (2C), 71.6, 71.3, 69.0, 68.8, 67.9, and 67.0 (2C) (C-2_B, 2_C, 3_A, 3_B, 3_C, 4_A, 4_B, 4_C, 5_A, 5_B, 5_C), 62.4, 61.2, and 60.2 (C-6_A, 6_B, 6_C), 56.2 (C-2_A), and 20.9–20.4 (CH_3CO); FABMS m/z 1050 [(M + K)⁺], 1034 [(M + Na)⁺], 1012 [(M + H)⁺], 1010 [(M + H – H₂)⁺]. Anal. ($\text{C}_{44}\text{H}_{53}\text{NO}_{26}$) C, H, N.

O-(2,3,4,6-Tetra-*O*-acetyl- α -D-galactopyranosyl)-(1 $\rightarrow$ 4)-*O*-(2,3,6-tri-*O*-acetyl- β -D-galactopyranosyl)-(1 $\rightarrow$ 4)-3,6-di-*O*-acetyl-2-deoxy-2-phthalimido- β -D-glucopyranosyl Trichloroacetimidate (**27**). To a stirred solution of **26** (2.38 g, 2.4 mmol) in CH_2Cl_2 (20 mL) were added at -30°C CCl_3CN (4.0 mL, 40 mmol) and 1,8-diazabicyclo[5.4.0]undec-7-ene (600 μL , 4 mmol) (21, 22). The solution was stirred at -20°C for 1 h and then was allowed to reach $\sim 20^\circ\text{C}$ in 2 h. The reaction mixture was applied to a column of silica gel suspended in hexane. Elution with hexane $\rightarrow$ hexane–EtOAc (1:2) followed by crystallization from diisopropyl ether–hexane gave **27** (2.22 g, 82%): mp 154°C dec; $[\alpha]_D^{+59^\circ}$ (c 0.5); NMR (CDCl_3) ^1H , 6.643 (d, 1 H, $J_{1,2} = 8.9$ Hz, H-1_A), 5.868 (dd, 1 H, $J_{2,3} = 10.5$ Hz, H-3_A), 5.577 (dd, 1 H, H-4_C), 5.380 (dd, 1 H, $J_{2,3} = 11$ Hz, $J_{3,4} = 3.3$ Hz, H-3_C), 5.170 (dd, 1 H, $J_{1,2} = 3.5$ Hz, H-2_C), 5.134 (dd, 1 H, $J_{1,2} = 7.7$ Hz, $J_{2,3} = 10.7$ Hz, H-2_B), 4.980 (d, 1 H, $J_{1,2} = 3.6$ Hz, H-1_C), 4.744 (dd, 1 H, $J_{3,4} = 2.5$ Hz, H-3_B), 4.578 (d, 1 H, H-1_B), 2.153–1.953 (8 s, 27 H, 9 CH_3CO); ^{13}C , δ 170.5–167.4 (C=O), 134.3, 131.2, and 123.5 (aromatic), 100.8 and 99.5 (C-1_B, 1_C), 93.5 (C-1_A), 76.9, 76.3, 73.5, 72.7, 71.8, 71.0, 68.9, 68.8, 67.9, 67.2, and 67.1 (C-2_B, 2_C, 3_A, 3_B, 3_C, 4_A, 4_B, 4_C, 5_A, 5_B, 5_C), 62.0, 61.4, and 60.3 (C-6_A, 6_B, 6_C), 53.9 (C-2_A), and 21.0–20.5 (CH_3CO); FABMS m/z 994 [(M + H-C₂H₂Cl₃NO)⁺], 934 [994 – AcOH]⁺. Anal. ($\text{C}_{46}\text{H}_{53}\text{Cl}_3\text{N}_2\text{O}_{26}$) C, H, N, Cl.

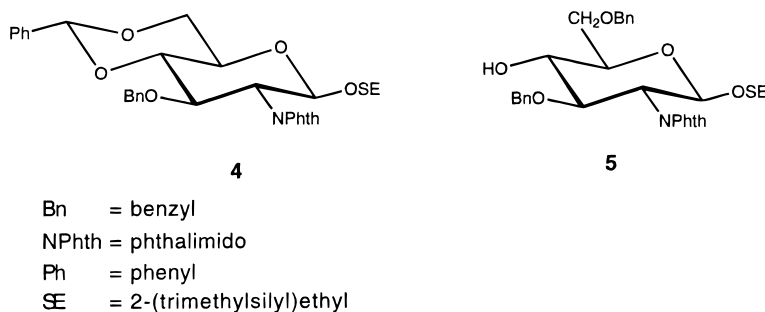
8-(Benzyloxy)octyl *O*-(2,3,4,6-Tetra-*O*-acetyl- α -D-galactopyranosyl)-(1 $\rightarrow$ 4)-*O*-(2,3,6-tri-*O*-acetyl- β -D-galactopyranosyl)-(1 $\rightarrow$ 4)-3,6-di-*O*-acetyl-2-deoxy-2-phthalimido- β -D-glucopyranoside (**28**). To a stirred mixture of **27** (2.05 g, 1.8 mmol), 8-(benzyloxy)octanol (10) (1.1 g, 4.7 mmol), and 4A molecular sieves (2 g) in CH_2Cl_2 (20 mL) was added $\text{BF}_3\cdot\text{Et}_2\text{O}$ (100 μL , 0.8 mmol) at -30°C . The mixture was allowed to reach 25°C in 1 h. Removal of the solids by filtration followed by extractive workup (CHCl_3 /aqueous NaHCO_3) and concentration under vacuum afforded a syrup which was crystallized from EtOH–diisopropyl ether to give **28** (1.75 g, 82%): mp 143 – 145°C ; $[\alpha]_D^{+49^\circ}$ (c 0.4); NMR (CDCl_3) ^1H , 5.737 (m, 1 H, $J_{2,3} = 10.5$ Hz, $J_{3,4} = 7.7$ Hz, H-3_A), 5.573 (br d, 1 H, H-4_C), 5.365 (dd, 1 H, $J_{2,3} = 11$ Hz, $J_{3,4} = 3.4$ Hz, H-3_C), 5.356 (d, 1 H, $J_{1,2} = 8.4$ Hz, H-1_A), 5.160 (dd, 1 H, $J_{1,2} = 3.5$ Hz, H-2_C), 5.116 (dd, 1 H, $J_{1,2} = 7.8$ Hz, $J_{2,3} = 10.6$ Hz, H-2_B), 4.971 (d, 1 H, $J_{1,2} = 3.4$ Hz, H-1_C), 4.735 (dd, 1 H, $J_{3,4} = 2.4$ Hz, H-3_B), 4.566 (d, 1 H, H-1_B), 3.991 (br d, 1 H, H-4_B), 2.134–1.944 (8 s, 27 H, 9 CH_3CO); ^{13}C , δ 170.6–168.8 (C=O of Ac), 167.7 (C=O of Phth), 138.6–123.4 (aromatic), 101.0, 99.6, and 97.9 (C-1_A, 1_B, 1_C), 76.6, 76.2, 72.83, 72.5, 71.7 (2C), 69.0, 68.8, 67.9, and 67.1 (2C) (C-2_B, 2_C, 3_A, 3_B, 3_C, 4_A, 4_B, 4_C, 5_A, 5_B, 5_C), 72.77 (CH_2 of Bn), 70.4 and 70.1 (C-1', 8'), 62.5, 61.2, and 60.2 (C-6_A, 6_B, 6_C),

55.0 (C-2_A), 29.7, 29.3 (2C), 29.1, 26.0, and 25.7 (C-2', 3', 4', 5', 6', 7'), and 20.9–20.5 (CH_3CO); FABMS m/z 1252 [(M + Na)⁺], 1228 [(M + H – H₂)⁺], 934 [(M + H – AcOH – HO(CH₂)₈OBn)⁺]. Anal. ($\text{C}_{59}\text{H}_{75}\text{NO}_{29}$) C, H, N.

8-(Benzyloxy)octyl *O*-(2,3,4,6-Tetra-*O*-acetyl- α -D-galactopyranosyl)-(1 $\rightarrow$ 4)-*O*-(2,3,6-tri-*O*-acetyl- β -D-galactopyranosyl)-(1 $\rightarrow$ 4)-2-acetamido-3,6-di-*O*-acetyl-2-deoxy- β -D-glucopyranoside (**29**). A solution of **28** (1.76 g, 1.5 mmol) and hydrazine hydrate (1.8 g, 56 mmol) in EtOH was stirred under reflux for 2 days. More hydrazine hydrate (0.65 g) was added, and stirring under reflux was continued for an additional day. The volatiles were removed under the vacuum of a water aspirator. The residual syrup was dissolved in pyridine (15 mL, 185 mmol) to which Ac_2O (15 mL, 160 mmol) and a catalytic amount of 4-(dimethylamino)pyridine were added. After 2 days, the solution was concentrated. Extractive workup ($\text{CHCl}_3/\text{H}_2\text{O}$) followed by chromatographic purification (4:1 EtOAc–hexane) afforded a syrup which was homogeneous by TLC when visualized by charring with H_2SO_4 , but contained additional compounds as detected by I_2 vapor. A solution of this syrup in MeOH was treated with a catalytic amount of NaOMe at 25°C for 24 h. The solution was neutralized (Dowex 50 $\times$ 2, H⁺) and concentrated. Column chromatographic purification of the residue (2:1 EtOAc–MeOH) afforded a syrup which was dissolved in pyridine (10 mL, 124 mmol) to which Ac_2O (10 mL, 106 mmol) and a catalytic amount of 4-(dimethylamino)pyridine were added. The usual workup followed by chromatographic purification (4:1 EtOAc–hexane) afforded **29** as a syrup (1.07 g, 63%): $[\alpha]_D^{+30^\circ}$ (c 0.2); NMR (CDCl_3) ^1H , 5.645 (d, 1 H, $J_{\text{H-2,NH}} = 9.4$ Hz, HN), 5.575 (br d, 1 H, H-4_C), 5.378 (dd, 1 H, $J_{2,3} = 11$ Hz, $J_{3,4} = 3.4$ Hz, H-3_C), 5.187 (dd, 1 H, $J_{1,2} = 3.6$ Hz, H-2_C), 5.115 (dd, 1 H, $J_{1,2} = 7.8$ Hz, $J_{2,3} = 10.8$ Hz, H-2_B), 5.080 (d, 1 H, $J_{2,3} + J_{3,4} = 18$ Hz, H-3_A), 4.989 (d, 1 H, $J_{1,2} = 3.5$ Hz, H-1_C), 4.754 (dd, 1 H, $J_{3,4} = 2.5$ Hz, H-3_B), 4.538 (d, 1 H, H-1_B), 4.418 (d, 1 H, H-1_A), and 2.129–1.948 (10 s, 30 H, 10 CH_3CO); ^{13}C , δ 170.5–169.0 (C=O), 128.2–127.4 (aromatic), 101.0 (2C) and 99.6 (C-1_A, 1_B, 1_C), 76.9, 75.9, 72.6 (2C), 72.5, 71.8, 68.9, 68.7, 67.8, and 67.1 (2C) (C-2_B, 2_C, 3_A, 3_B, 3_C, 4_A, 4_B, 4_C, 5_A, 5_B, 5_C), 72.8 (CH_2 of Bn), 70.4 and 69.6 (C-1', 8'), 62.5, 61.3, and 60.3 (C-6_A, 6_B, 6_C), 53.3 (C-2_A), 29.7, 29.4 (2C), 29.2, 26.1, and 25.8 (C-2', 3', 4', 5', 6', 7'), 23.2 (CH_3CON), and 20.9–20.6 (CH_3COO); FABMS m/z 1142 [(M + H)⁺], 906 [(M + H – HO(CH₂)₈OBn)⁺], 846 [(906 – AcOH)⁺]. Anal. ($\text{C}_{53}\text{H}_{75}\text{NO}_{26}$) C, H, N.

8-Hydroxyoctyl *O*-(2,3,4,6-Tetra-*O*-acetyl- α -D-galactopyranosyl)-(1 $\rightarrow$ 4)-*O*-(2,3,6-tri-*O*-acetyl- β -D-galactopyranosyl)-(1 $\rightarrow$ 4)-2-acetamido-3,6-di-*O*-acetyl-2-deoxy- β -D-glucopyranoside (**30**). A mixture of **29** (430 mg, 0.38 mmol), 10% Pd–C (100 mg), EtOH (10 mL), and AcOH (2 mL) was stirred under H_2 at 25°C at 345 kPa for 1 day. Removal of the catalyst by filtration and the volatiles under vacuum afforded amorphous **30** (380 mg, 96%): $[\alpha]_D^{+45^\circ}$ (c 0.2); NMR (CDCl_3) ^1H , 5.983 (d, 1 H, $J_{\text{H-2,NH}} = 9.3$ Hz, HN), 5.574 (br d, 1 H, H-4_C), 5.382 (dd, 1 H, $J_{2,3} = 11$ Hz, $J_{3,4} = 3.3$ Hz, H-3_C), 5.186 (dd, 1 H, $J_{1,2} = 3.3$ Hz, H-2_C), 5.115 (dd, 1 H, $J_{1,2} = 7.8$ Hz, $J_{2,3} = 10.8$ Hz, H-2_B), 5.058 (dd, 1 H, $J_{2,3} + J_{3,4} = 18$ Hz, H-3_A), 4.987 (d, 1 H, H-1_C), 4.751 (dd, 1 H, $J_{3,4} = 2.5$ Hz, H-3_B), 4.537 (d, 1 H, H-1_B), 4.404 (d, 1 H, H-1_A), 2.129–1.948 (9 s, 30 H, 10 CH_3CO); ^{13}C , δ 171.0–169.2 (C=O), 101.1, 101.0, and 99.7 (C-1_A, 1_B, 1_C), 76.9, 76.0, 72.7, 72.6, 72.5, 71.9, 68.9, 68.7, 67.8, 67.1, and 67.0 (C-2_B, 2_C, 3_A, 3_B, 3_C, 4_A, 4_B, 4_C, 5_A, 5_B, 5_C), 69.3 (C-1'), 62.8, 62.4, 61.3, and 60.2 (C-6_A, 6_B, 6_C, 8'), 53.2 (C-2_A), 32.4, 28.9, 28.8, 28.6, 25.2, and 25.1 (C-2', 3', 4', 5', 6', 7'), 23.1 (CH_3CON), and 20.8–20.5 (CH_3COO); FABMS m/z 1052 [(M + H)⁺], 906 [(M + H

Chart 2



– (CH₂)₈(OH)₂]⁺, 846 [(906 – AcOH)⁺]. Anal. (C₄₆H₆₉NO₂₆) C, H, N.

8,8-Dimethoxyoctyl O-(2,3,4,6-Tetra-O-acetyl- α -D-galactopyranosyl)-(1 $\rightarrow$ 4)-O-(2,3,6-tri-O-acetyl- β -D-galactopyranosyl)-(1 $\rightarrow$ 4)-2-acetamido-3,6-di-O-acetyl-2-deoxy- β -D-glucopyranoside (31**).** (CH₃)₂SO (70 μ L, 1 mmol) was added through a microsyringe to a stirred solution of (COCl)₂ (58 μ L, 0.66 mmol) in CH₂Cl₂ at –50 °C over a period of 1 min (23). To this solution was added a solution of **30** (240 mg, 0.23 mmol) in CH₂Cl₂ (2 mL) through a syringe. The vial containing **30** was rinsed with CH₂Cl₂ (2 $\times$ 0.5 mL), and the washing liquids were transferred to the reaction mixture. Stirring was continued for 10 min at –25 to –30 °C, and then the reaction mixture was allowed to reach –10 °C and was treated with *N*-ethyl-diisopropylamine (300 μ L, 1.72 mmol). The solution was concentrated. The residue was dissolved in 2,2-dimethoxypropane (3 mL, 24.4 mmol). To this solution was added a catalytic amount of *p*-toluenesulfonic acid monohydrate at 5 °C. After 15 min, the solution was treated with Et₃N (1 mL, 7.2 mmol) and the mixture was concentrated under vacuum. The residue was equilibrated between CHCl₃ and H₂O. The organic layer was concentrated. Column chromatographic purification of the residue (9:1 EtOAc–hexane) gave **31** (165 mg, 66%) as a syrup: [α]_D +28° (c 0.6); NMR (CDCl₃) ¹H, 5.577 (br d, 1 H, H-4_C), 5.383 (dd, 1 H, *J*_{2,3} = 11 Hz, *J*_{3,4} = 3.3 Hz, H-3_C), 5.188 (dd, 1 H, *J*_{1,2} = 3.6 Hz, H-2_C), 5.119 (dd, 1 H, *J*_{1,2} = 7.8 Hz, *J*_{2,3} = 10.8 Hz, H-2_B), 5.084 (dd, 1 H, *J*_{2,3} + *J*_{3,4} = 18 Hz, H-3_A), 4.990 (d, 1 H, H-1_C), 4.752 (dd, 1 H, *J*_{3,4} = 2.7 Hz, H-3_B), 4.535 (d, 1 H, H-1_B), 2.132–1.958 (9 s, 30 H, 10 CH₃CO); ¹³C, δ 170.6–169.0 (C=O), 104.5 (C-8'), 101.0 (2C) and 99.7 (C-1_A, 1_B, 1_C), 76.0, 72.7 (2C), 72.6, 71.9, 69.7, 68.9, 68.7, 68.1, 67.9, 67.13, and 67.09 (C-1', 2_B, 2_C, 3_A, 3_B, 3_C, 4_A, 4_B, 4_C, 5_A, 5_B, 5_C), 62.6, 61.4, and 60.3 (C-6_A, 6_B, 6_C), 53.3 (C-2_A), 32.5, 29.4 (2C), 29.2, 25.7, and 24.5 (C-2', 3', 4', 5', 6', 7'), 23.3 (CH₃CON), and 20.9–20.7 (CH₃COO); FAB/MS *m/z* 1094 [(M + H – H₂)⁺], 1064 [(M + H – CH₃OH)⁺]. Anal. (C₄₈H₇₁NO₂₇) C, H.

7-Formylheptyl O- α -D-Galactopyranosyl-(1 $\rightarrow$ 4)-O- β -D-galactopyranosyl-(1 $\rightarrow$ 4)-2-acetamido-2-deoxy- β -D-glucopyranoside (32**).** A solution of **31** (180 mg, 0.165 mmol) in MeOH (10 mL) was treated with NaOMe as described for **13**. After 12 h the solution was carefully neutralized (Dowex 50 $\times$ 2 H⁺) and then concentrated under vacuum. The solution of the residue in H₂O was applied to a Sep Pak 18 cartridge (24), which was eluted with a gradient of H₂O–MeOH until the concentration of MeOH reached 20%. The fractions giving a positive phenol–sulfuric acid reaction (26) were combined and concentrated to a volume of ~20 mL. To this solution was added at 25 °C AcOH (200 μ L, 3.5 mmol). After 24 h, the solution was freeze-dried to give amorphous **32** (95 mg, 86%): [α]_D +24° (c 0.4, H₂O); NMR (D₂O) ¹H, 9.669 (br t, H-8'), 5.019 (t, *J* = 5.8 Hz, H-8'), 4.946 (d, 1 H, *J*_{1,2} = 4.0 Hz, H-1_C), 2.525 (dt, *J*_{7',8'} = 1.6 Hz, *J*_{6',7'} = 7 Hz, H-7'), 2.03 (CH₃–

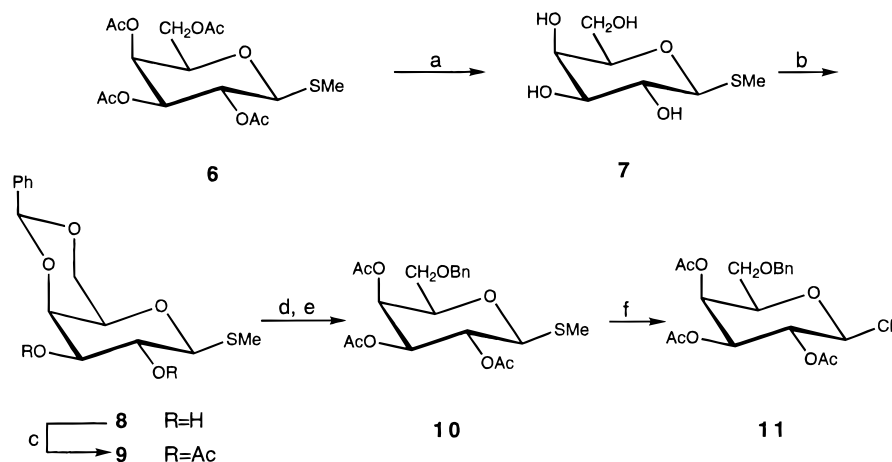
CON); ¹³C, δ 210.2 (C-8'), 175.0 (C=O of Ac), 104.0, 101.7, and 101.0 (C-1_A, 1_B, 1_C), 91.9 (C-8'), 79.6, 78.0, 76.2, 75.5, 73.2, 72.9, 71.7, 71.6, 69.9, 69.7, and 69.3 (C-2_B, 2_C, 3_A, 3_B, 3_C, 4_A, 4_B, 4_C, 5_A, 5_B, 5_C), 71.2 (C-1'), 61.3, 61.1, and 60.8 (C-6_A, 6_B, 6_C), 56.0 (C-2_A), 43.9 (C-7'), 37.7 (C-7'), 29.3, 29.0, and 28.8 (C-2', 3', 4'), 25.73 (C-6'), 25.64 (C-6'), 24.7 (C-5'), 23.0 (CH₃CON), and 22.0 (C-5'); CIMS *m/z* 672 [(M + H)⁺].

Coupling of **32** to Polyacrylhydrazinoagarose. Approximately 10 mL (bed volume) of a commercial polyacrylhydrazino-agarose gel (Sigma, cat. no. P9282) was washed with 50 mM phosphate buffer, pH 7.5 (3 $\times$ 15 mL) and then was mixed with a solution of **32** (20 mg, 0.03 mmol) in 15 mL of the same buffer. The mixture was gently stirred at 25 °C for 5 h and then was treated with NaCNBH₃ (22 mg, 0.35 mmol) (25). Stirring was continued for 48 h. The gel was transferred to a plastic column (~10 $\times$ 1 cm) which was subsequently eluted with H₂O (50 mL). Phenol–sulfuric acid assay (26) of the combined washings indicated that ~15 mg of **32** is bound to the solid matrix.

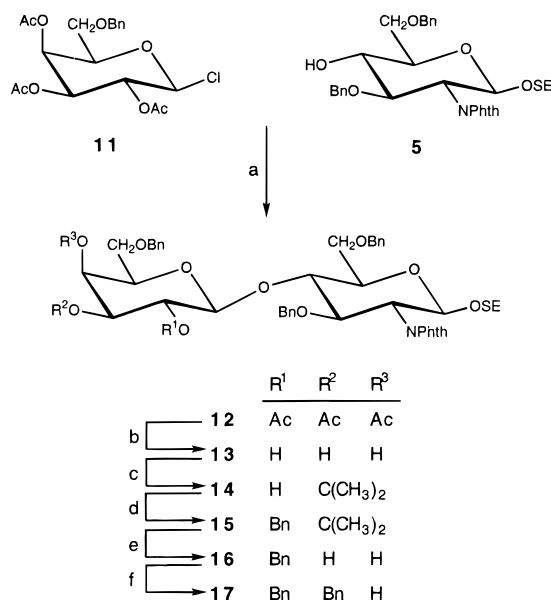
Production and Isolation of Subunit B of Shiga Toxin. Fermentation of *V. cholerae* (Strain 0395-N1, pSBC32), containing the gene of subunit B of Shiga toxin but lacking in the gene of subunit A, was carried out in a computer-controlled 10.0 L Magnaferm fermenter (New Brunswick Scientific, Edison, NJ) as described previously (9). After an overnight growth, the culture was harvested with an air-driven, continuous flow centrifuge (Alfa Laval Sharples, Warminster, PA). The cell pellet was washed once with PBS to remove residual medium and treated with polymyxin B to 2 mg/mL final concentration. The suspension was incubated at 4 °C for 20 min and centrifuged at 13200*g* for 20 min. Supernatant was collected as periplasmic material (360 mL) and stored at –70 °C. The total protein content of the periplasmic extract was 2.63 g. Twenty mL of the periplasmic material was loaded on a column of the affinity sorbent (2.6 $\times$ 10 cm) at 2 mL/min flow rate. The column was washed with 130 mL of PBS and then eluted with 3.5 M guanidine·HCl. In preliminary experiments, fractions containing subunit B were identified by their reaction with the anti-subunit B antibody. These fractions were subsequently identified by photometry at 280 nm. The fractions containing subunit B of Shiga toxin were pooled and dialyzed in a 3500 D molecular weight cutoff tubing against several changes of 4 L PBS for 2 days. Protein assay (27) indicated that 10 mg of subunit B was recovered in each run. The combined yield of pure subunit B from one fermentation was 175 mg.

RESULTS AND DISCUSSION

Stepwise syntheses of the P₁ trisaccharide (28) and its propyl glycoside have been described by the groups of Anderson and Sinaÿ (28, 29). Using block-synthetic approaches, preparation of spacer-linked P₁ trisaccharide

Scheme 1^a

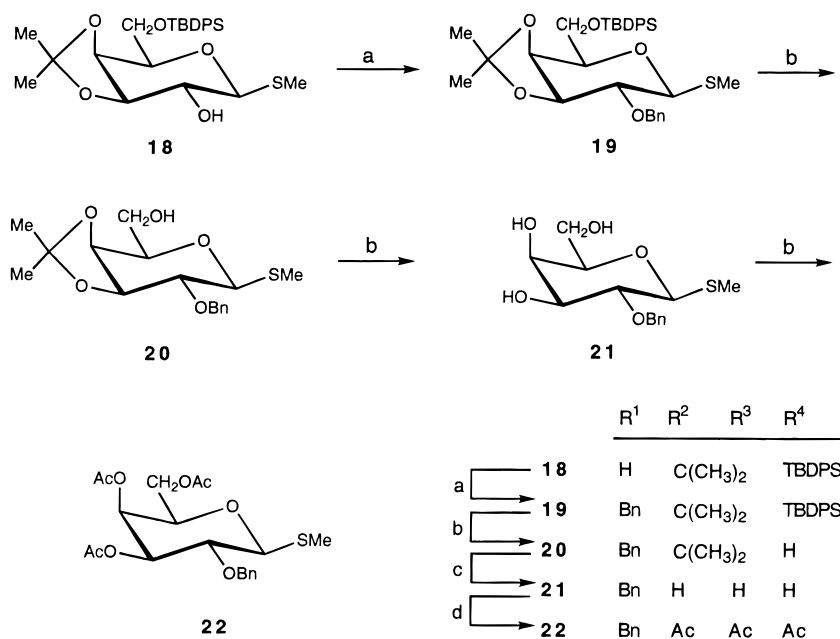
^a Abbreviations, reagents and conditions: Ac = acetyl, Bn = benzyl, Me = methyl, Ph = phenyl; (a) MeOH, cat NaOMe, 23 °C, 2 h, 92%; (b) C₆H₅CH(OMe)₂ (excess), DMF, CSA, 23 °C, 12 h, 76%; (c) Ac₂O, C₅H₅N, 0 °C, 3 h, 85; (d) NaCNBH₃, HCl, Et₂O, 0 °C; (e) Ac₂O, C₅H₅N, 25 °C, 12 h, 74% for two steps; (f) Cl₂, CH₂Cl₂, 0 °C, 20 min, 88%.

Scheme 2^a

^a Abbreviations, reagents, and conditions: Ac = acetyl, Bn = benzyl, SE = 2-(trimethylsilyl)ethyl; Phth = phthalyl; (a) 2.7 equiv of **11**, 2.2 equiv of 2,6-di-*tert*-butyl-4-methylpyridine, 5.5 equiv of CF₃SO₂OAg, CH₂Cl₂, 4A molecular sieves, -40 → +25 °C, 1 h 77%; (b) MeOH, cat. NaOMe, 25 °C, 96%; (c) Me₂C(OMe)₂ (excess), CSA, 25 °C, 2 h, 94%; (d) 3.2 equiv of Bn-Br, DMF, NaH, 0 °C, 82%; (e) AcOH-H₂O, 80 °C, 2 h, 78%; (f) 1.05 equiv of Bu₂SnO, PhMe, reflux 2 h, then 1.04 equiv of Bu₄NBr 1.35 equiv of Bn-Br, 40 °C, 3 h, 98%.

derivatives has also been reported (30, 31), which enables the covalent attachment of the P₁ trisaccharide to proteins (31). Our approach targeted the glycotriosyl donor **27** as the key intermediate which can be linked to a heterobifunctional aglycon suitable for covalent coupling to macromolecular matrices and also can be used as a building block for the synthesis of extended oligosaccharides. Retrosynthetic analysis indicated that the 2-(trimethylsilyl)ethyl (SE) glycoside **25** can be a suitable precursor to the glycotriosyl imidate **27**, according to a well-documented sequence (32) involving acid-catalyzed removal of the SE group (33) followed by base-catalyzed reaction with CCl₃CN (21, 22). SE glycosides are particularly suitable for temporary protection of the anomeric hydroxyl group since the glycosidic linkage of such compounds is stable in glycosylation reactions and under numerous transformations commonly used in protecting

group manipulations and can be selectively cleaved under conditions that leave most protecting groups and the interglycosidic linkages intact (33). Exceptions to this were recently reported by Pozsgay and Pannell (32) and Zhiyuan and Magnusson (34), who observed that the interglycosidic linkage of deoxysugar residues is unstable under attempted, direct conversion of oligosaccharide SE glycosides into glycosyl chlorides. Since the target in this study contains no such residue, the SE glycoside-based approach was followed. The starting material was the known alcohol **5** (**11**) obtained from the benzylidene derivative **4** (**11**) (Chart 1). The precursor to the interchain galactose synthon could be advantageous to prevent side reactions at a later conversion in the synthetic sequence. The benzyl group was selected for this purpose. Thus, **6** was deacetylated [→ **7** (13)] and then benzylidened according to Evans (14) (C₆H₅CH(OMe)₂/ H⁺/DMF) to afford **8** (**12**) in 76% yield, which was acetylated (Ac₂O/ C₅H₅N, → **9**) (Scheme 1). Reductive opening of the benzylidene ring according to Garegg *et al.* (16) (NaCNBH₃/ H⁺/THF) followed by acetylation afforded compound **10** in 74% yield. Chlorinolysis of the thioglycoside **10** with a solution of Cl₂ in CCl₄ (17) afforded the β chloride **11** (*J*_{H-1,H-2} = 8.7 Hz) as a crystalline compound. Condensation of the β-galactosyl donor **11** with the nucleophile **5** using silver trifluoromethanesulfonate as promoter in the presence of the non-nucleophilic base 2,6-di-*tert*-butyl-4-methylpyridine (18) gave the disaccharide **12** in 77% yield (Scheme 2). Compound **12** was converted to the triol **13** by treatment with sodium methoxide in MeOH (96%). Subsequent reaction with 2,2-dimethoxypropane under catalysis by camphorsulfonic acid (19) afforded the 3,4-*O*-isopropylidene derivative **14** in 94% yield. Such high yields of 3,4-isopropylidene derivatives of galactopyranosides cannot usually be achieved without previous protection of the HO-6 group due to side reactions that lead to 4,6-*O*-isopropylidene and 6-*O*-(1-methoxy-1-methylethyl) derivatives (13c, 19, 35). After the completion of this work, Nishida and Thiem (36) reported that reaction of β-galactosides with acetone and

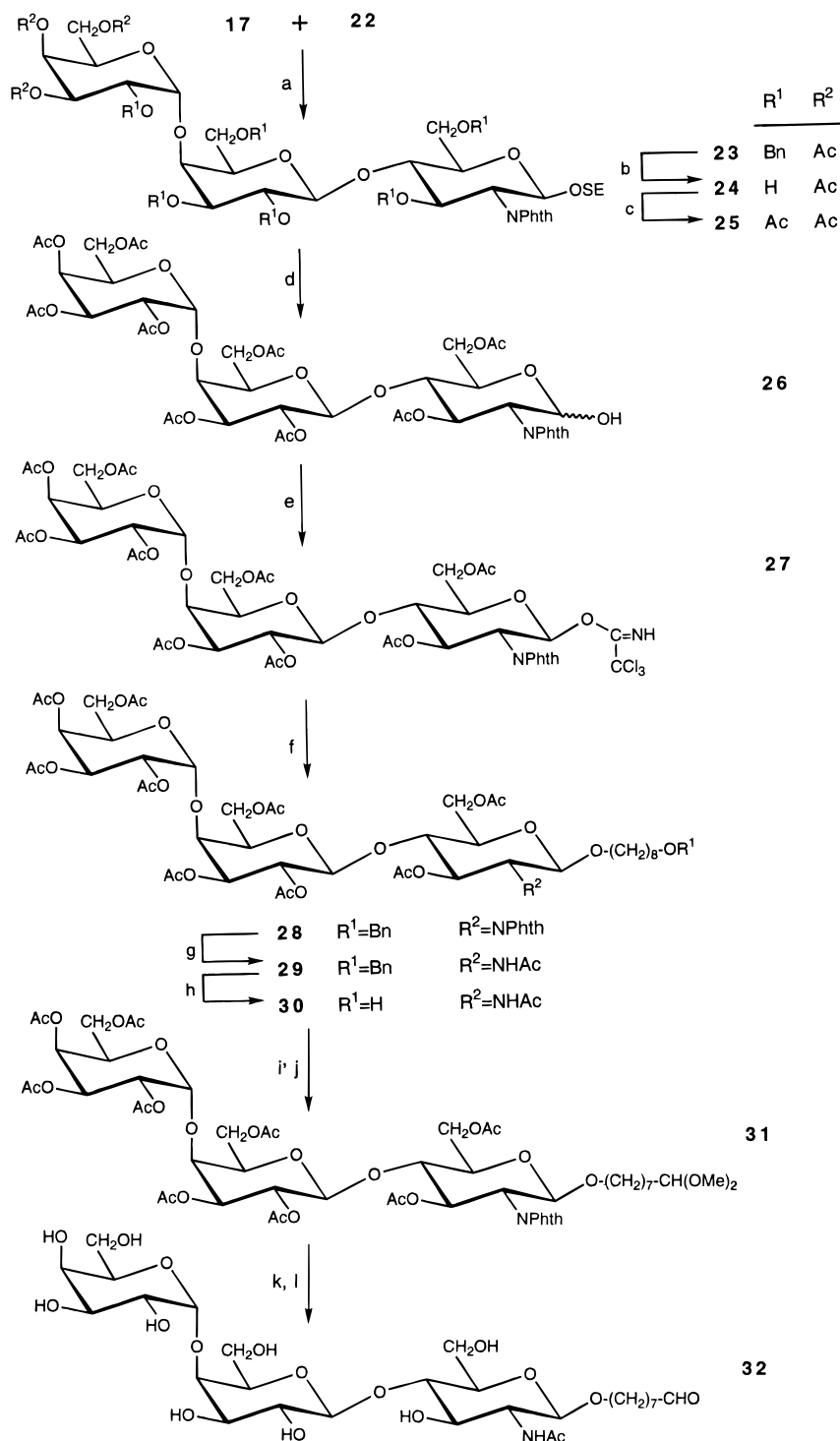
Scheme 3^a

^a Abbreviations, reagents, and conditions: Ac = acetyl, Bn = benzyl, Me = methyl, TBDPS = *tert*-butyldiphenylsilyl; (a) 2.7 equiv of 11, 2.2 equiv of 2,6-di-*tert*-butyl-4-methylpyridine, 5.5 equiv of CF₃SO₂OAg, CH₂Cl₂, 4A molecular sieves, -40 → +25 °C, 1 h, 77%; (b) MeOH, cat. NaOMe, 25 °C, 96%; (c) Me₂C(OMe)₂ (excess), CSA, 25 °C, 2 h, 94%; (d) 3.2 equiv of Bn-Br, DMF, NaH, 0 °C, 82%; (e) AcOH-H₂O, 80 °C, 2 h, 78%; (f) 1.05 equiv of Bu₂SnO, PhMe, reflux 2 h, then 1.04 equiv of Bu₄NBr 1.35 equiv of Bn-Br, 40 °C, 3 h, 98%.

chlorotrimethylsilane (37) leads exclusively to 3,4-*O*-isopropylidene derivatives. Their discovery may make the multistep process that was necessary to avoid side reactions at the HO-6 hydroxyl group during isopropylideneation unnecessary in future work. Conventional benzylation of **14** (BnBr/NaH/DMF → **15**) followed by acid hydrolysis afforded the diol **16** which was regioselectively benzylation at HO-3' according to David *et al.* (38) (Bu₂SnO/BnBr/Bu₄NBr/toluene) to afford the acceptor **17** for coupling with the nonreducing end terminal galactose moiety. 1-Thiogalactoside **22** was selected as the synthetic equivalent for the nonreducing terminal unit in which the *O*-benzyl group next to the anomeric center was expected to facilitate the 1,2-*cis* coupling of this moiety to the disaccharide **17**. Precursor to **22** was the known alcohol **18** (20), which was benzylation at *O*-2 (BnBr/NaH/DMF) to provide **19** in 83% yield (Scheme 3). Sequential removal of the silyl group by Bu₄NF in THF (39) (→ **20**, 97%) and the isopropylidene group by acidolysis afforded the triol **21** in 95% yield. Conventional acetylation of **21** (Ac₂O/C₅H₅N) afforded the triacetate **22** in 97% yield. Condensation of the 1-thiogalactoside donor **22** with the disaccharide acceptor **17** under promotion by methyl trifluoromethanesulfonate according to Lönn (40), in the presence of the hindered base 2,6-di-*tert*-butyl-4-methylpyridine, afforded the trisaccharide **23** in 73% yield (Scheme 4). The benzyl groups in **23** were replaced by acetyl groups [(i) H₂/Pd-C → **24**; (ii) Ac₂O/C₅H₅N → **25**] to make the protecting group scenario compatible with the subsequent transformations. Sequential treatment of **25** with CF₃CO₂H (11) (→ **26**, 77%) and then with trichloroacetonitrile and 1,8-diazabicyclo[5.4.0]undec-1-ene (21, 22) furnished the key trisaccharide donor **27** in 82% yield. Condensation of compound **27** with 8-(benzyloxy)octanol (10) in the presence of BF₃·Et₂O afforded the glycoside **28** in 82% yield. The aglycon employed was used previously as a spacer for covalent attachment of mono- and disaccharides to macromolecular matrices (10). Replacement of the phthalyl group in **28** by acetyl [(i) NH₂NH₂/EtOH; (ii) Ac₂O/C₅H₅N) afforded compound **29** in 63% yield. Lib-

eration of the *ω*-hydroxyl group of the aglycon by hydrolytic removal of the *O*-benzyl protecting group furnished the alcohol **30**. Swern-oxidation [Me₂SO, (COCl)₂] (23) of **30** to an aldehyde that was treated *in situ* with 2,2-dimethoxypropane afforded the dimethyl acetal **31** in 66% yield. The *O*-acetyl groups in **31** were removed by treatment with NaOMe in MeOH. Purification of the intermediate so obtained through a Sep Pak 18 cartridge (24) followed by exposure to mild acid afforded the spacer-equipped P₁ trisaccharide **32** in 86% yield. The purity and the structure of **32** and those of the intermediates described in this work were verified by ¹H and ¹³C NMR spectroscopy at 300 and 75.5 MHz, respectively, and by chemical ionization and fast atom bombardment mass spectroscopy that gave data consistent with the proposed structures (Experimental Procedures). Thus, the presence of the formyl group in **32** is indicated by the resonance at 9.669 ppm in its ¹H NMR spectrum in D₂O (HC=O), which is corroborated by the corresponding signal in the ¹³C NMR spectrum at 175 ppm (HC=O). The hydrated form is also present as indicated by a triplet at 5.019 ppm in the ¹H NMR spectrum [CH(OH)₂] and by a signal at 91.9 ppm in the ¹³C NMR spectrum [CH(OH)₂]. Compound **32** is therefore suitable for covalent attachment to proteins and other, amino group-containing matrices by reductive amination (25). Indeed, coupling of compound **32** with commercial polyacrylhydrazinoagarose in the presence of NaCNBH₃ afforded the P₁ trisaccharide-containing affinity sorbent. Carbohydrate analysis (26) indicated that approximately 75% of the trisaccharide was bound under the conditions reported in the Experimental Procedures.

Subunit B of Shiga toxin was produced by fermentation of a nonvirulent *V. cholerae* strain (Strain 0395-N1) containing the gene of subunit B but lacking in the gene of subunit A as described previously (9). An aliquot of the periplasmic extract containing ~150 mg of protein was loaded directly on the affinity column. This was the maximum amount that could be applied while maintaining complete adsorption of subunit B. Subsequent washing

Scheme 4^a

^a Abbreviations, reagents, and conditions: Bn = benzyl, SE = 2-(trimethylsilyl)ethyl; Phth = phthalyl; (a) 2.1 equiv of **22**, 2.1 equiv of 2,6-di-*tert*-butyl-4-methylpyridine, 5.1 equiv of CF₃SO₂OMe, Et₂O, 4A molecular sieves, +25 °C, 96 h 73%; (b) H₂/Pd-C, EtOH, AcOH, 23 °C, 16 h 92%; (c) Ac₂O, C₅H₅N 25 °C, 12 h, 94%; (d) CF₃CO₂H, CH₂Cl₂, 25 °C, 4 h, 77%; (e) CCl₃CN, 1,8-diazabicyclo[5.4.0]undec-7-ene, CH₂Cl₂, -20 → +20 °C, 2 h, 82%; (f) 2.6 equiv of C₆H₅CH₂OH, cat. BF₃·Et₂O, CH₂Cl₂, -30 → +25 °C, 1 h, 82%; (g) 57 equiv of NH₂NH₂, EtOH, reflux, 48 h, Ac₂O, C₅H₅N, 25 °C, 63%; (h) H₂/Pd-C, EtOH, AcOH, 23 °C, 24 h 96%; (i) 4.3 equiv of Me₂SO, 2.9 equiv of (COCl)₂, CH₂Cl₂, -25 °C, 10 min; (j) Me₂C(OMe)₂, PTS, 5 °C, 66% for two steps; (k) MeOH, cat. NaOMe, 25 °C, 12 h; (l) MeOH, AcOH, H₂O, 24 h, 86% for two steps.

with PBS eluted the accompanying proteins (Figure 1). Our initial attempts to elute subunit B with 1 M galactose in saline, 1.7 M NaCl, or 3 M MgCl₂ were unsuccessful. On the other hand, 3.5 M guanidine·HCl (9) completely eluted subunit B from the affinity matrix. (Figure 1). Bound subunit B could also be eluted with 4.5 M MgCl₂. In preliminary experiments, the fractions containing subunit B were identified by their reaction with a monoclonal antisubunit B antibody. After the

conditions for elution were established, the process was performed in a semiautomatic mode in a Pharmacia liquid chromatograph, using photometry at 280 nm for detection. Following elution, subunit B was further purified by dialysis against PBS to give 10 mg of pure subunit B of shiga toxin in each run. The affinity sorbent was used for over 20 cycles with unchanged capacity for subunit B. The identity and purity of the subunit B so prepared was assessed by SDS-PAGE electrophoresis

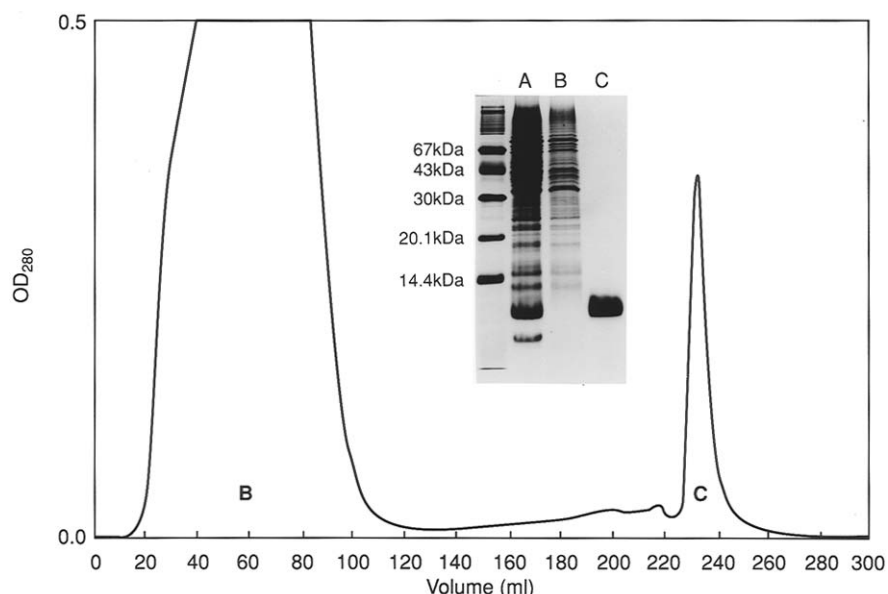


Figure 1. Elution of the proteins in the periplasmic extract from the P_1 trisaccharide-hydrazinoagarose column. The periplasmic extract (20 mL) was loaded on the affinity column (2.6×10 cm) equilibrated with PBS buffer. The column was washed with 150 mL of PBS and then was eluted with 3.5 M guanidine-HCl. Fraction B: proteins eluted from the column with the buffer. Fraction C: subunit B of Shiga toxin. Inset shows the sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the proteins in the periplasmic extract. Left lane: molecular weight markers. Lane A: periplasmic extract. Lane B: proteins eluted from the column with the buffer. Lane C: subunit B of Shiga toxin eluted with 3.5 M guanidine-HCl.

(Figure 1) and its antigenicity was demonstrated by its reaction with a monoclonal antibody directed against subunit B. The monoclonal antibody has been described in ref 9.

CONCLUSION

We have described a method for the preparative-scale isolation of subunit B of Shiga toxin, using a P_1 trisaccharide-containing affinity matrix. The approach presented here is based on a chemically synthesized derivative (27) of the P_1 trisaccharide that permits its covalent attachment to a variety of linking arms. We selected an ω -aldehydealkyl spacer (10) for covalent attachment of the P_1 trisaccharide to the insoluble matrix under the mild conditions of reductive amination. The geometry of the linking arm provided a sufficient distance of the hapten from the polymeric support that can avoid possible unfavorable steric interactions between the lectin and the supportive matrix. Our protocol represents an improvement over existing methods since it obviates the need for laborious, multiple chromatographic procedures (9) and maintains the simplicity of the one-step, receptor analog affinity chromatography (8). The ability of the affinity matrix to be used repeatedly in a semiautomatic fashion makes this approach suitable for the isolation of subunit B on the 100 mg scale. Furthermore, our method avoids the reliance on affinity material isolated from biological sources (8), which may be objectionable if the subunit B so purified is intended for human or animal experiments. The pure subunit B is currently being evaluated as a possible protein component of saccharide-protein conjugate vaccines (41). This work will be the subject of a future publication.

ACKNOWLEDGMENT

We thank Dr. Lewis Pannell and Mr. Noel Whittaker for the mass spectra.

LITERATURE CITED

(1) (a) Takeda, Y. (1995) In *Bacterial Toxins and Virulence Factors in Diseases* (J. Moss, B. Iglewski, M. Vaughan, and

A. T. Tu, Eds.) pp 313–326, Marcel Dekker, Inc.: New York.
 (b) O'Brien, A. D., and Holmes, R. K. (1987) *Microbial Rev.* 51, 206–220.
 (2) Olsnes, S., Reisbing, R., and Eiklid, K. (1981) *J. Biol. Chem.* 256, 8732–8738.
 (3) Lindberg, A. A., Brown, J. E., Strömberg, N., Westling-Ryd, M., Schultz, J. E., and Karlsson, K.-A. (1987) *J. Biol. Chem.* 262, 1779–1785.
 (4) Marcus, D. M., Kundu, S. K., and Suzuki, A. (1981) *Semin. Hematol.* 18, 63–71.
 (5) Armstrong, G. D., Fodor, E., and R. Vanmaele, (1991) *J. Infect. Dis.* 164, 1160–1167.
 (6) St. Hilaire, P. M., Isbister, B. D., Boyd, M., and Toone, E. J. (1994) *Biochemistry* 33, 14452–14463.
 (7) Donohue-Rolfe, A., Jacewicz, M., and Keusch, G. T. (1989) *Mol. Microbiol.* 3, 1231–1236.
 (8) Donohue-Rolfe, A., Acheson, D. W. K., Kane, A. V., and Keusch, G. T. (1989) *Infect. Immun.* 57, 3888–3893.
 (9) Acheson, D. W. K., Calderwood, S. B., Boyko, S. A., Lincicome, L. L., Kane, A. V., Donohue-Rolfe, A., and Keusch, G. T. (1993) *Infect. Immun.* 61, 1098–1104.
 (10) Pozsgay, V. (1993) *Glycoconjugate J.* 10, 133–141.
 (11) Jansson, K., Ahlfors, S., Frejd, T., Kihlberg, J., and Magnusson, G. (1988) *J. Am. Chem. Soc.* 53, 5629–5647.
 (12) Ferrier, R. J., and Furneaux, R. H. (1980) *Methods Carbohydr. Chem.* 8, 251–253.
 (13) (a) Helferich, B., Grünwald, H., and Langenhoff, F. (1953) *Chem. Ber.* 86, 873–875. (b) Koike, K., Sugimoto, M., Sato, S., Ito, Y., Nakahara, Y., and Ogawa, T. (1987) *Carbohydr. Res.* 163, 189–208. (c) Pozsgay, V., and Jennings, H. J. (1988) *Carbohydr. Res.* 179, 61–75.
 (14) Evans, M. E. (1972) *Carbohydr. Res.* 21, 473–475.
 (15) Leontin, K., Nilsson, M., and Norberg, T. (1985) *Carbohydr. Res.* 144, 231–240.
 (16) Garegg, P. J., Hultberg, H., and Wallin, S. (1982) *Carbohydr. Res.* 108, 97–101.
 (17) Wolfrom, M. L., and Groebke, W. (1963) *J. Org. Chem.* 28, 2986–2988.
 (18) Kronzer, F. J., and Schuerch, C. (1973) *Carbohydr. Res.* 27, 379–390.
 (19) Lipták, A., Fügedi, P., Kerékgyártó, J., and Nánási, P. (1983) *Carbohydr. Res.* 113, 225–231.
 (20) Pozsgay, V., Glaudemans, C. P. J., Robbins, J. B., and Schneerson, R. (1993) *Carbohydr. Res.* 244, 259–273.
 (21) Schmidt, R. R., and Michel, J. (1980) *Angew. Chem., Int. Ed. Engl.* 19, 731–732.

- (22) Numata, M., Sugimoto, M., Koike, K., and Ogawa, T. (1987) *Carbohydr. Res.* 163, 209–225.
- (23) Mancuso, A. J., Huang, S. L., and Swern, D. (1978) *J. Org. Chem.* 43, 2480–2482.
- (24) (a) Merkelson-Watson, L. J., Kanemitsu, K., and Sweeley, C. C. (1987) *Glycoconjugate J.* 4, 7–16. (b) Lowary, T. L., Swiedler, S. J., and Hindsgaul, O. (1994) *Carbohydr. Res.* 256, 257–273.
- (25) Gray, G. R. (1974) *Arch. Biochem. Biophys.* 163, 426–428.
- (26) Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., and Smith, F. (1956) *Anal. Chem.* 28, 350–356.
- (27) Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J., and Klenk, D. C. (1985) *Anal. Biochem.* 150, 76–85.
- (28) Avram Zollo, P.-H., Jacquinet, J.-C., and Sinaï, P. (1983) *Carbohydr. Res.* 122, 201–208.
- (29) Nashed, M. A., and Anderson, L. (1983) *Carbohydr. Res.* 114, 43–52.
- (30) Dahmén, J., Frejd, T., Magnusson, G., Noori, G., and Carlström, A. (1984) *Carbohydr. Res.* 129, 63–71.
- (31) Nilsson, S., Lönn, H., and Norberg, T. (1991) *Glycoconjugate J.* 8, 9–15.
- (32) Pozsgay, V., and Pannell, L. (1994) *Carbohydr. Res.* 258, 105–122.
- (33) Magnusson, G. (1992) *Trends Glycosci. Glycotechnol.* 4, 358–367.
- (34) Zhiyuan, Z., and Magnusson, G. (1994) *Carbohydr. Res.* 262, 79–101.
- (35) (a) Barili, P. L., Berti, G., Catelani, G., Colonna, F., and Marra, A. (1986) *Tetrahedron Lett.* 27, 2307–2310. (b) Pozsgay, V., Jennings, H. J., and Kasper, D. L. (1987) *J. Carbohydr. Chem.* 6, 41–55. (c) Knapp, S., Jaramillo, C., and Freeman, B. (1994) *J. Org. Chem.* 59, 4800–4804.
- (36) Nishida, Y., and Thiem, J. (1994) *Carbohydr. Res.* 263, 295–301.
- (37) Chan, T. H., Brook, M. A., and Chaly, T. (1983) *Synthesis* 203–205.
- (38) David, S., Thieffry, A., and Veyrières, A. (1981) *J. Chem. Soc., Perkin Trans. 1* 1796–1801.
- (39) Hanessian, S., and Lavalley, P. (1975) *Can. J. Chem.* 53, 2975–2977.
- (40) Lönn, H. (1985) *Carbohydr. Res.* 139, 105–113.
- (41) Robbins, J. B., and Schneerson, R. (1990) *J. Infect. Dis.* 161, 821–832.
- BC9500711

CORRECTIONS

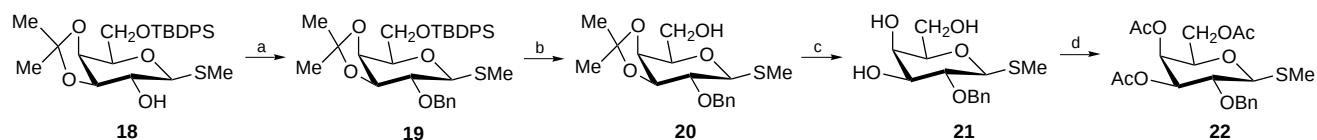
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PURIFICATION OF SUBUNIT B OF SHIGA TOXIN USING A SYNTHETIC TRISACCHARIDE-BASED AFFINITY MATRIX

Page 52. There were several errors in Scheme 3.

Scheme 3^a



^a Abbreviations, reagents, and conditions: Ac = acetyl, Bn = benzyl, Me = methyl, TBDPS = *tert*-butyldiphenylsilyl; (a) 1.9 equiv of BnBr, DMF, NaH, 0 °C, 83%; (b) 0.7 equiv of Bu₄NF, THF, 25 °C, 12 h, 97%; (c) AcOH–H₂O, 80 °C, 2 h, 95%; (d) Ac₂O, C₅H₅N, 0 °C, 1 h, 97%.

BC960192G

Synthesis, Purification, and Tumor Cell Uptake of ^{67}Ga -Deferoxamine–Folate, a Potential Radiopharmaceutical for Tumor Imaging

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The vitamin folic acid was covalently linked to the chelating agent deferoxamine (DF) via an amide bond using a simple carbodiimide coupling reaction. A mixture of two isomers, DF–folate(α) and DF–folate(γ), was produced involving the α - and γ -carboxyl group of folic acid, respectively. These two isomers were separated by anion-exchange chromatography using a NH_4HCO_3 gradient. Competitive binding studies revealed that only the DF–folate(γ) is recognized by the folate receptor on KB cells, interacting with an affinity comparable to unconjugated folic acid. The DF–folate conjugates were radiolabeled with the γ -emitting radionuclide $^{67}\text{Ga}^{3+}$ and tested for uptake by cultured KB cells overexpressing the folate receptor. The cellular accumulation of ^{67}Ga -DF–folate(γ) complex was found to be time-, temperature-, and concentration-dependent. The ^{67}Ga -DF–folate(γ) tracer exhibited rapid uptake kinetics in cell culture with a $t_{1/2}$ of ~ 3 min. The KB cell association of ^{67}Ga -DF–folate(γ) was competitively blocked by free folic acid, indicating that uptake of the ^{67}Ga -DF–folate(γ) was specifically mediated by the folate receptor. Since the folate receptor is overexpressed on the surfaces of many neoplastic cells, these results suggest that ^{67}Ga -DF–folate(γ) complex might be useful as a diagnostic agent for noninvasive imaging of folate receptor-positing tumors.

INTRODUCTION

Early detection and classification of human tumors is one of the major goals in the fight to overcome cancer. One strategy for noninvasively detecting the location and nature of neoplastic tissue involves imaging patients following administration of γ -emitting radiopharmaceuticals designed to localize specifically in the tumor mass. Consequently, substantial interest has developed in designing methods for selective delivery of radionuclides to tumors, for example, through the use of monoclonal antibodies directed to tumor-associated antigens and the use of peptides that are bound by tumor membrane receptors (Wilbur, 1992; Griffiths et al., 1992; Serafini, 1993; Fischman, 1993; Lamki, 1995).

Recent studies have identified the folate receptor as a tumor marker that is overexpressed on many cancer cell surfaces but highly restricted in most normal tissues (Rettig et al., 1988; Campbell et al., 1991; Coney et al., 1991; Weitman et al., 1992; Garin-Chesa et al., 1993; Holm et al., 1994; Franklin et al., 1994; Ross et al., 1994). For example, immunohistochemical examination of ovarian carcinomas derived from coelomic epithelium showed that 52 out of 56 cases were strongly folate-receptor-positive (Garin-Chesa et al., 1993). For breast cancers, the corresponding frequency was 11 out of 53. Other

epithelial cancers showing folate-receptor-positive immunostaining included endometrial (10/11), colorectal (6/27), lung (6/18), and renal (9/18). Additionally, 4 out of 5 brain metastases derived from epithelial cancers were found to be strongly folate-receptor-positive. This measured folate receptor overexpression frequency might have increased even further if staining for other folate receptor isoforms had been conducted (Ross et al., 1994).

The folate receptor is a glycosylphosphatidylinositol-anchored glycoprotein (Elwood, 1989; Lacey et al., 1989; Verma et al., 1992) with high affinity for the vitamin folic acid ($K_d \sim 10^{-9}$ M) (McHugh and Cheng, 1979; Kamen and Capdevila, 1986; Kane and Waxman, 1989; Luhrs et al., 1992; Matsue et al., 1992). Membrane-associated folate receptor on cultured KB cells, a human nasopharyngeal epidermal carcinoma cell line, has been shown to mediate the specific internalization of folates at physiological concentrations (McHugh and Cheng, 1979; Antony et al., 1985). We have previously observed that when folate is covalently linked to a macromolecule via its γ -carboxylate moiety, its affinity for the cell surface receptors remains essentially unaltered (Leamon and Low, 1991, 1993). This property has been successfully exploited to deliver folate-conjugated protein toxins and drug/antisense oligonucleotide-carrying liposomes into cultured tumor cells overexpressing the folate receptor (Leamon and Low, 1992; Leamon et al., 1993; Lee and Low, 1994; Wang et al., 1995). Such folate–macromolecule conjugates were shown to be internalized via a nondestructive receptor-mediated endocytic pathway and delivered into the cytoplasm of the targeted cells in a biologically active form.

In the present work, it was hypothesized that noninvasive diagnostic imaging of folate-receptor-bearing tumors might be feasible using radiolabeled folate conjugates obtained by folate derivatization of chelating agents that exhibit high affinity for γ -emitting metal radionuclides. We report here the synthesis, purification, and ^{67}Ga -labeling of a deferoxamine–folate conjugate (DF–

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¹ Abbreviations: acac, acetylacetone; DCC, dicyclohexylcarbodiimide; DEAE, (diethylamino)ethyl; DF, deferoxamine (desferrioxamine); FAB-MS, fast-atom bombardment mass spectroscopy; FDMEM, folate-deficient modified Eagle's medium; HPLC, high-pressure liquid chromatography; PBS, phosphate-buffered saline (136.9 mM NaCl, 2.68 mM KCl, 8.1 mM Na_2HPO_4 , 1.47 mM KH_2PO_4 , pH 7.4).

folate) and the specific accumulation of the ^{67}Ga -DF-folate in cultured tumor cells overexpressing the folate receptor.

EXPERIMENTAL PROCEDURES

Materials. Folic acid, deferoxamine (DF) mesylate, DEAE-trisacryl anion-exchange resin, and carboxypeptidase G_2 were purchased from Sigma (St. Louis, MO). Bicinchoninic acid (BCA) protein assay kit was obtained from Pierce (Rockford, IL). Acetonitrile (HPLC grade) and dicyclohexylcarbodiimide (DCC) were purchased from Aldrich (Milwaukee, WI). Gallium-67 chloride was purchased from Mallinckrodt Medical, Inc. (St. Louis, MO). Tissue culture products were obtained from GIBCO (Grand Island, NY), and cultured cells were received as a gift from the Purdue Cancer Center (West Lafayette, IN).

Cell Culture. KB cells, a human nasopharyngeal epidermal carcinoma cell line that greatly overexpresses the folate binding protein, and WI38 cells, a human lung fibroblast primary cell line used as a control, were cultured continuously as a monolayer at 37 °C in a humidified atmosphere containing 5% CO_2 in folate-deficient modified Eagle's medium (FDMEM) (a folate-free modified Eagle's medium supplemented with 10% (v/v) heat-inactivated fetal calf serum as the only source of folate) containing penicillin (50 units/mL), streptomycin (50 $\mu\text{g}/\text{mL}$), and 2 mM L-glutamine. The final folate concentration in the complete FDMEM is in the physiological range (~ 2 nM). Forty-eight h prior to each experiment, the cells were transferred to 35 mm culture dishes at 5×10^5 cells per dish and grown to $\sim 80\%$ confluence.

Synthesis and Purification of Folate-Deferoxamine Conjugate. One hundred mg of DF mesylate was dissolved in 3 mL of dimethyl sulfoxide containing 200 μL of pyridine. A 10-fold excess of folic acid (672 mg) dissolved in 15 mL of warm (~ 40 °C) dimethyl sulfoxide plus 5 molar equiv of DCC (157 mg) were then added. The reaction mixture was stirred at 40 °C in the dark, during which time ninhydrin assay and thin layer chromatography were used to follow the reaction progress. After the coupling was complete, the DF-folate conjugate and excess folic acid were precipitated with 200 mL of cold acetone and pelleted by centrifugation. The pellet was washed once with cold acetone, dried under vacuum, and then redissolved in 5 mL of deionized water. The pH of the solution was adjusted to 8.0 to facilitate dissolution of the solid.

The crude product contained a mixture of folate linked to DF via either its α -carboxyl or γ -carboxyl group, as well as unreacted folic acid. The two isomers of DF-folate were isolated and purified on a weak anion-exchange column. Briefly, the product mixture was loaded onto a 1.5 cm $\times$ 15 cm DEAE-trisacryl column preequilibrated in 10 mM NH_4HCO_3 buffer (pH 7.9). The column was washed with 50 mL of 10 mM NH_4HCO_3 and then eluted with a 500 mL gradient of 80–180 mM NH_4HCO_3 followed by 150 mL of 500 mM NH_4HCO_3 . Three folate-containing peaks were obtained as detected by UV absorbance at 363 nm. Each peak was collected, lyophilized, and redissolved in deionized water. The purity of each component was confirmed by reversed-phase high-pressure liquid chromatography (HPLC) with a 10 mm $\times$ 250 mm Licosorb RP-18 column (Alltech, Deerfield, IL), and the molecular weights of the conjugates were evaluated by fast-atom bombardment mass spectroscopy (FAB-MS). The characteristic pK_a values of the two DF-folate isomers were obtained by titration on a pH/ion

analyzer (Corning, Corning, NY). Carboxypeptidase G_2 analysis of the folate linker chemistry was performed as reported (Levy and Goldman, 1967; McCullough et al., 1971) using a UV-Vis spectrophotometer.

Comparison of the Abilities of the Two DF-Folate Isomers To Bind the Cell Surface Folate Receptors. The affinities of the DF-folate isomers for cell surface receptors were determined by adding different concentrations of tritium-labeled folate conjugates in 1 mL of FDMEM to cultured KB cells. The cells were incubated at 4 °C for 30 min, washed thoroughly with cold phosphate-buffered saline (PBS), and suspended in 1 mL of PBS by scraping. Cell-associated [^3H]folate conjugates were measured by liquid scintillation counting, and the cellular protein content was evaluated by the BCA protein assay. The abilities of the two DF conjugates to interact with the folate receptors were also compared using a [^3H]folic acid binding competition assay. Briefly, 100 pmol of [^3H]folic acid and 100 pmol of either DF-folate(α) or DF-folate(γ) dissolved in 0.1 mL of PBS were added to KB cells in 1 mL of FDMEM. The cells were then processed and analyzed for folate binding by the same procedures.

Preparation of the ^{67}Ga -DF-Folate. A dilute HCl solution of $^{67}\text{Ga}^{3+}$ was evaporated to dryness with heating under a stream of N_2 and the tracer reconstituted in ~ 300 μL of ethanol containing 0.002% acetylacetone (acac). The ethanolic $^{67}\text{Ga}(\text{acac})_3$ solution (3.2 mCi) was diluted with an equal volume of TRIS-buffered saline (pH 7.4) (Bates, 1978) followed by addition of 2.25×10^{-6} mol of aqueous DF-folate(γ) conjugate. Labeling was complete after the mixture stood at room temperature for 24 h. For control experiments, $^{67}\text{Ga}(\text{III})$ -citrate was prepared by evaporating a ^{67}Ga -chloride solution to dryness and reconstituting with 0.1 mL of 3% sodium citrate (pH 7.4). A portion of the resulting ^{67}Ga -citrate solution (50 μL) was mixed with 0.1 mg of deferoxamine to obtain ^{67}Ga -deferoxamine (^{67}Ga -DF), which was also employed in control experiments. The radiochemical purity of the ^{67}Ga -tracers was determined by thin layer chromatography on commercial C_{18} reversed phase plates eluted with methanol and in all cases was found to exceed 98%. The radiochromatograms were evaluated using a Berthold (Wildbad, Germany) Tracemaster 20 Automatic TLC linear analyzer. R_f values of 0.93; 0.0; 0.1; and 0.74 were obtained for ^{67}Ga -DF-folate(γ) $^{67}\text{Ga}(\text{acac})_3$, ^{67}Ga -DF, and ^{67}Ga -citrate, respectively. All experiments employing the ^{67}Ga -DF-folate(γ) tracer were performed within 1–2 days of preparation.

Uptake of ^{67}Ga -DF-Folate by Cultured KB Cells and WI38 Cells. In the time-dependent uptake study, ^{67}Ga -DF-folate(γ) prepared as described was diluted in 0.5 mL of FDMEM, and 0.15 μCi (100 pmol) was added to monolayers of KB cells grown in 35 mm tissue culture dishes. The cells were incubated at 4 or 37 °C for different lengths of time, washed with 3×1 mL of cold PBS, and then suspended in 1 mL of PBS by scraping. The amount of cell-associated radioactivity was determined using an automatic γ -counter. Cellular protein content was measured by the BCA protein assay. To determine the concentration dependence of uptake, KB cells were incubated with various amounts of the ^{67}Ga -DF-folate(γ) complex at 4 or 37 °C for 30 min. In free folate competition experiments, the same protocol was used except that different concentrations of free folic acid were also included in the incubation medium containing 200 nM DF-folate(γ) plus ^{67}Ga -DF-folate(γ). To examine cell-type selectivity of the ^{67}Ga -labeled conjugates, WI38 cells, as well as KB cells, were incubated with 100

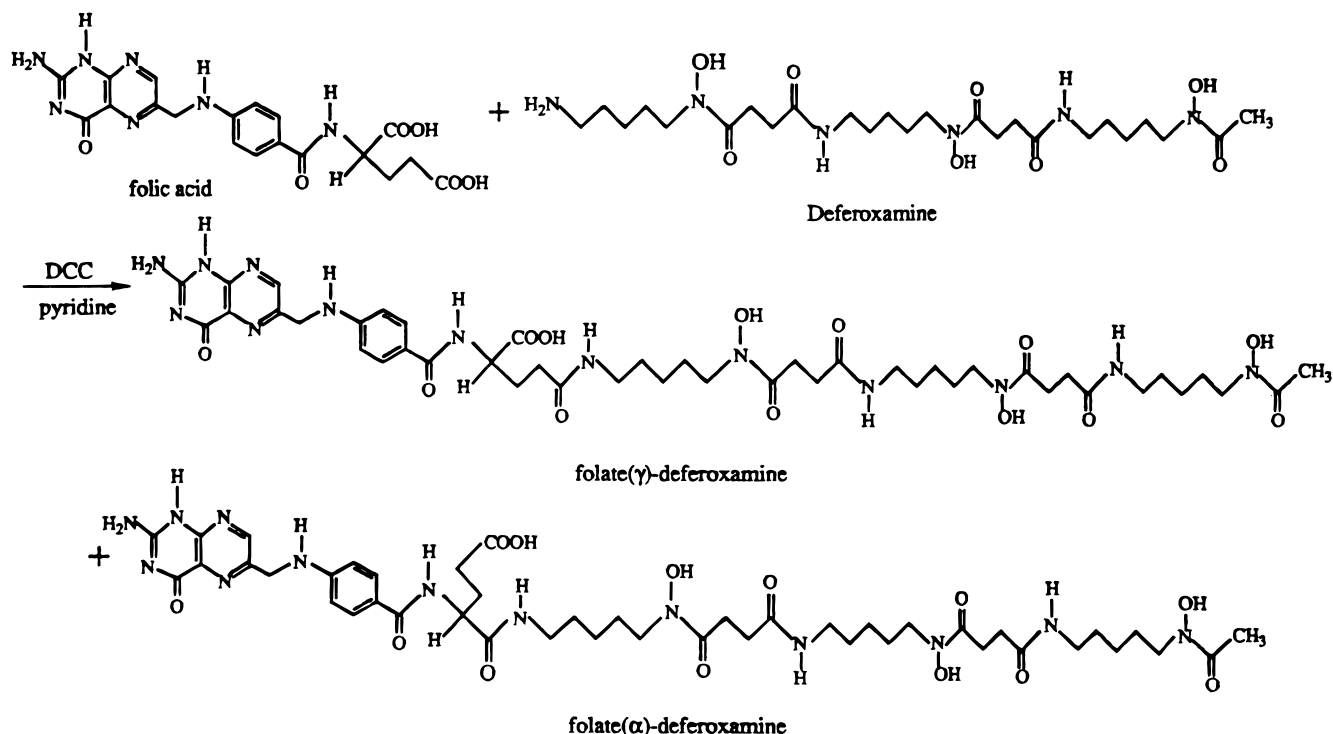


Figure 1. Reaction scheme illustrating the synthesis of the DF-folate conjugates.

pmol of various complexes in 1 mL of FDMEM at 37 °C for 30 min, and cell-associated radioactivity was evaluated similarly.

RESULTS

Synthesis and Purification of Folate-Deferoxamine Conjugate. The synthesis of folate-deferoxamine is outlined in Figure 1. The carboxyl groups of the folic acid were activated by DCC and the coupling reaction reached completion after 2 h incubation, as indicated by the disappearance of free amino groups in a ninhydrin assay and by the disappearance of DF analyzed by thin layer chromatography.

The acetone-precipitated crude product can be purified by ion-exchange chromatography. Three folate-containing peaks were collected from the DEAE-trisacryl column as shown in Figure 2. The first two peaks were identified as DF-folate conjugates, both giving a molecular weight of 984.0 in their FAB-MS spectra (Figure 3), indicating the expected 1:1 ratio between folic acid and DF. Fragmentation of the molecules at the CH₂-NH bond in folate resulted in a pteridiny radical and complementary fragment of molecular weight 809. The third peak on the elution profile had the same molecular weight as free folic acid.

Since the two isoforms of DF-folate conjugate retain either a free γ-carboxyl or free α-carboxyl, they can be distinguished from each other and from unreacted folic acid by their characteristic pK_a values, which were determined by titration (Figure 4). The DF-folate(α) conjugate (pK_a = 4.5, constituting ~20% of total DF-folate) eluted in 140–260 mL fractions (peak 1), the DF-folate(γ) conjugate (pK_a = 2.5, constituting ~80% of total DF-folate) eluted in 340 to 420 mL fractions (peak 2), and the free folic acid (pK_{a1} = 2.5, pK_{a2} = 4.5) eluted between 580 and 680 mL (peak 3). Trace amounts of the unreacted DF and the side product bis(DF)-folate were removed during the 10 mM NH₄HCO₃ wash (Figure 2, inset). The purity of the two folate conjugates was

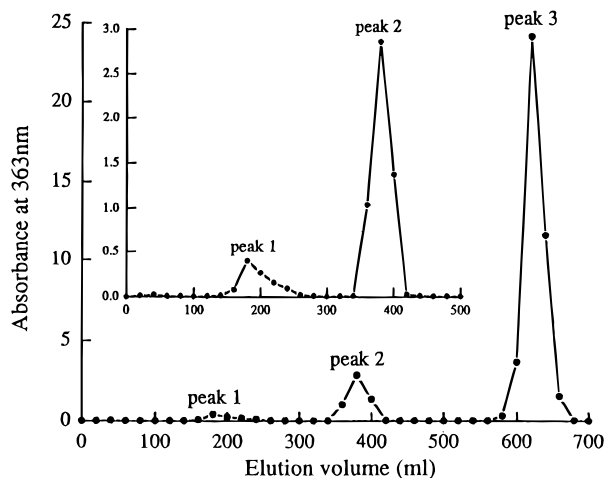


Figure 2. Purification of DF-folate by DEAE-trisacryl anion-exchange chromatography. Thirty mg of the reaction product precipitated by acetone was dissolved in 1 mL of H₂O (pH 9) and applied to the 1.5 × 15 cm column. The column was washed with 50 mL of 10 mM NH₄HCO₃, and the isomers were eluted with a gradient of 500 mL of 80–180 mM NH₄HCO₃. Unreacted folic acid was removed with 150 mL of 500 mM NH₄HCO₃. The inset contains a magnified elution profile of the first 500 mL off the column.

confirmed by reversed-phase HPLC. The α- and γ-isomers were eluted as single peaks at 7.0 and 7.8 min, respectively, with a linear gradient of 0–50% acetonitrile in 10 min.

The assignment of the structures of the two DF-folate conjugates was further confirmed by experiments with carboxypeptidase G₂, an enzyme which hydrolyzes folate compounds at the pteroyl-glutamate linkage (Levy and Goldman, 1967). It has been shown that derivatization of the α-carboxyl of folate prevents hydrolysis by carboxypeptidase G₂, but derivatization of the γ-carboxyl has little effect on enzyme activity (Fan et al., 1991). When the two isomers of DF-folate were tested as substrates (at 0.1 mM) for this enzyme (7.5 milliunits), peak 1 was inert while peak 2 was hydrolyzed at a rate of 0.029 ΔA₃₀₃

JT11090041 Scan 2 RT=0:32 100%=39219 mv 10 Nov 94 7:21
Compacted SLRP +EI SW204 NBA

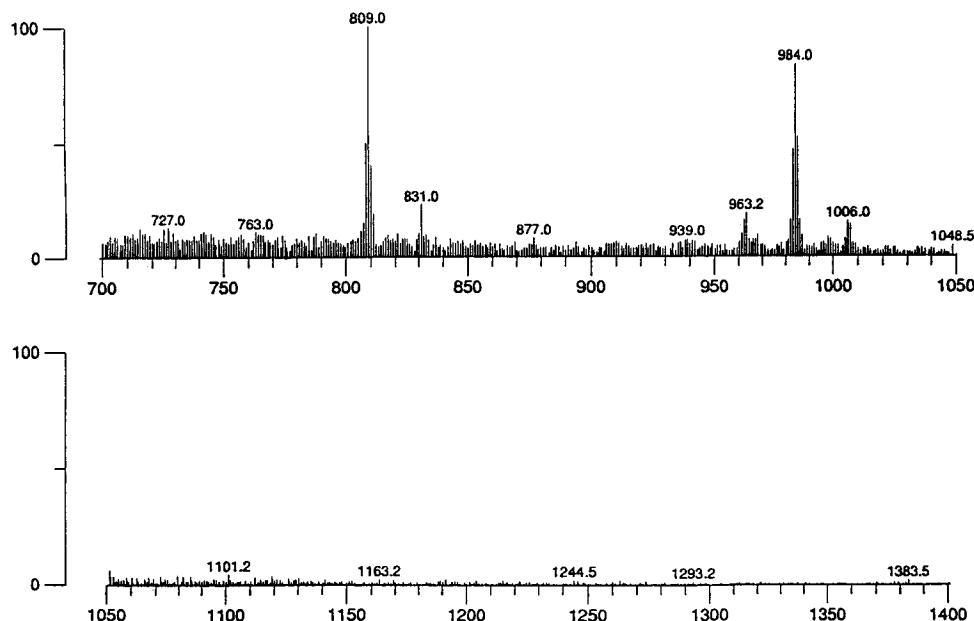


Figure 3. FAB-MS spectrum of DF-folate(γ) conjugate. The m/z peak at 984.0 corresponds to the expected molecular weight of the complex, while the peak at 809 derives from the major fragmentation product (see text for details).

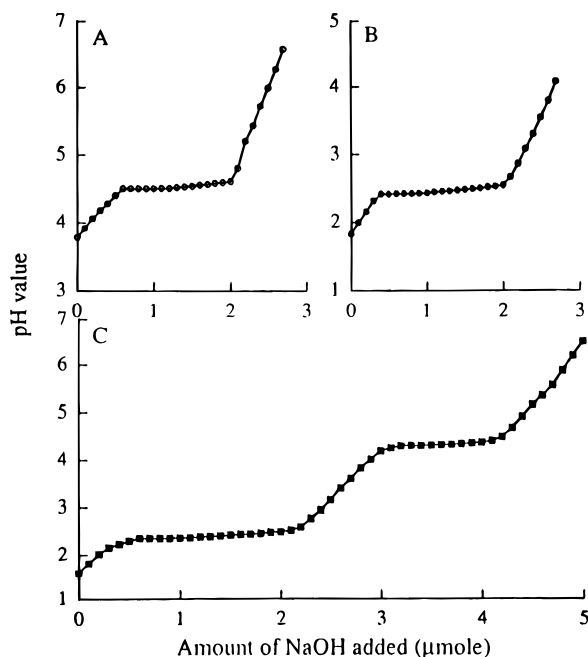


Figure 4. Determination of the pK_a values of DF-folate(α), DF-folate(γ), and free folic acid by NaOH titration. Each folate-containing peak eluted from the DEAE-trisacryl column was acidified to the indicated pH and titrated with 10 mM NaOH; (A) peak 1, $pK_a = 4.5$, identified as DF-folate(α); (B) peak 2, $pK_a = 2.5$, identified as DF-folate(γ); (C) peak 3, $pK_{a1} = 2.5$, $pK_{a2} = 4.5$, identified as free folic acid.

units/min, which is comparable to the rate for free folic acid ($0.063 \Delta A_{303}$ units/min).

Determination of the Affinities of the Two DF-Folate Isomers for Cell Surface Folate Receptors. The affinities of the DF-folate(α) and DF-folate(γ) conjugates for the KB cell folate-binding protein was both directly evaluated and also examined by a competitive binding assay using [^3H]folic acid as the receptor ligand. As shown in Figure 5, binding of DF-folate(γ) by cultured KB cells was saturable at 4 °C. Assuming a

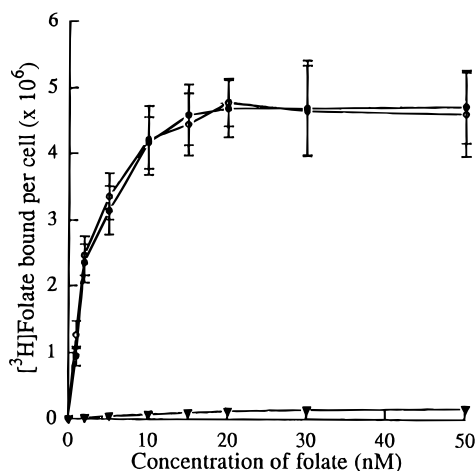


Figure 5. Determination of the affinities of the DF-folate isomers for cell surface folate receptors. KB cells were incubated with different concentrations of tritium-labeled DF-folate(α) (▼), DF-folate(γ) (●), or folic acid (○) at 4 °C for 30 min. The cells were then washed thoroughly by cold PBS and suspended in 1 mL of PBS by scraping. Cell-associated [^3H]folates was measured by liquid scintillation counting, and the cellular protein content was evaluated by the BCA protein assay. Error bars represent standard deviations of three parallel experiments.

cellular protein content of 2.0×10^{-7} mg (Lee and Low, 1994), approximately 4.8×10^6 molecules were associated with each cell when maximum binding was achieved. Half-maximal binding was found at 2.0 nM, which is similar to that of free folic acid (1.8 nM). DF-folate(α), on the other hand, exhibited almost no affinity for the cell surface folate receptors.

In competition assays, a 50% decrease in bound [^3H]folic acid was observed in the presence of an equimolar amount of the DF-folate(γ) conjugate, while the DF-folate(α) conjugate displayed virtually no ability to compete with the radiolabeled vitamin (Table 1). Importantly, competition by DF-folate(γ) was similar to that of unlabeled folic acid, again indicating that covalent

Table 1. Competitive Inhibition of [³H]Folic Acid Binding to KB Cells by α - and γ -Carboxylate DF-Folate Isomers

amt of [³ H]folic acid added (pmol)	amt of unlabeled folate compd added (pmol)	no. of bound [³ H]folic acid per cell (10 ⁶)	(%) inhibition
100	0	4.8 $\pm$ 0.4 ^a	0
100	100 (α -isomer)	4.7 $\pm$ 0.5 ^a	1.5
100	100 (γ -isomer)	2.5 $\pm$ 0.3 ^a	49
100	100 (folic acid)	2.4 $\pm$ 0.2 ^a	50

^a Data represent the average of three parallel experiments $\pm$ standard deviation. Labeled folic acid and unlabeled folate compound were added simultaneously to the KB cells.

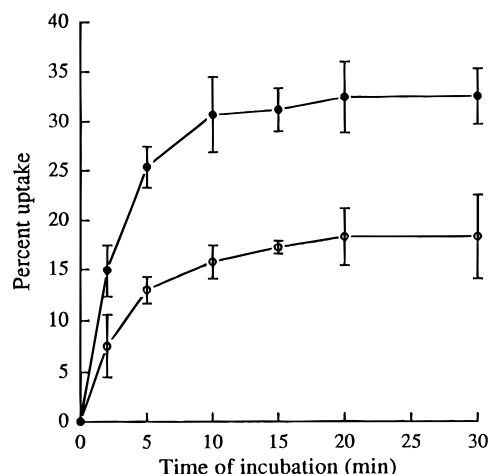


Figure 6. Kinetics of association of ⁶⁷Ga-DF-folate(γ) with cultured KB cells at 4 and 37 °C. KB cells were incubated for the indicated times with 200 nM ⁶⁷Ga-DF-folate(γ) at 4 °C (○) or at 37 °C (●). Due to the high radioactivity and short half-life of the ⁶⁷Ga-DF-folate(γ) complex, no attempt was made in this study to separate it from the nonradioactive metal-free conjugate. Thus, some competition with uncomplexed DF-folate(γ) conjugate will undoubtedly exist. After the cells were washed with 3 $\times$ 1 mL of cold PBS, the cells were suspended in 1 mL of PBS by scraping. The amount of cell-associated radioactivity was determined using a γ -counter, and cellular protein content was determined by BCA assay. Error bars represent standard deviations of three parallel experiments.

conjugation of DF to the γ -carboxyl of folic acid does not compromise the latter's high affinity for the membrane-associated folate binding protein. These data demonstrate that the cell surface folate receptor recognizes folates derivatized at the γ - but not α -carboxylate.

Uptake of ⁶⁷Ga-DF-Folate Complex by Cultured KB Cells. Because folate and its conjugates bind to cell surface receptors at 4 °C, but are capable of endocytosis only at higher temperatures (Antony et al., 1985; Leamon and Low, 1991), it is possible to separately evaluate the kinetics of binding and internalization by measuring the rates of folate conjugate uptake at the two temperatures. As shown in Figure 6, half maximal binding of ⁶⁷Ga-DF-folate(γ) was achieved in ~3 min at 4 °C, suggesting rapid association of the conjugate with unoccupied receptors. By the end of the 30 min incubation, binding approached saturation with 18% of the initial radioactivity found associated with the cell surface.

Incubation at 37 °C, a temperature which permits both binding and endocytosis, yielded similar kinetics, only maximal uptake reached 32% of the total conjugate added. Presumably, the difference in magnitude of the two cellular uptake curves reflects the ability of the folate receptor to internalize the conjugate at 37 °C and then recycle to the cell surface in its unoccupied form (Kamen et al., 1988). As controls, ⁶⁷Ga-DF lacking the folate group did not show any significant cell association (Table

Table 2. Cellular Uptake of Various ⁶⁷Ga Complexes by Cultured KB Cells and WI 38 Cells

	% uptake by each KB cell ^a	rel uptake by KB cells	% uptake by each WI38 cell ^a
⁶⁷ Ga-DF-folate(γ)	32.3 $\pm$ 2.4 ^b	100	0.22 $\pm$ 0.04 ^b
⁶⁷ Ga-DF-folate(γ) + 100 $\times$ folic acid	0.18 $\pm$ 0.05 ^b	0.56	0.15 $\pm$ 0.02 ^b
⁶⁷ Ga-DF-folate(α)	0.64 $\pm$ 0.07 ^b	2.0	0.27 $\pm$ 0.05 ^b
⁶⁷ Ga-DF-folate(α) + 100 $\times$ folic acid	0.14 $\pm$ 0.03 ^b	0.43	0.20 $\pm$ 0.02 ^b
⁶⁷ Ga-citrate	0.31 $\pm$ 0.05 ^b	0.96	0.29 $\pm$ 0.04 ^b
⁶⁷ Ga-DF	0.09 $\pm$ 0.02 ^b	0.28	0.16 $\pm$ 0.03 ^b

^a Percent uptake = (cell-associated CPM per cell/total added CPM per cell) $\times$ 100%. ^b Data represent the average of three parallel experiments $\pm$ standard deviation.

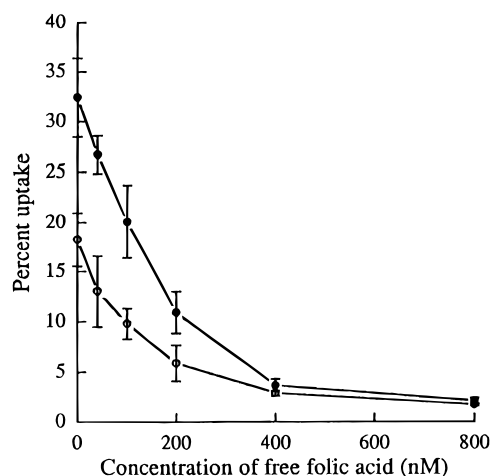


Figure 7. Competition for KB cell uptake between ⁶⁷Ga-DF-folate(γ) and folic acid. KB cells were incubated for 30 min with 200 nM ⁶⁷Ga-DF-folate(γ) as described in Figure 6 in the presence of the indicated concentrations of free folic acid at 4 °C (○) or at 37 °C (●). The cells were then processed and analyzed for complex association as described in Figure 6. Error bars represent standard deviations of three parallel experiments.

2). Also, the ⁶⁷Ga-DF-folate(α) complex was taken up with only 2% of the efficiency of ⁶⁷Ga-DF-folate(γ), probably due to the inability of the α -conjugate to be recognized by the folate receptor (Figure 5 and Table 1). When ⁶⁷Ga-citrate was added to the culture medium and incubated at 37 °C for 30 min, cell-associated ⁶⁷Ga-radioactivity was 106-fold lower than observed with ⁶⁷Ga-DF-folate(γ) (Table 2). Finally, the uptake of ⁶⁷Ga-DF-folate(γ) was observed to be cell selective, since WI38 cells, a normal cell line that does not express the folate receptor, displayed no capacity to take up the complex (Table 2).

To further verify the involvement of a cell surface folate receptor in mediating the uptake of ⁶⁷Ga-DF-folate(γ), competition between the complex and free folic acid for uptake by KB cells was examined (Figure 7). As the concentration of the unligated vitamin was increased, binding of ⁶⁷Ga-DF-folate(γ) decreased, falling to only ~10% of its initial value by 800 nM free folate. In fact, only 0.5% of initial cellular uptake was retained in the presence of 100-fold molar excess of free folate (Table 2). Taken together, these results suggest that the ⁶⁷Ga-DF-folate(γ) complex enters KB cells via the folate receptor in much the same manner as free folic acid.

DISCUSSION

Gallium-67 has been used for many years as a radiopharmaceutical for detection of certain tumors, particu-

larly broncogenic carcinoma, Hodgkin's and non-Hodgkin's lymphoma, and primary hepatomas (Hayes, 1978; Fritzberg et al., 1988; Front and Israel, 1995). In systemic circulation, $^{67}\text{Ga}^{3+}$ administered in the form of the aqueous citrate complex is rapidly bound by the plasma protein, transferrin (Harris, 1983; Green and Welch, 1989), and slowly delivered to tissue and tumor cells expressing elevated levels of transferrin receptors (Hayes, 1978; van Leeuwen-Stok et al., 1993; Sohn et al., 1993). However, the clinical use of ^{67}Ga -citrate for tumor-imaging is plagued by slow tracer clearance from non-target tissues, leading to delays in imaging, as well as poor tumor/background contrast (Hayes, 1978; Lentle, 1986). The linear trihydroxamate ligand, deferoxamine (DF), forms a highly-stable octahedral coordination complex with trivalent cations like ferric ion (Richardson and Baker, 1992) or $^{67}\text{Ga}^{3+}$ (Weiner, 1979; Yokoyama et al., 1982; Smith-Jones et al., 1994) that can resist ligand exchange with plasma transferrin. A free amino group separated by five methylene units from the closest dehydroxamate provides a site for covalent coupling of deferoxamine to targeting agents without interfering with its chelating properties. Thus, ^{67}Ga -, ^{68}Ga -, and/or ^{66}Ga -labeled deferoxamine conjugates with antibodies and peptides have been frequently used for γ scintigraphy, positron emission tomography (PET), or other radioassays of receptor-positing tumors (Yokoyama et al., 1982; Presant et al., 1988; Furukawa et al., 1991; Vera, 1992; Green, 1993; Smith-Jones et al., 1994).

In this study, we synthesized the DF-folate conjugate by a simple one-step coupling reaction. A large excess of folic acid and a controlled amount of DCC were used to avoid leaving unreacted DF in the product mixture and to also minimize the production of (DF)₂-folate. (Contamination of the conjugate with unreacted DF must be avoided, since its presence will adversely affect the efficiency of conjugate radiolabeling with ^{67}Ga and diminish the radiochemical purity of the ^{67}Ga -labeled DF-folate.) By using DEAE-trisacryl chromatography, we were able to separate unreacted DF from the α - and γ -carboxyl-linked conjugates and obtain pure DF-folate(γ) for use in tumor cell targeting.

In vitro, the cellular uptake of ^{67}Ga -citrate was less than 1/100 as efficient as uptake of the ^{67}Ga -DF-folate(γ) complex, but was nevertheless higher than that of nontargeted ^{67}Ga -DF. This uptake could be due to the transfer of ^{67}Ga from citrate to transferrin present in the serum supplement to the cell culture medium, allowing tracer to be bound to cells *via* the transferrin receptor. As noted above, this targeting mechanism has been already exploited in the clinical use of ^{67}Ga -citrate as a radiopharmaceutical for tumor imaging.

The study of KB cell uptake of ^{67}Ga -DF-folate(γ) revealed that the complex is specifically bound by the folate receptor and probably enters cells *via* folate-receptor-mediated endocytosis. The uptake kinetics are fast, reaching 50% maximal cell association in ~ 3 min. This half-time is comparable to that reported for folate-conjugated serum albumin ($M_r \sim 68\,000$), but much shorter than the 1–2 h half-life observed for folate-conjugated liposomes (Leamon and Low, 1991; Lee and Low, 1994).

Although barriers to effective tumor-specific drug delivery are more pronounced *in vivo* than in cell culture, our studies suggest that ^{67}Ga -DF-folate(γ) might be useful as a radiopharmaceutical for tumor imaging. The affinity of the conjugate is comparable to that of folic acid, i.e., 10^{-9} M, for receptor-bearing cells. Furthermore, the complex is small in size, allowing for facile diffusion to receptor-bearing cells, and the conjugate shows little

nonspecific adhesion to cell surfaces (e.g., free folate blocks cell surface binding almost quantitatively and the α -carboxyl derivative shows very low affinity for KB cells). Finally, the folate receptor is present in a large fraction of human tumors, suggesting the complex might be helpful in imaging numerous types of metastatic disease.

ACKNOWLEDGMENT

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LITERATURE CITED

- Antony, A. C., Kane, M. A., Portillo, R. M., Elwood, P. C., and Kolhouse, J. F. (1985) Study of the role of a particulate folate-binding protein in the uptake of 5-methyltetrahydrofolate. *J. Biol. Chem.* 260, 14911–14917.
- Bates, R. G., Vega, C. A., and White, D. R. (1978) Standards for pH measurements in isotonic saline media of ionic strength $I=0.16$. *Anal. Chem.* 50, 1295–1300.
- Campbell, I. G., Jones, T. A., Foulkes, W. D., and Trowsdale, J. (1991) Folate-binding protein is a marker for ovarian cancer. *Cancer Res.* 51, 5329–5338.
- Coney, L. R., Tomassetti, A., Carayannopoulos, L., Frasca, V., Kamen, B. A., Colnaghi, M. I., and Zurawski, V. R., Jr. (1991) Cloning of a tumor-associated antigen: MOv18 and MOv19 antibodies recognize a folate-binding protein. *Cancer Res.* 51, 6125–6132.
- Elwood, P. C. (1989) Molecular cloning and characterization of the human folate-binding protein cDNA from placenta and malignant tissue culture (KB) cells. *J. Biol. Chem.* 264, 14893–14901.
- Fan, J. G., Pope, L. E., Vitols, K. S., and Huennekens, F. M. (1991) Affinity labeling of folate transport proteins with the N-hydroxysuccinimide ester of the γ -isomer of fluorescein-methotrexate. *Biochemistry* 30, 4573–80.
- Fischman, A. J., Babich, J. W., and Strauss, H. W. (1993) A ticket to ride: peptide radiopharmaceuticals. *J. Nucl. Med.* 34, 2253–2263.
- Franklin, W. A., Waintrub, M., Edwards, D., Christensen, K., Prendergast, P., Woods, J., Bunn, P. A., and Kolhouse, J. F. (1994) New anti-lung-cancer antibody cluster 12 reacts with human folate receptors present on adenocarcinoma. *Int. J. Cancer* 8 (Suppl.), 89–95.
- Fritzberg, A. R., Berninger, R. W., Hadley, S. W., and Wester, D. W. (1988) Approaches to radiolabeling of antibodies for diagnosis and therapy of cancer. *Pharm. Res.* 5, 325–334.
- Front, D., and Israel, O. (1995) The role of Ga-67 scintigraphy in evaluating the results of therapy of lymphoma patients. *Semin. Nucl. Med.* 25, 60–71.
- Furukawa, T., Fujibayashi, Y., Fukunaga, M., Saga, T., Endo, K., and Yokoyama, A. (1991) An approach for immunoradiometric assay with metallic radionuclides: gallium-67–deferoxamine–dialdehyde starch–IgG. *J. Nucl. Med.* 32, 825–829.
- Garin-Chesa, P., Campbell, I., Saigo, P., Lewis, J., Old, L., and Rettig, W. (1993) Trophoblast and ovarian cancer antigen LK26. Sensitivity and specificity in immunopathology and molecular identification as a folate-binding protein. *Am. J. Pathol.* 142, 557–567.
- Green, M. A. (1993) Metal radionuclides in diagnostic imaging by positron emission tomography (PET). *Adv. Metals Med.* 1, 75–114.
- Green, M. A., and Welch, M. J. (1989) Gallium radiopharmaceutical chemistry. *Int. J. Rad. Appl. Instrum.* 16, 435–448.
- Griffiths, G. L., Goldenberg, D. M., Jones, A. L., and Hansen, H. J. (1992) Radiolabeling of monoclonal antibodies and fragments with technetium and rhenium. *Bioconjugate Chem.* 3, 91–99.
- Harris, W. R., and Pecoraro, V. L. (1983) Thermodynamic binding constants for gallium transferrin. *Biochemistry* 22, 292–299.
- Hayes, R. L. (1978) The medical use of gallium radionuclides: a brief history with some comments. *Semin. Nucl. Med.* 8, 183–192.

- Holm, J., Hansen, S. I., Hoier-Madsen, M., Sondergaard, K., and Bzorek, M. (1994) Folate receptor of human mammary adenocarcinoma. *APMIS* 102, 413–419.
- Kamen, B. A., and Capdevila, A. (1986) Receptor-mediated folate accumulation is regulated by the cellular folate content. *Proc. Natl. Acad. Sci. U.S.A.* 83, 5983–5987.
- Kamen, B. A., Wang, M. T., Streckfuss, A. J., Peryea, X., and Anderson, R. G. W. (1988) Delivery of folates to the cytoplasm of MA104 cells is mediated by a surface membrane receptor that recycles. *J. Biol. Chem.* 263, 13602–13609.
- Kane, M. A., and Waxman, S. (1989) Role of folate binding proteins in folate metabolism. *Lab. Invest.* 60, 737–746.
- Lacey, S. W., Sanders, J. M., Rothberg, K. G., Anderson, R. G. W., and Kamen, B. A. (1989) Complementary DNA for the folate binding protein correctly predicts anchoring to the membrane by glycosyl-phosphatidylinositol. *J. Clin. Invest.* 84, 715–720.
- Lamki, L. M. (1995) Tissue characterization in nuclear oncology: its time has come. *J. Nucl. Med.* 36, 207–210.
- Leamon, C. P., and Low, P. S. (1991) Delivery of macromolecules into living cells: a method that exploits folate receptor endocytosis. *Proc. Natl. Acad. Sci. U.S.A.* 88, 5572–5576.
- Leamon, C. P., and Low, P. S. (1992) Cytotoxicity of momordin-folate conjugates in cultured human cells. *J. Biol. Chem.* 267, 24966–24971.
- Leamon, C. P., and Low, P. S. (1993) Membrane folate-binding proteins are responsible for folate-protein conjugate endocytosis into cultured cells. *Biochem. J.* 291, 855–860.
- Leamon, C. P., Pastan, I., and Low, P. S. (1993) Cytotoxicity of folate-pseudomonas exotoxin conjugates toward tumor cells. *J. Biol. Chem.* 268, 24847–24854.
- Lee, R. J., and Low, P. S. (1994) Delivery of liposomes into cultured KB cells via folate receptor-mediated endocytosis. *J. Biol. Chem.* 269, 3198–3204.
- Lentle, B. C. (1986) Tumor detection with radiolabeled agents. *Current Applications in Radiopharmacology* (M. W. Billingham, Ed.) pp 48–61, Pergamon Press, Canada.
- Levy, C. C., and Goldman, P. (1967) The enzymatic hydrolysis of methotrexate and folic acid. *J. Biol. Chem.* 242, 2933–2938.
- Luhers, C. A., Raskin, C. A., Durbin, R., Wu, B., Sadasivan, E., McAllister, W., and Rothenberg, S. P. (1992) Transfection of a glycosylated phosphatidylinositol-anchored folate-binding protein complementary DNA provides cells with the ability to survive in low folate medium. *J. Clin. Invest.* 90, 840–847.
- Matsue, H., Rothberg, K. G., Takashima, A., Kamen, B. A., Anderson, R. G. W., and Lacey, S. W. (1992) Folate receptor allows cells to grow in low concentrations of 5-methyltetrahydrofolate. *Proc. Natl. Acad. Sci. U.S.A.* 89, 6006–6009.
- McCullough, J. L., Chabner, B. A., and Bertino, J. R. (1971) Purification and properties of carboxypeptidase G₁. *J. Biol. Chem.* 246, 7207–7213.
- McHugh, M., and Cheng, Y. C. (1979) Demonstration of a high affinity folate binder in human cell membranes and its characterization in cultured human KB cells. *J. Biol. Chem.* 254, 11312–11318.
- Presant, C. A., Proffitt, R. T., Turner, A. F., Williams, L. E., Winsor, D., Werner, J. L., Kennedy, P., Wiseman, C., Gala, K., McKenna, R. J., Smith, J. D., Bouzaglou, S. A., Callahan, R. A., Baldeschwieler, J., and Crossley, R. J. (1988) Successful imaging of human cancer with indium-111-labeled phospholipid vesicles. *Cancer* 62, 905–911.
- Rettig, W., Garin-Chesa, P., Beresford, H., Oettgen, H., Melamed, M., and Old, L. (1988) Cell-surface glycoproteins of human sarcomas: differential expression in normal and malignant tissues and cultured cells. *Proc. Natl. Acad. Sci. U.S.A.* 85, 3110–3114.
- Richardson, D., and Baker, E. (1992) Two mechanisms of iron uptake from transferrin by melanoma cells. The effect of desferrioxamine and ferric ammonium citrate. *J. Biol. Chem.* 267, 13972–13979.
- Ross, J. F., Chaudhuri, P. K., and Ratnam, M. (1994) Differential regulation of folate receptor isoforms in normal and malignant tissues in vivo and in established cell lines. Physiologic and clinical implications. *Cancer* 73, 2432–2443.
- Serafini, A. N., Ed. (1993) Monoclonal antibody imaging: crossing the research/clinical barrier. *Semin. Nucl. Med.* 23(2), 87–179.
- Smith-Jones, P. M., Stolz, B., Bruns, C., Albert, R., Reist, H. W., Fridrich, R., and Macke, H. R. (1994) Gallium-67/gallium-68-[DFO]-octreotide—a potential radiopharmaceutical for PET imaging of somatostatin receptor-positive tumors: synthesis and radiolabeling in vitro and preliminary in vivo studies. *J. Nucl. Med.* 35, 317–325.
- Sohn, M. H., Jones, B. J., Whiting, J. H., Jr., Datz, F. L., Lynch, R. E., and Morton, K. A. (1993) Distribution of gallium-67 in normal and hypotransferrinemic tumor-bearing mice. *J. Nucl. Med.* 34, 2135–2143.
- van Leeuwen-Stok, A. E., Drager, A. M., Schuurhuis, G. J., Platier, A. W., Teule, G. J., and Huijgens, P. C. (1993) Gallium 67 in the human lymphoid cell line U-715: uptake, cytotoxicity and intracellular localization. *Int. J. Radiat. Biol.* 64, 749–759.
- Verma, D. R. (1992) Gallium-labeled deferoxamine-galactosyl-neoglycoalbumin: a radiopharmaceutical for regional measurement of hepatic receptor biochemistry. *J. Nucl. Med.* 33, 1160–1166.
- Wagner, S. J., and Welch, W. J. (1979) Gallium-68 labeling of albumin and albumin microspheres. *J. Nucl. Med.* 20, 428–433.
- Wang, S., Lee, R. J., Cauchon, G., Gorenstein, D. G., and Low, P. S. (1995) Delivery of antisense oligonucleotides against the human epidermal growth factor receptor into cultured KB cells with liposomes conjugated to folate via polyethyleneglycol. *Proc. Natl. Acad. Sci. U.S.A.* 92, 3318–3322.
- Weiner, R. E., Thakur, M. L., Goodman, M., and Hoffer, P. B. (1979) Relative stability of In-111 and Ga-67 desferrioxamine and human transferrin complexes. *Radiopharmaceuticals II*, pp 331–340, Society of Nuclear Medicine, New York.
- Weitman, S. D., Lark, R. H., Coney, L. R., Fort, D. W., Frasca, V., Zurawski, V. R., Jr., and Kamen, B. A. (1992) Distribution of the folate receptor (GP38) in normal and malignant cell lines and tissues. *Cancer Res.* 52, 3396–3401.
- Wilbur, D. S. (1992) Radiohalogenation of proteins: an overview of radionuclides, labeling methods, and reagents for conjugate labeling. *Bioconjugate Chem.* 3, 433–470.
- Yokoyama, A., Ohmomo, Y., Horiuchi, K., Saji, H., Tanaka, H., Yamamoto, K., and Ishii, Y. (1982) Deferoxamine, a promising bifunctional chelating agent for labeling proteins with gallium: Ga-67-DF-HSA: concise communication. *J. Nucl. Med.* 23, 909–914.

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Labeling a Hydrazino Nicotinamide-Modified Cyclic I Ib/IIIa Receptor Antagonist with ^{99m}Tc Using Aminocarboxylates as Coligands

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A series of ^{99m}Tc complexes containing a hydrazinonicotinamide-conjugated cyclic I Ib/IIIa receptor antagonist, cyclo(D-Val-NMeArg-Gly-Asp-Mamb-(hydrazinonicotinyl-5-(6-aminocaproic acid))), were synthesized in high yield using tricine or other aminocarboxylates as coligands. These ^{99m}Tc complexes have the potential to be used as thrombus imaging agents. The radiolabeling of the HYNIC-conjugated cyclic I Ib/IIIa peptide (HYNICTide) was carried out by reaction with pertechnetate in the presence of excess tricine and stannous chloride at pH 4–5. The reaction time and temperature depend on the amount of the HYNICTide and pertechnetate used for the radiolabeling. Very high specific activity ($\geq 20\,000\text{ mCi}/\mu\text{mol}$) can be achieved for the complex $[^{99m}\text{Tc}(\text{HYNICTide})(\text{tricine})_2]$ without postlabeling purification. The complex $[^{99m}\text{Tc}(\text{HYNICTide})(\text{tricine})_2]$ was found by two reversed phase HPLC methods to exist as multiple species, some of which interconvert, depending on the temperature, reaction time, and pH of the reaction mixture. The presence of these multiple species is most likely due to different bonding modalities of either the hydrazine moiety of the HYNICTide or the two tricine coligands. The complex $[^{99m}\text{Tc}(\text{HYNICTide})(\text{EDDA})]$ (EDDA = ethylenediamine-*N,N'*-diacetic acid) was prepared either by reacting the cyclic I Ib/IIIa HYNICTide with pertechnetate, excess EDDA, and stannous chloride at pH 4–5 and 75 °C for 30 min or by reacting excess EDDA with $[^{99m}\text{Tc}(\text{HYNICTide})(\text{tricine})_2]$. The complex $[^{99m}\text{Tc}(\text{HYNICTide})(\text{EDDA})]$ was found to be stable for at least 12 h in the reaction mixture. Three major species were detected in the radio-HPLC chromatograms, presumably due to the more limited number of possible coordination isomers. Similar results were obtained using other polydentate aminocarboxylates (such as HEDTA, *N*-(2-hydroxyethyl)ethylenediaminetriacetic acid) as coligands. It is clear that the replacement of tricine by other polydentate aminocarboxylates produces ^{99m}Tc -HYNICTide complexes with higher stability and fewer coordination isomers.

INTRODUCTION

There is currently considerable interest in labeling proteins and small peptides with ^{99m}Tc for the development of target specific imaging agents (1–4). In the last decade, a large number of radiolabeling techniques have been developed and extensively reviewed (5–10). They can be classified into three main categories: direct labeling, the preformed chelate approach, and the indirect labeling approach. The direct labeling approach uses a reducing agent to convert a number of disulfide linkages in a protein into free thiols, which are able to bind the Tc very efficiently. The advantage of this approach is that it is easy to carry out. However, very little is known about the coordination chemistry of the Tc. There is little control over the stability of the ^{99m}Tc complex or the nonspecific binding. In addition, this method applies only to proteins or their fragments because many small peptides do not have any disulfide bonds, or in some cases the disulfide bond is too critical for maintaining their biological properties to be reduced. The preformed chelate approach involves formation of the ^{99m}Tc complex with a bifunctional coupling agent (BFCA) and conjugation of the ^{99m}Tc -BFCA complex to a protein or peptide in a separate step on the tracer level. In this approach, the chemistry is better defined, and the peptide or protein is not exposed to the sometimes forcing

conditions used to prepare the complex. However, the multistep tracer level synthesis limits its clinical application. In the indirect labeling approach, a BFCA is first attached to the peptide or protein to form a BFCA-peptide (protein) conjugate. The radiolabeling can be achieved either by direct reduction of $^{99m}\text{TcO}_4^-$ in the presence of BFCA-peptide (protein) conjugate or by ligand exchange with an intermediate ^{99m}Tc complex such as ^{99m}Tc -glucoheptonate. This approach combines the ease of direct labeling with the well-defined chemistry of the preformed chelate approach. Therefore, the indirect labeling is the most practical approach for the development of peptide-based target specific radiopharmaceuticals.

We have been actively pursuing a research program toward developing thrombus imaging agents based on cyclic I Ib/IIIa receptor antagonists (11–13). Since these small peptides are very potent, the ^{99m}Tc labeling must be accomplished with high specific activity. Therefore, we are particularly interested in a BFCA, which is able to form stable ^{99m}Tc complexes in high yield at very low concentrations of the BFCA-peptide conjugate. Recently, Abrams and co-workers reported the use of aromatic hydrazines in labeling polyclonal IgG with ^{99m}Tc for imaging focal sites of infection (14, 15). The HYNIC group is of particular interest because it can be easily labeled with high efficiency (rapid and high yield radiolabeling), and the resulting complexes have high in vivo stability (16). Since the HYNIC group can only occupy one or two sites in the technetium coordination sphere, a coligand such as glucoheptonate is required. It

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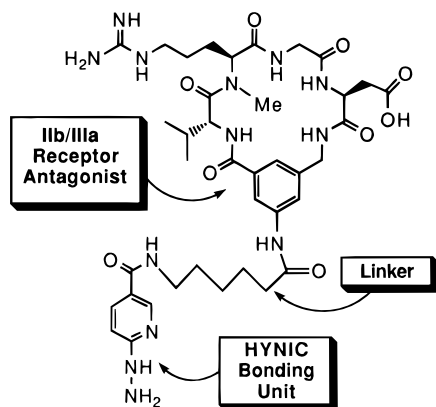


Figure 1. Cyclic Iib/IIIa HYNICTide.

has been reported that when glucoheptonate is used as coligand, a specific activity of $>100\,000$ mCi/ μ mol can be obtained after HPLC purification on a routine basis in labeling chemotactic peptides with ^{99m}Tc (4, 16). It was also found that the nature of the coligand has significant effects on the biodistribution of the radiolabeled chemotactic peptides (17). Tricine, tris(hydroxymethyl)methylglycine, has also been reported as a coligand in labeling HYNIC-conjugated polyclonal IgG with ^{99m}Tc (18, 19). The specific activity (>150 mCi/mg protein) of the radiolabeled HYNIC-IgG conjugate using tricine as coligand is higher than that (<25 mCi/mg protein) obtained using glucoheptonate.

We have chosen to use HYNIC as the bonding unit for labeling the cyclic Iib/IIIa antagonist (HYNICTide in Figure 1) with ^{99m}Tc at very low concentrations using tricine as the coligand. Using different radio-HPLC methods, we also demonstrated that the complex [^{99m}Tc -(HYNICTide)(tricine) $_2$] exists as multiple species, which we attribute to coordination isomerism about the Tc center. In order to reduce the number of coordination isomers, we have studied a variety of other polydentate aminocarboxylates as coligands. The results of these studies are described in this paper.

EXPERIMENTAL PROCEDURES

Chemicals were purchased from either Aldrich Chemical Co. or Sigma Chemical Co. and were used as received. ^1H NMR spectra were recorded on a 270 MHz Bruker spectrometer. NMR data were reported as δ (ppm) relative to TMS. Infrared spectra were recorded as KBr disks in the range of $4000\text{--}400\text{ cm}^{-1}$ on a Nicolet 5DXB IR spectrophotometer. FAB-MS and elemental analyses were performed by Oneida Research Services, Inc., Whitesboro, NY. $\text{Na}^{99m}\text{TcO}_4$ was obtained from a commercial DuPont $^{99}\text{Mo}/^{99m}\text{Tc}$ generator. Deionized water was obtained from a Millipore MilliQ water system and was of $>18\text{ M}\Omega$ quality. Synthesis and biological properties of the cyclic Iib/IIIa receptor HYNICTide will be reported elsewhere (20).

Analytical Methods. Three radio-HPLC methods were used. The TFA method used a Hewlett-Packard Model 1050 instrument and a Vydac C_{18} column ($4.6\text{ mm} \times 25\text{ cm}$) at a flow rate of 1.0 mL/min with a gradient mobile phase from 100% A (0.1% TFA in H_2O) to 100% B (0.1% TFA in acetonitrile) over 25 min. The phosphate method used a Hewlett-Packard Model 1050 instrument and a Vydac C_{18} column ($4.6\text{ mm} \times 25\text{ cm}$) at a flow rate of 1.0 mL/min with a gradient mobile phase from 100% A (0.01 M pH 6 phosphate buffer) to 30% B (acetonitrile) at 15 min and 75% B at 25 min. The ion-pair method used a Hewlett-Packard Model 1090 HPLC instrument and a Zorbax Rx C_{18} column ($4.6\text{ mm} \times 25\text{ cm}$) at a flow

rate of 1.0 mL/min with a gradient mobile phase from 100% A (95:5 (0.025 M pH 3.7 phosphate buffer/0.005 M TBAP):acetonitrile) to 90% A and 10% B (20:80 (0.025 M pH 3.7 phosphate buffer/0.005 M tetrabutylammonium phosphate):acetonitrile) at 20 min, 40% B at 30 min and 100% B at 33 min. The ITLC method used Gelman Sciences silica gel paper strips and 1:1 mixture of acetone and saline as eluant.

N,N' -Et $_2$ EDDA $\cdot 2\text{HCl}$. To a suspension of potassium carbonate (15 g, 0.11 mol) in dry acetonitrile (150 mL) was added N,N' -diethylethylenediamine (5.8 g, 0.05 mol) followed by *tert*-butyl bromoacetate (20 g, 0.10 mol). The reaction mixture was stirred at room temperature for 24 h. Solvent was removed under reduced pressure, and the residue was extracted with dichloromethane ($2 \times 150\text{ mL}$). The organic phases were combined, washed with H_2O ($2 \times 50\text{ mL}$), and dried over anhydrous MgSO_4 . Removal of dichloromethane afforded a pale yellow liquid. The liquid (*tert*-butyl ester of Et $_2$ EDDA) was dissolved in concentrated HCl (100 mL) and the resulting solution stirred at room temperature for 5–6 h. Solvent was removed under the reduced pressure to give a white solid, which was dried under vacuum overnight. The yield was 10.2 g (68%). Anal. Calcd (found) for $\text{C}_{10}\text{H}_{20}\text{N}_2\text{O}_4 \cdot 2\text{HCl} \cdot 1.5\text{H}_2\text{O}$: C, 36.15 (36.35); H, 7.58 (7.39); N, 8.43 (8.54). IR (cm^{-1} , KBr disk): 3500–2000 (br, ν_{OH}), 1740 (vs, $\nu_{\text{C=O}}$). FAB-MS: $m/z = 233$ ($M + 1$, $[\text{C}_{10}\text{H}_{21}\text{N}_2\text{O}_4]^+$). ^1H NMR (D_2O): 1.20 (t, 6H, CH_3 , $^3J_{\text{H-H}} = 7.3\text{ Hz}$); 3.29 (q, 4H, CH_2 , $^3J_{\text{H-H}} = 7.2\text{ Hz}$); 3.65 (s, 4H, CH_2CH_2); and 4.07 (s, 4H, CH_2COOH).

N -MeEDTA $\cdot 2\text{HCl}$. *N*-Methylethylenediaminetriacetic acid dihydrochloride (*N*-MeEDTA $\cdot 2\text{HCl}$) was prepared in a fashion similar to that for Et $_2$ EDDA $\cdot 2\text{HCl}$ using *N*-methylethylenediamine (1.5 g, 0.02 mol), potassium carbonate (8.5 g, 0.06 mol), and *tert*-butyl bromoacetate (12 g, 0.06 mol). The yield was 5.6 g (87%). Anal. Calcd (found) for $\text{C}_9\text{H}_{16}\text{N}_2\text{O}_6 \cdot 2\text{HCl} \cdot 0.25\text{C}_4\text{H}_8\text{O}$: C, 35.36 (35.70); H, 6.08 (5.87); N, 8.25 (8.00). IR (cm^{-1} , KBr disk): 3500–2000 (br, ν_{OH}), 1740 (vs, $\nu_{\text{C=O}}$). FAB-MS: $m/z = 249$ ($M + 1$, $[\text{C}_9\text{H}_{17}\text{N}_2\text{O}_6]^+$). ^1H NMR (D_2O): 2.88 (s, 3H, CH_3); 3.56 (m, 4H, CH_2CH_2); and 3.85–4.20 (m, 6H, CH_2COOH).

N -BzlEDTA $\cdot 2\text{HCl}$. *N*-Benzylethylenediaminetriacetic acid dihydrochloride (*N*-BzlEDTA $\cdot 2\text{HCl}$) was prepared similarly to Et $_2$ EDDA $\cdot 2\text{HCl}$ using *N*-benzylethylenediamine (3.0 g, 0.02 mol), potassium carbonate (8.5 g, 0.06 mol), and *tert*-butyl bromoacetate (12 g, 0.06 mol). The yield was 7.0 g (88%). Anal. Calcd (found) for $\text{C}_{15}\text{H}_{20}\text{N}_2\text{O}_6 \cdot 2\text{HCl}$: C, 45.35 (45.42); H, 5.58 (5.58); N, 7.05 (7.00). IR (cm^{-1} , KBr disk): 3500–2000 (br, ν_{OH}), 1745 (vs, $\nu_{\text{C=O}}$). FAB-MS: $m/z = 325$ ($M + 1$, $[\text{C}_{15}\text{H}_{21}\text{N}_2\text{O}_6]^+$). ^1H NMR (D_2O): 3.30, 3.50 (m, 4H, CH_2CH_2); 3.67 (s, 4H, CH_2COOH); 4.08 (s, 2H, CH_2COOH); 4.43 (s, 2H, PhCH_2); and 7.42 (m, aromatic H).

Radiolabeling of Cyclic Iib/IIIa HYNICTide Using Tricine as Coligand in One Step. To a 10 mL vial was added 0.5 mL of $^{99m}\text{TcO}_4^-$ solution (20–200 mCi/mL) in saline, followed by 0.5 mL of tricine solution (20–75 mg/mL, pH ~ 5.0) in H_2O , 0.1 mL of HYNICTide solution (5–500 $\mu\text{g/mL}$) in H_2O , and 20 μL of $\text{SnCl}_2 \cdot 2\text{H}_2\text{O}$ solution (0.5–5.0 mg/mL) in 0.1 N HCl. The reaction mixture was allowed to stand at room temperature for 20–60 min depending on the amount of HYNICTide used for the radiolabeling and then analyzed by radio-HPLC. ITLC was used to determine the amount of ^{99m}Tc -colloid.

Radiolabeling of Cyclic Iib/IIIa HYNICTide Using Tricine as Coligand in Two Steps. To a 10 mL vial was added 0.5 mL of $^{99m}\text{TcO}_4^-$ solution (20–200 mCi/mL) in saline, followed by 0.5 mL of tricine solution (20–75 mg/mL, pH ~ 5.0) in H_2O , and 10 μL of $\text{SnCl}_2 \cdot 2\text{H}_2\text{O}$

solution (10 mg/mL) in 1 N HCl. After the mixture was allowed to stand at room temperature for 10 min, 0.1 mL of HYNICTide solution (100 μ g/mL) in H₂O was added. Upon standing at room temperature for another 30–60 min, the reaction mixture was analyzed by radio-HPLC and ITLC. The complex prepared in two steps was found to be the same as that prepared in one step.

Radiolabeling of Cyclic IIb/IIIa HYNICTide Using EDDA as Coligand in One Step. To a 10 mL vial was added 0.4 mL of ^{99m}TcO₄[−] solution (50–125 mCi/mL) in saline, followed by 0.5 mL of EDDA solution (10 mg/mL, pH 7–7.5) in H₂O, 0.1 mL of HYNICTide solution (100 μ g/mL) in H₂O, and 10 μ L of SnCl₂·2H₂O solution (10 mg/mL) in 1 N HCl. The reaction mixture was heated at 75 °C for 30 min and then analyzed by radio-HPLC and ITLC. The same procedure was used for the N-substituted EDDA analogs.

Radiolabeling of Cyclic IIb/IIIa HYNICTide Using EDDA as Coligand in Two Steps. To a 10 mL vial was added 0.4 mL of ^{99m}TcO₄[−] solution (50–125 mCi/mL) in saline, followed by 0.5 mL of EDDA solution (10 mg/mL, pH 7–7.5) in H₂O and 10 μ L of SnCl₂·2H₂O solution (10 mg/mL) in 1 N HCl. After the mixture was allowed to stand at room temperature for 5 min, 0.1 mL of HYNICTide solution (100 μ g/mL) in H₂O was added to the reaction mixture. It was heated at 75 °C for 30 min and then analyzed by radio-HPLC and ITLC. The complex prepared in two steps was found to be the same as that prepared in one step.

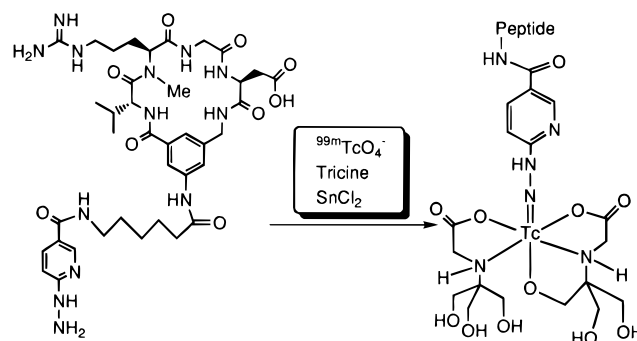
Radiolabeling of Cyclic IIb/IIIa HYNICTide Using Polydentate Aminocarboxylates by Exchange Labeling. The complex [^{99m}Tc(HYNIC)(tricine)₂] was prepared in one step as described above. To resulting [^{99m}Tc(HYNIC)(tricine)₂] kit was added 1.0 mL of aminocarboxylate solution (~20 mg/mL, pH ~8). The mixture was heated at 75 °C for 30 min. The radio-HPLC analysis showed that the tricine complex [^{99m}Tc(HYNIC)(tricine)₂] was completely converted into [^{99m}Tc(HYNICTide)(L)] (L = EDDA, N,N'-Et₂EDDA, N-BzLEDTA, N-MeEDTA) in 90–95% yield.

RESULTS AND DISCUSSION

The high specificity of biologically active molecules, such as antibodies and peptides, has been used in the development of target specific radiopharmaceuticals in both diagnostic and therapeutic nuclear medicine (2, 3). Compared to antibodies with low molecular weight, naturally occurring peptides may better serve as target molecules for radiopharmaceutical development because peptides are necessary elements in more fundamental biological processes than any other class of molecule (3) and in many cases the affinities of peptides for their receptors are significantly greater than that of monovalent antibody fragments (3). Radiopharmaceuticals based on small peptides may not suffer some common problems, such as slow blood clearance and low target-to-background ratio, encountered with radiolabeled antibodies. The faster blood clearance makes it practical to use ^{99m}Tc, which has a half-life of 6.02 h and is the preferred radionuclide for diagnostic nuclear medicine.

The preferred technetium radiolabeling method is the one-step synthesis, in which the biologically active molecule conjugated with a BFCA is labeled by reduction of pertechnetate with a reducing agent such as stannous chloride. For proteins and their fragments, the conjugation and radiolabeling do not significantly change their chemical and biological properties because the ^{99m}Tc complex is attached to only a small portion of these macromolecules. For small peptides, however, modification with a BFCA and radiolabeling may dramatically

Scheme 1



change their chemical and biological properties. In addition, due to the high potency of many peptides, it is often critical to have high specific activity, which is dependent on the BFCA used. Therefore, the choice of BFCA is very important to the success of the radiolabeling.

Various BFCAs have been used in labeling proteins and peptides with ^{99m}Tc. These include DTPA (21), N₂S₂ diamidedithiols (22, 23), N₃S triamidethiols (24), BATOs (25, 26), and N₂S₂ diaminedithiols (27). Among them, only N₂S₂ diaminedithiols show radiolabeling efficiency comparable to the HYNIC group (24, 27). When HYNIC is used as the bonding group for the Tc, the necessity of a coligand offers a wide range of options to modify the biological characteristics of ^{99m}Tc-HYNICTide complexes, such as improving the blood clearance of the imaging agent. This, in return, may result in the improved target-to-background ratios.

The cyclic IIb/IIIa HYNICTide (Figure 1) is composed of three parts: a cyclic IIb/IIIa platelet receptor antagonist, a linker, and the HYNIC bonding unit. The cyclic IIb/IIIa receptor antagonist itself has very high potency, the IC₅₀ for the inhibition of platelet aggregation is 20 nM and 6 nM against fibrinogen binding to human platelets (28). Therefore, it must be labeled in high specific activity in order to be used without having a biological effect. The use of the 6-aminocaproic acid linker keeps the Tc center far from the peptide backbone, to minimize loss of binding affinity toward the receptor.

Labeling the Cyclic IIb/IIIa HYNICTide with ^{99m}Tc Using Tricine as Coligand. The cyclic IIb/IIIa HYNICTide can be easily labeled (Scheme 1) either in one step by reaction of ^{99m}TcO₄[−] with a reducing agent such as stannous chloride in the presence of excess tricine or in two steps by reacting the cyclic IIb/IIIa HYNICTide with ^{99m}Tc-tricine. The reaction is carried out at room temperature for 30–60 min (depending on the amount of HYNICTide). Radiolabeling yields are ≥90% using 5 μ g (4.69 × 10^{−9} mol) of HYNICTide (FW = 1066 for HYNICTide·2TFA) and 100 mCi of pertechnetate (~7 × 10^{−10} mol of ^{99m}Tc and ^{99m}Tc for 24 h generator) resulting in a specific activity of ≥20 000 mCi/ μ mol. The HYNICTide/Tc ratio is only ~7:1 under these conditions. Since the ^{99m}Tc-labeled IIb/IIIa HYNICTide requires no post-labeling purification, this procedure is amenable for clinical application.

In order to fully characterize the ^{99m}Tc-HYNICTide–tricine complex, we used three different radio-HPLC methods: the TFA method, which uses acetonitrile–water (containing 0.1% TFA) as the mobile phase and a flow rate of 1.0 mL/min, the phosphate gradient method, which uses 10 mM phosphate buffer (pH 6)–acetonitrile as the mobile phase, and the ion-pair method, which uses tetra(*n*-butyl)ammonium cation as the ion pair agent and 30 mM phosphate buffer (pH 3.7)–acetonitrile as the

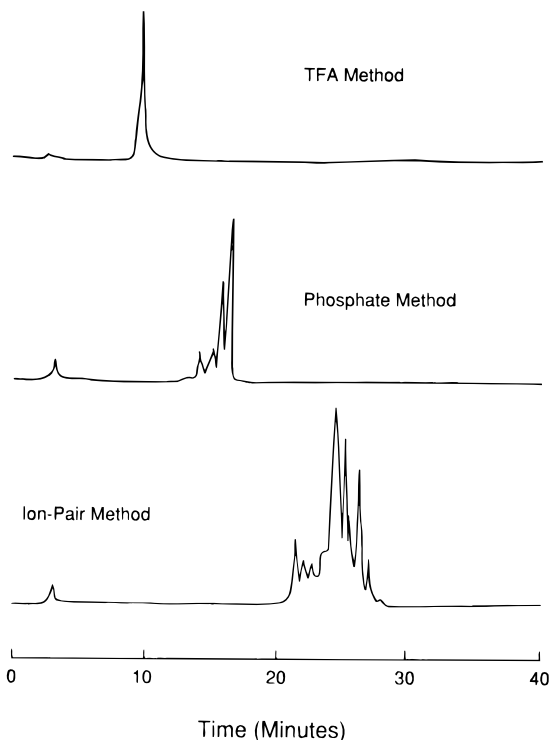


Figure 2. Radio-HPLC chromatogram for $[^{99m}\text{Tc}(\text{HYNICtide})-(\text{tricine})_2]$ by the TFA gradient method (top), the phosphate gradient method (middle), and the ion-pair method (bottom).

mobile phase. The advantage of using the ion-pair method is that the cationic ion pair agent enhances the resolution of species that differ in charge by preferentially retaining anionic species. We found that when the TFA method was used only one peak was detected at 9.6 min (Figure 2). However, the same sample analyzed by the phosphate method shows a cluster of peaks between 11 and 14 min (Figure 2). By the ion pair method, the radio-HPLC chromatogram shows at least 10 partially resolved peaks with retention times ranging from 20 to 28 min (Figure 2).

In order to determine the number of HYNICtides in the ^{99m}Tc -HYNICtide-tricine complex, a mixed-ligand experiment was carried out. In this experiment, the cyclic I Ib/IIIa HYNICtide and a model compound, 2-hydrazinopyridine (HYPY), were used in the same reaction mixture. If there is only one HYNICtide in the ^{99m}Tc -HYNICtide-tricine complex, the radio-HPLC chromatogram is expected to show two sets of peaks, one set due to the ^{99m}Tc -HYNICtide-tricine complex and the other due to the ^{99m}Tc -HYPY-tricine complex. If there were two HYNICtides bonding to the Tc center, a third set of peaks from the mixed-ligand complex, ^{99m}Tc -HYNICtide-HYPY-tricine, is expected. The presence of only two sets of peaks from the ^{99m}Tc -HYNICtide-tricine complex and the ^{99m}Tc -HYPY-tricine complex in the radio-HPLC chromatogram (Figure 3) demonstrates clearly that there is only one HYNIC group bonded to the Tc.

If one assumes that the coordination geometry around the Tc is distorted octahedral and HYNICtide occupies only one site by forming either a Tc(V)-hydrazido or Tc(V)-diazenido bond, it requires at least two tricine ligands to complete the coordination sphere. This assumption is supported by the work from Abrams and co-workers (19), who found that the ^{99m}Tc -tricine complex, which is a precursor for the synthesis of the ^{99m}Tc -HYNICtide-tricine complex, is chromatographically and electrophoretically equivalent to the complex $[^{99m}\text{TcO}(\text{tricine})_2]^-$ prepared from the reaction of TcOCl_4^- with excess

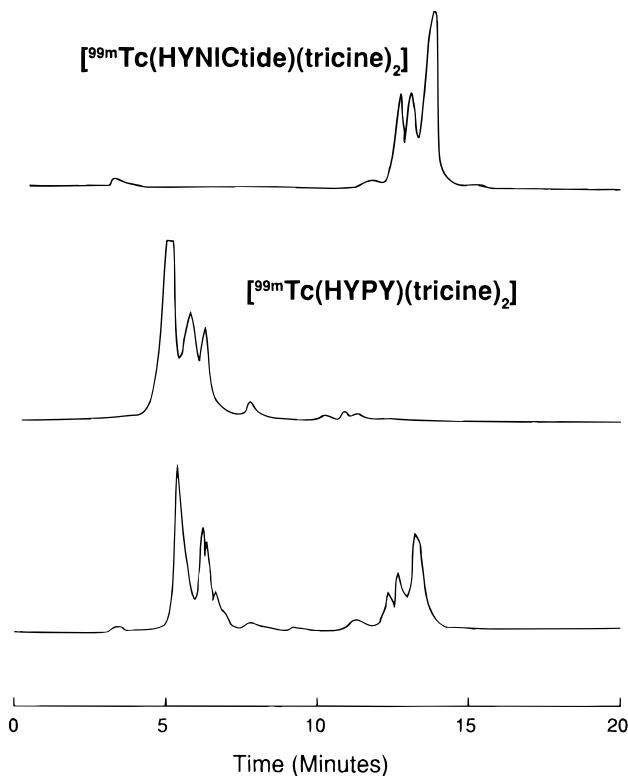


Figure 3. Radio-HPLC chromatograms for $[^{99m}\text{Tc}(\text{HYNICtide})-(\text{tricine})_2]$, $[^{99m}\text{Tc}(\text{HYPY})-(\text{tricine})_2]$, and their mixture (bottom) by the phosphate gradient method.

tetrabutylammonium salt of tricine. Therefore, the composition for the ^{99m}Tc -HYNICtide-tricine complex is most likely $[^{99m}\text{Tc}(\text{HYNICtide})(\text{tricine})_2]$ (Scheme 1).

The presence of the multiple species is most likely attributed to the resolution of some of the many possible isomers that can result from different bonding modalities of the hydrazine functionality of the HYNICtide and the two tricine coligands (Figure 4). Both Tc-hydrazido and Tc-diazenido bonds have been previously reported and characterized by X-ray crystallography (29–33). These different bonding modalities from the tricine and hydrazine ligands are expected to produce numerous structural and stereo isomers. Therefore, it is not surprising that the radio-HPLC chromatogram of $[^{99m}\text{Tc}(\text{HYNICtide})-(\text{tricine})_2]$ by the ion-pair method shows at least 10 partially resolved peaks. The observation of a single peak in the radio-HPLC chromatogram in Figure 2 is a result of the low resolution of the TFA method.

The labeling studies also show that the retention times for different ^{99m}Tc -labeled HYNICtides vary systematically with lipophilicity while the peak patterns in the radio-HPLC chromatograms remain the same. This strongly suggests that the formation of the multiple species is, in fact, due to coordination isomerism and not due to peptide related rearrangements or degradation or the formation of radioimpurities. This conclusion is supported by the fact that replacement of tricine in $[^{99m}\text{Tc}(\text{HYNICtide})(\text{tricine})_2]$ by other polydentate aminocarboxylates dramatically decreases the number of coordination isomers.

The ratios of the peak areas in the radio-HPLC chromatograms change with time, reaction temperature, and pH. Similar phenomena were observed for other ^{99m}Tc -labeled HYNICtides using tricine as coligand. This is probably caused by the conversion from a kinetically favored mixture of coordination isomers to a more thermodynamically favored mixture of isomers. In order to prove this assumption, we have successfully isolated

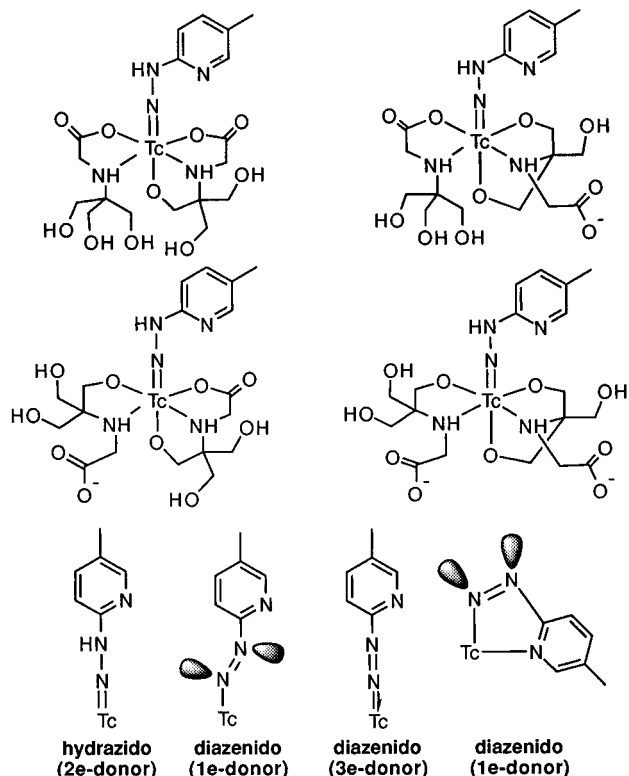


Figure 4. Possible coordination isomers from the tricine coligand and the hydrazine moiety.

several major peaks (Figure 5) and found that most of them interconvert and the rate of interconversion depends on the matrix. The fact that reinjection of the isolated peaks 8 and 9 (E in Figure 5) produces most other peaks in the radio-HPLC chromatogram (A in Figure 5) indicates clearly that the speciations in the radio-HPLC chromatograms are actually due to the isomerism in the complex $[\text{}^{99m}\text{Tc}(\text{HYNICTide})(\text{tricine})_2]$ and none are artifacts of the chromatographic conditions.

We also found $[\text{}^{99m}\text{Tc}(\text{HYNICTide})(\text{tricine})_2]$ to be unstable in dilute solutions (Figure 6). The freshly prepared $[\text{}^{99m}\text{Tc}(\text{HYNICTide})(\text{tricine})_2]$ kit was diluted 1:10 and 1:100 with 10 mM phosphate (pH 7.4), 10 mM sodium acetate (pH 5.5), and 2% tricine in 10 mM sodium acetate solution (pH 5.5). The resulting solutions were analyzed by radio-HPLC and ITLC. It was found that the complex $[\text{}^{99m}\text{Tc}(\text{HYNICTide})(\text{tricine})_2]$ is stable only in the 2% tricine solution (Figure SI, supporting information). In either 10 mM phosphate solution (pH 7.4) or 10 mM sodium acetate solution (pH 5.5) with 100-fold dilution, 10–15% of the complex $[\text{}^{99m}\text{Tc}(\text{HYNICTide})(\text{tricine})_2]$ decomposes to unidentified hydrophilic ^{99m}Tc species over a period of 2 h (Figure 6). Apparently, a large excess of tricine in the diluted solutions is needed to stabilize the ^{99m}Tc -HYNICTide core.

A platelet-binding assay (34) was used to determine if all these species contain the ^{99m}Tc -labeled HYNICTide and bind to the platelets. We isolated five fractions (instead of single peaks) of the complex $[\text{}^{99m}\text{Tc}(\text{HYNICTide})(\text{tricine})_2]$ with retention times from 20 to 30 min using the ion pair method. We found that all five fractions are able to bind to the platelets, but with various amounts of platelet-binding. These data clearly demonstrated that all five fractions of the complex $[\text{}^{99m}\text{Tc}(\text{HYNICTide})(\text{tricine})_2]$ contain the radiolabeled HYNICTide. This is consistent with our *in vivo* studies in two canine thrombosis models for the whole kit (13). Like the individual peaks, all five isolated fractions are not quite stable in the HPLC mobile phase, particularly when these frac-

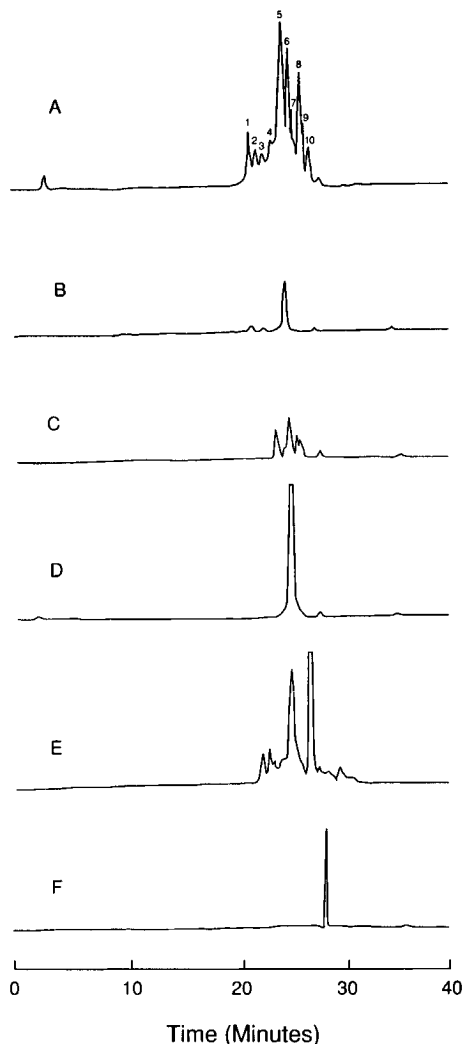


Figure 5. Radio-HPLC chromatograms for $[\text{}^{99m}\text{Tc}(\text{HYNICTide})(\text{tricine})_2]$ (A), and the isolated peaks (peak 1 (B), peaks 2 and 3 (C), peak 5 (D), peaks 8 and 9 (E), and peak 10 (F)).

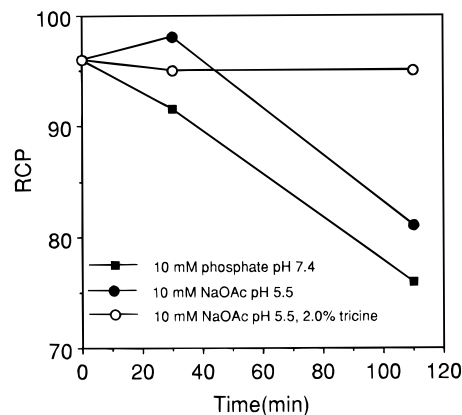
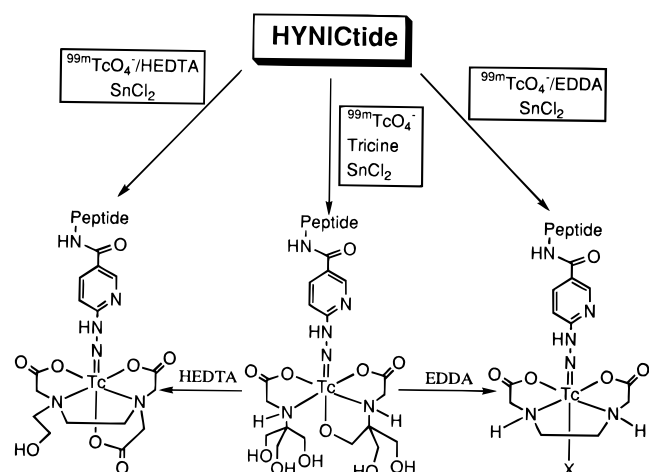


Figure 6. Instability of $[\text{}^{99m}\text{Tc}(\text{HYNICTide})(\text{tricine})_2]$ in dilute solution (100-fold).

tions are diluted (5–10-fold) with saline before the platelet-binding assay. In the process of isolation and dilution, parts of the isolated fractions decompose to pertechnetate and parts of them interconvert. The differences in the solution instability of these five fractions may contribute to their different level of platelet-binding.

Labeling Cyclic IIb/IIIa HYNICTide with ^{99m}Tc Using EDDA as Coligand. Since the complex $[\text{}^{99m}\text{Tc}(\text{HYNICTide})(\text{tricine})_2]$ is not stable and exists in many

Scheme 2



isomeric forms, some of which interconvert depending the reaction conditions, it would be difficult to develop for clinical use, even though the biological data from two animal models show that [$^{99m}\text{Tc}(\text{HYNICtide})(\text{tricine})_2$] can be used to image arterial, and deep venous thrombus (13). Therefore, our research is directed toward preparing ^{99m}Tc -HYNICtide complexes with higher stability and a minimum number of coordination isomers using other polydentate aminocarboxylates as coligands.

EDDA is of particular interest as coligand because it is potentially tetradentate and is expected to form a more symmetrical and stable complex. The higher symmetry should result in fewer coordination isomers. We have prepared a ^{99m}Tc -HYNICtide complex using EDDA as coligand either by a one-step synthesis (Scheme 2) or by a two-step approach, in which a ^{99m}Tc -EDDA precursor is formed first and is then allowed to react with the cyclic I Ib/IIIa HYNICtide. Using the one-step approach, the reaction was carried out by direct reduction of $^{99m}\text{TcO}_4^-$ in the presence of the cyclic I Ib/IIIa HYNICtide and excess EDDA with heating at 75–80 °C for 30 min. The use of shorter reaction times (particularly at HYNICtide concentrations $\leq 20 \mu\text{g/mL}$) dramatically decreases the labeling yield. If the reaction temperature is too high ($\geq 95^\circ\text{C}$), the ^{99m}Tc -HYNICtide complex is not stable and decomposes by forming ^{99m}Tc -colloid and $^{99m}\text{TcO}_4^-$. The specific activity ($\leq 10\,000 \text{ mCi}/\mu\text{mol}$) obtained using EDDA as coligand is slightly lower than that using tricine ($\geq 20\,000 \text{ mCi}/\mu\text{mol}$). This is probably due to the higher stability of the ^{99m}Tc -EDDA precursor. The EDDA concentration also affects the radiolabeling of the cyclic I Ib/IIIa HYNICtide. If the EDDA concentration is too high, it will require a higher HYNICtide concentration for a successful labeling. If the EDDA concentration is too low, there is not enough EDDA to stabilize the ^{99m}Tc -HYNICtide complex and to prevent the formation of ^{99m}Tc -colloid. It was found that the lowest acceptable EDDA concentration is $\sim 5 \text{ mg/mL}$.

Using the phosphate method, the radio-HPLC chromatogram shows two main peaks at 11.3 and 12.2 min (Figure 7). By the ion-pair method, three major peaks are observed (Figure 7). This method clearly demonstrates that there are at least three HYNICtide related species in the [$^{99m}\text{Tc}(\text{HYNICtide})(\text{EDDA})$] kit. Considering the potential isomerism from the hydrazine moiety and the possibility of a ligand X either trans or cis to the Tc–hydrazido bond, it is not surprising that there are still three or more coordination isomers for the complex [$^{99m}\text{Tc}(\text{HYNICtide})(\text{EDDA})$]. However, compared to the > 10 isomeric species from [$^{99m}\text{Tc}(\text{HYNICtide})(\text{tricine})_2$],

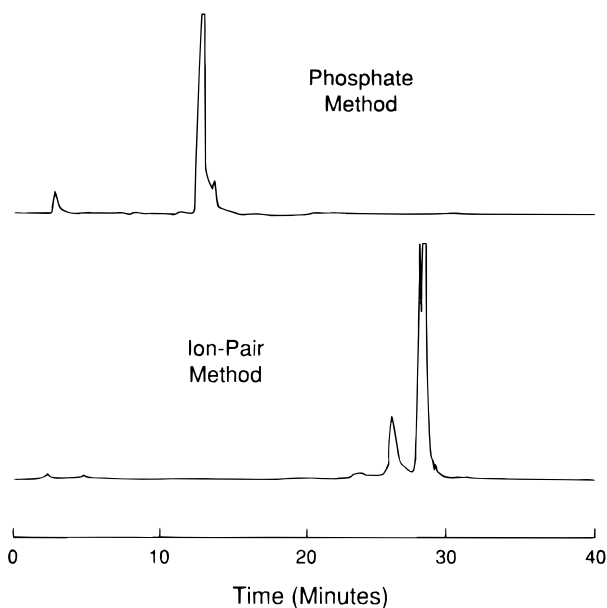


Figure 7. Radio-HPLC chromatogram for [$^{99m}\text{Tc}(\text{HYNICtide})$ -(EDDA)] by the phosphate gradient method (top) and the ion-pair method (bottom).

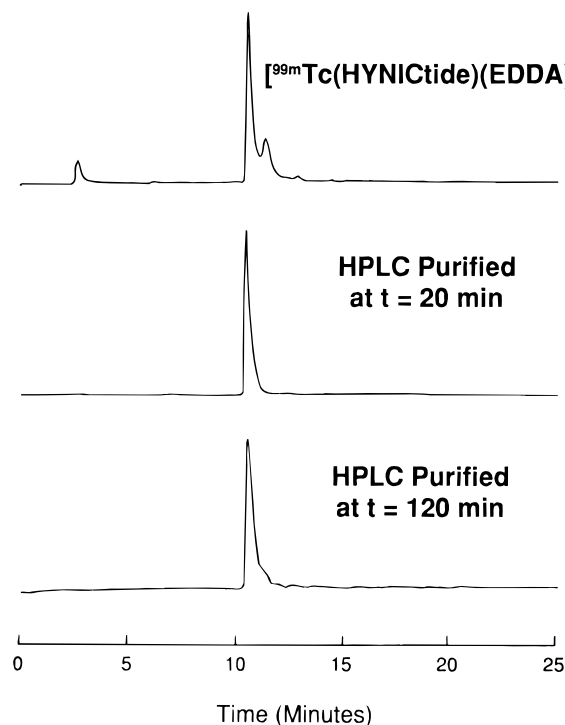


Figure 8. Radio-HPLC chromatograms for [$^{99m}\text{Tc}(\text{HYNICtide})$ -(EDDA)] in the original kit (top), the HPLC purified [$^{99m}\text{Tc}(\text{HYNICtide})(\text{EDDA})$] in saline at $t = 20 \text{ min}$ (middle), and the HPLC purified [$^{99m}\text{Tc}(\text{HYNICtide})(\text{EDDA})$] in saline at $t = 180 \text{ min}$ (bottom).

the dramatic decrease in coordination isomers for [$^{99m}\text{Tc}(\text{HYNICtide})(\text{EDDA})$] is clearly a big improvement.

Another advantage of using EDDA as coligand is the higher stability of the corresponding ^{99m}Tc complex. This is demonstrated by the fact that the complex [$^{99m}\text{Tc}(\text{HYNICtide})(\text{EDDA})$] is stable for at least 12 h in the kit matrix and the peak ratios remain relatively constant. The major isomer can be easily isolated and is stable for 2 h in saline solution (Figure 8) and 3 h in 1% EDDA solution. No decomposition was observed. However, there is evidence of conversion to the other species as seen by the growing shoulder on the main peak (Figure

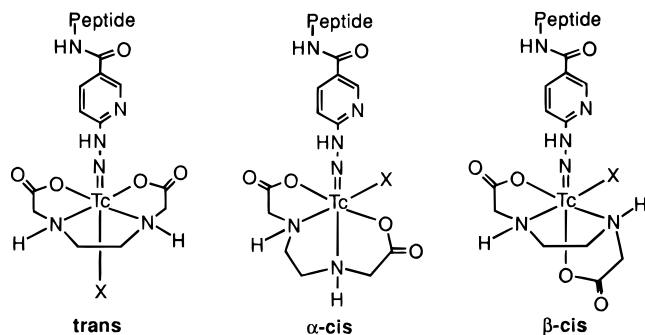


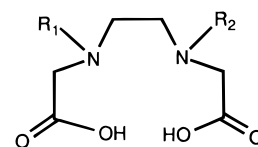
Figure 9. Three possible bonding modalities of EDDA.

8), suggesting that there is an equilibrium between different isomeric forms. The interconversion also shows that these peaks in the radio-HPLC chromatogram are actually from the complex [$^{99m}\text{Tc}(\text{HYNICTide})(\text{EDDA})$], not artifacts of the chromatographic conditions. On the basis of these data, it is quite clear that the use of EDDA to replace tricine as coligand produces a ^{99m}Tc complex with higher solution stability and less isomerism.

Using EDDA as Coligand by the Exchange Labeling Approach. The major advantage of using tricine as coligand is the high labeling efficiency and favorable labeling kinetics. The labeling efficiency using EDDA as coligand is not as high as that obtained using tricine even though it forms more stable ^{99m}Tc -HYNICTide complexes with fewer coordination isomers. In order to achieve higher specific activity using EDDA as coligand while retaining the stability of the ^{99m}Tc -HYNICTide complex, the exchange labeling approach was used to label the cyclic IIb/IIIa HYNICTide. In this approach, the tricine complex [$^{99m}\text{Tc}(\text{HYNICTide})(\text{tricine})_2$] is formed first and is then allowed to undergo ligand exchange (Scheme 2) with a large excess of EDDA (EDDA:tricine ≥ 2) and prolonged (≥ 30 min) heating at 75 °C. The radio-HPLC shows that the complex [$^{99m}\text{Tc}(\text{HYNICTide})(\text{EDDA})$] prepared by this approach is the same as that prepared by the one-step synthesis.

The exact nature of the bonding between the Tc-HYNICTide core and EDDA is not quite clear. Very little is known about the coordination chemistry at the carrier-added (^{99}Tc) level. It has been reported that polydentate aminocarboxylates such as EDTA and TCTA (1,4,7-triazacyclononane- N,N',N'' -triacetate) form stable Tc(III) and Tc(IV) complexes (35, 36). However, EDTA is used as a transfer ligand in Neurolite kits (37). In the labeling process, the complex [$^{99m}\text{TcO}(\text{EDTA})\text{]}^{n-}$ is formed first and then undergoes ligand exchange with a stronger chelator, ECD (N,N' -ethylenediybis-L-cysteinate diethyl ester), to form a neutral complex [$^{99m}\text{TcO}(\text{ECD})$]. It is difficult to imagine that the Tc is reduced to Tc(III) or Tc(IV) and then reoxidized back to Tc(V)=O for ligand exchange in the presence of excess reducing agent. Since the complex [$^{99m}\text{Tc}(\text{HYNICTide})(\text{EDDA})$] can be prepared by the two-step approach, it is reasonable to assume that the oxidation state for technetium is Tc(V), with only one HYNIC group bonding to the Tc center. If EDDA acts as a tetradentate ligand, another monodentate ligand X (such as Cl⁻) is required to complete the Tc coordination sphere. Figure 9 shows several possible bonding modalities for the EDDA ligand.

Labeling the Cyclic IIb/IIIa HYNICTide with ^{99m}Tc Using HEDTA as Coligand. HEDTA (N -(2-hydroxyethyl)ethylenediaminetriacetic acid) is potentially pentadentate using its N_2O_3 donor atoms in bonding to the Tc to form a six-coordinated ^{99m}Tc -HYNICTide complex. Either hydroxyl-O or carboxylate-O may coordinate to the Tc center trans to the Tc-hydrazido or Tc-diazenido



Et₂EDDA: R₁ = R₂ = ethyl
Bzl-EDTA: R₁ = Benzyl, R₂ = CH₂COOH
Me-EDTA: R₁ = methyl, R₂ = CH₂COOH

Figure 10. N-Substituted EDDA analogs.

Table 1. Radiolabeling Results Using EDDA and Its N-Substituted Analogs

complex ^a	yield (%)	t _R ^b (min)
$^{99m}\text{Tc}(\text{HYNICTide})(\text{EDDA})$	90–95	11.3, 12.2
$^{99m}\text{Tc}(\text{HYNICTide})(\text{HEDTA})$	90–93	11.5, 12.8, 13.5
$^{99m}\text{Tc}(\text{HYNICTide})(\text{N},\text{N}'\text{-Et}_2\text{EDDA})$	90	12.1, 12.5, 13.0
$^{99m}\text{Tc}(\text{HYNICTide})(\text{N-BzlEDTA})$	93	15.6, 16.5
$^{99m}\text{Tc}(\text{HYNICTide})(\text{N-MeEDTA})$	85	13.1, 13.8

^a ^{99m}Tc -HYNICTide complexes were prepared by the one-step approach. ^b Peaks ($\geq 2\%$) observed in radio-HPLC chromatograms (by the phosphate method).

bond. Radiolabeling studies show that it can be used as a coligand to label the cyclic IIb/IIIa HYNICTide either by a one-step reaction or by the ligand exchange labeling method (Scheme 2). The labeling efficiency is comparable to that of EDDA by the one-step approach. The radio-HPLC data by both the phosphate (Figure SII, supporting information) and ion-pair methods show more isomerism for the complex [$^{99m}\text{Tc}(\text{HYNICTide})(\text{HEDTA})$] probably due to the asymmetric character of HEDTA.

N-Substituted EDDA and HEDTA Analogs. The results from studies on [$^{99m}\text{Tc}(\text{HYNICTide})(\text{EDDA})$] and [$^{99m}\text{Tc}(\text{HYNICTide})(\text{HEDTA})$] clearly demonstrate that these polydentate aminocarboxylates are promising coligands because of the increased stability and less isomerism. ¹H NMR studies on vanadium(V) complexes showed that in aqueous solution the complex [$\text{VO}_2(\text{EDDA})$]⁻ exists as a mixture of isomers (α -cis and β -cis) at room temperature while complexes [$\text{VO}_2(\text{L})$]⁻ (L = N,N' -Me₂-EDDA and EDTA) exist only in the α -cis configuration because of the steric effect of the N-substituents (38, 39). In order to explore the effect of N-substituents on isomerism in ^{99m}Tc -HYNICTide complexes, we have prepared several N-substituted EDDA analogs (Figure 10) and used them to label the cyclic IIb/IIIa HYNICTide.

Synthesis of N-substituted EDDA analogs is straightforward. The alkylation is carried out by reacting N-substituted ethylenediamine with *tert*-butyl bromoacetate in the presence of anhydrous potassium carbonate in acetonitrile. The acid hydrolysis of the *tert*-butyl ester can be easily achieved by dissolving the corresponding *tert*-butyl ester in concentrated hydrochloric acid. These ligands were isolated as their hydrochloride salts and have been characterized by IR, FAB-MS, ¹H NMR, and elemental analysis.

Radiolabeling studies (Table 1) clearly show that N-substituted EDDA analogs can be used as coligands to synthesize ^{99m}Tc -HYNICTide complexes. Like EDDA, they typically give lower labeling efficiency. The introduction of different N-substituents produces ^{99m}Tc -HYNICTide complexes with different hydrophilicity, but it offers no improvement with respect to the reduction of the number of coordination isomers.

CONCLUSION

In conclusion, the cyclic IIb/IIIa HYNICTide, cyclo(D-Val-NMeArg-Gly-Asp-Mamb(hydrazinonicotinyl-5-(6-ami-

nocaproic acid))), was labeled with ^{99m}Tc using polydentate aminocarboxylates as coligands. A high specific activity ($\geq 20\,000\text{ mCi}/\mu\text{mol}$) can be achieved using tricine as coligand. The complex [$^{99m}\text{Tc}(\text{HYNICTide})(\text{tricine})_2$] was unstable in solution and was found to exist in multiple forms that we attribute to coordination isomerism. It should be emphasized that the isomerism becomes observable only in the radiolabeling of small peptides. For proteins such as polyclonal IgG, the chemical and biological properties are dominated by the polypeptide structure and should not be affected by coordination isomerism at the Tc center. The use of EDDA to replace tricine as a coligand produces the complex [$^{99m}\text{Tc}(\text{HYNICTide})(\text{EDDA})$] with higher solution stability and less isomerism. The N-substituted analogs of EDDA can also be used as coligands but show no superiority over EDDA with respect to the solution stability and reduction of isomers for the ^{99m}Tc -HYNICTide complexes.

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Supporting Information Available: Diagram showing the instability of [$^{99m}\text{Tc}(\text{HYNICTide})(\text{tricine})_2$] in three different media upon dilution. Radio-HPLC chromatogram for the complex [$^{99m}\text{Tc}(\text{HYNICTide})(\text{HEDTA})$] prepared by the one-step synthesis (2 pages). Ordering information is available on any current masthead page.

LITERATURE CITED

- Goldenberg, D. M. (1989) Future role of radiolabeled monoclonal antibodies in oncological diagnosis and therapy. *Seminars Nucl. Med.* 19, 332–339.
- Eckelman, W. C. (1994) The application of receptor theory to receptor-binding and enzyme-binding oncologic radiopharmaceuticals. *Nucl. Med. Biol.* 21, 759–769.
- Fischman, A. J., Babich, J. W., and Strauss, H. W. (1993) A ticket to ride: peptide radiopharmaceuticals. *J. Nucl. Med.* 34, 2253–2263.
- Fischman, A. J., Babich, J. W., and Rubin, H. R. (1993) Infection imaging with technetium-99m-labeled chemotactic peptides analogs. *Semin. Nucl. Med.* 24, 154–168.
- Eckelman, W. C., Paik, C. H., and Steigman, J. (1989) Three approaches to radiolabeling antibodies with ^{99m}Tc . *Nucl. Med. Biol.* 16, 171–176.
- Fritzberg, A. R., Berninger, R. W., Hadley, S. W., and Wester, D. W. (1988) Approaches to radiolabeling of antibodies for diagnosis and therapy of cancer. *Pharm. Res.* 5, 325–334.
- Otsuka, F. L., and Welch, M. J. (1987) Methods to label monoclonal antibodies for use in tumor imaging. *Nucl. Med. Biol.* 14, 243–249.
- Hnatowich, D. J. (1990) Antibody radiolabeling, problems and promises. *Nucl. Med. Biol.* 17, 49–55.
- Hnatowich, D. J. (1990) Recent developments in radiolabeling of antibodies with iodine, indium, and technetium. *Semin. Nucl. Med.* 20, 80–91.
- Srivastava, S. C., and Mease, R. C. (1991) Progress in research on ligands, nuclides and techniques for labeling monoclonal antibodies. *Nucl. Med. Biol.* 18, 589–603.
- Barrett, J. A., Heminway, S. J., Damphousse, D. J., Thomas, J. R., Looby, R. J., Edwards, D. S., Harris, T. D., Rajopadhye, M., Liu, S., and Carroll, T. R. (1994) Platelet GP IIb/IIIa antagonists in the canine arteriovenous shunt: potential thrombus imaging agents. *J. Nucl. Med.* 35, 52P (Abstract 202).
- Harris, T. D., Barrett, J. A., Bourque, J. P., Carroll, T. R., Damphousse, P. R., Edwards, D. S., Glowacka, D., Liu, S., Looby, R. J., Poirier, M. J., Rajopadhye, M., and Yu, K. (1994) Design and synthesis of radiolabeled GPIIb/IIIa receptor antagonists as potential thrombus imaging agents. *J. Nucl. Med.* 35, 245P (Abstract 1005).
- Barrett, J. A., Bresnick, M., Crocker, A., Damphousse, D. J., Hampson, J. R., Heminway, S. J., Mazaika, T. J., Kagan, M., Lazewatsky, J., Edwards, D. S., Liu, S., Harris, T. D., Rajopadhye, M., and Carroll, T. R. (1995) RP-431: a potential thrombus imaging agent. *J. Nucl. Med.* 36, 16P (Abstract 55).
- Schwartz, D. A., Abrams, M. J., Hauser, M. M., Gaul, F. E., Larsen, S. K., Rauh, D., and Zubietta, J. A. (1991) Preparation of hydrazino-modified proteins and their use for the synthesis of ^{99m}Tc -protein conjugate. *Bioconjugate Chem.* 2, 334–336.
- Abrams, M. J., Juweid, M., tenKate, C. I., Schwartz, D. A., Hauser, M. M., Gaul, F. E., Fuccello, A. J., Rubin, R. H., Strauss, H. W., and Fischman, A. J. (1990) Technetium-99m-human polyclonal IgG radiolabeled via the hydrazino nicotinamide derivative for imaging focal sites of infection in rats. *J. Nucl. Med.* 31, 2022–2028.
- Babich, J. W., Solomon, H., Pike, M. C., Kroon, D., Graham, W., Abrams, M. J., Tompkins, R. G., Rubin, R. H., and Fischman, A. J. (1993) Technetium-99m-labeled hydrazino nicotinamide derivatized chemotactic peptide analogs for imaging focal sites of bacterial infection. *J. Nucl. Med.* 34, 1967–1974.
- Babich, J. W., and Fischman, A. J. (1995) Effect of “coligand” on the biodistribution of ^{99m}Tc -labeled hydrazino nicotinamide derivatized chemotactic peptides. *Nucl. Med. Biol.* 22, 25–30.
- Larson, S. K., Abrams, M. J., Higgins, J. D., III, Solomon, H. F., Babich, J. W., and Fischman, A. J. (1994) Technetium complexes of tricine: useful precursor for the ^{99m}Tc labeling of hydrazino nicotinamide modified human polyclonal IgG. *J. Nucl. Med.* 35, 105P.
- Larson, S. K., Caldwell, G., Higgins, J. D., III, Abrams, M. J., and Solomon, H. F. (1994) Technetium complex of tricine: useful precursor for the ^{99m}Tc labeling of hydrazino nicotinamide modified proteins. *J. Labeled Compds. Radiopharm.* 35, 1–2.
- Rajopadhye, M., Edwards, D. S., and Liu, S. Unpublished data.
- Lanteingne, D., and Hnatowich, D. J. (1984) The labeling of DTPA coupled proteins. *Int. J. Radiat. Isot.* 35, 617–621.
- Kasina, S., Rao, T. N., Srinivasan, A., Sanderson, J. A., Fitner, J. N., Reno, J. M., Beaunier, P. L., and Fritzberg, A. R. (1991) Development and biologic evaluation of a kit for preformed chelate technetium-99m radiolabeling of an antibody Fab fragment using a diamide dimercaptide chelating agent. *J. Nucl. Med.* 32, 1445–1451.
- Fritzberg, A. R., Abrams, P. G., Beaumier, P. L., Kasina, S., Morgan, A. C., Rao, T. N., Reno, J. M., Sanderson, J. A., Srinivasan, A., and Wilbur, D. S. (1988) Specific and stable labeling of antibodies with technetium-99m with a diamide dithiolate chelating agent. *Proc. Natl. Acad. Sci. U.S.A.* 85, 4025–4029.
- Liu, S., and Edwards, D. S. (1994) New N_2S_2 diamidedithiol and N_3S triamidethiols as bifunctional chelating agents for labeling small peptides with ^{99m}Tc . In *Technetium and Rhenium in Chemistry and Nuclear Medicine* (M. Nicolini, G. Bandoli, and U. Mazzi, Eds.), pp 383–393, SGEditional, Padova.
- Linder, K. E., Wen, M. D., Nowotnik, D. P., Malley, M. F., Goufoutas, J. Z., Nunn, A. D., and Eckelman, W. C. (1991) Technetium labeling of monoclonal antibodies with functionalized BATO's: 1. $\text{TcCl}(\text{DMG})_3$ PITC. *Bioconjugate Chem.* 2, 160–170.
- Linder, K. E., Wen, M. D., Nowotnik, D. P., Ramalingam, K., Sharkey, R. M., Yost, F., Narra, R. K., Nunn, A. D., and Eckelman, W. C. (1991) Technetium labeling of monoclonal antibodies with functionalized BATO's: 2. $\text{TcCl}(\text{DMG})_3$ CPITC labeling of B72.3 and NP-4 whole antibodies and NP-4 F(ab')₂. *Bioconjugate Chem.* 2, 407–414.
- Baidoo, K. E., Scheffel, U., Finley, P., and Lever, S. Z. (1994) No carrier added (NCA) Tc-99m-diaminedithiolate derivatives of chemotactic peptides for imaging inflammation. *J. Nucl. Med.* 35, 19P (Abstract 67).

- (28) Jackson, S., DeGrado, W., Dwivedi, A., Parthasarathy, A., Hifley, A., Krywko, J., Rockwell, A., Markwalder, J., Wells, G., Wexler, R., Mousa, S., and Harlow R. (1994) Template-constrained cyclic peptides: design of high-affinity ligands for GP IIb/IIIa. *J. Am. Chem. Soc.* **116**, 3220–3230.
- (29) Archer, C. M., Dilworth, J. R., Jobanputra, P., Thompson, R. M., McPartlin, M., Povey, D. C., Smith, G. W., and Kelly, J. D. (1990) Development of new technetium cores containing technetium-nitrogen multiple bonds. Synthesis and characterization of some diazenido-, hydrazido- and imido-complexes of technetium. *Polyhedron* **9**, 1497–1502.
- (30) Dilworth, J. R., Jobanputra, P., Thompson, R. M., Archer, C. M., Povey, D. C., Kelly, J. D., and Hiller, W. (1992) Crystal structure of a diazenidodithiocarbamate complex of technetium, [Tc(NNC₆H₄Cl)((CH₃)₂NCS₂)₂(PPh₃)]. *Z. Naturforsch.* **46**, 449–452.
- (31) Archer, C. M., Dilworth, J. R., Jobanputra, P., Thompson, R. M., McPartin, M., and Hiller, W. (1993) Technetium diazenido complexes. Part 1. Synthesis and structures of [TcCl(NNC₆H₄Cl-4)₂(PPh₃)₂] and [TcCl(NNPh)(Ph₂PCH₂CH₂-PPh₂)₂][PF₆]·H₂O. *J. Chem. Soc., Dalton Trans.*, 897–904.
- (32) Dilworth, J. R., Jobanputra, P., Thompson, R. M., Povey, D. C., Archer, C. M., and Kelly, J. D. (1994) Technetium diazenido complexes. Part 2. Substitution chemistry of structures of [TcCl(NNC₆H₄Cl-4)₂(PPh₃)₂] and the synthesis of technetium diazenido-complexes directly from [NH₄][TcO₄]. *J. Chem. Soc., Dalton Trans.*, 1251–1256.
- (33) Abrams, M. J., Larsen, S. K., Shaikh, S. N., and Zubietta, J. (1991) Investigation of technetium-organohydrazine coordination chemistry. The crystal and molecular structures of [TcCl₂(C₈H₅N₄)(PPh₃)₂]·0.75C₇H₈ and [TcNCl₂(PPh₃)₂]·0.25CH₂-Cl₂. *Inorg. Chim. Acta* **185**, 7–15.
- (34) Plow, E. F., Marguerie, G., and Ginsberg, M. (1987) Fibrinogen, fibrinogen receptors and the peptides that inhibit these interactions. *Biochem. Pharmacol.* **36**, 4035–4041.
- (35) Burgi, H. B., Anderegg, G., and Blauenstein, P. (1981) Preparation, characterization, and crystal, molecular, and electronic structure of (H₂EDTA)⁹⁹Tc^{IV}(μ-O)₂⁹⁹Tc(H₂EDTA)·5H₂O. A 2.33-Å Tc–Tc distance which may represent a σ²π²δ*² bond. *Inorg. Chem.* **20**, 3829–3834.
- (36) Linder, K. E., Dewan, J. C., and Davison, A. (1989) Technetium bis(μ-oxo) dimers of 1,4,7-triazacyclononane-N,N',N''-triacetate (TCTA). Synthesis and characterization of [(TCTA)Tc(μ-O)₂Tc(TCTA)]ⁿ⁻ (n = 2, 3) and the crystal structure of Ba₂[(TCTA)Tc(μ-O)₂Tc(TCTA)](ClO₄)·9H₂O. *Inorg. Chem.* **28**, 3820–3825.
- (37) Dewanjee, M. K. (1990) The chemistry of ^{99m}Tc-labeled radiopharmaceuticals. *Semin. Nucl. Med.* **20**, 5–7.
- (38) Amos, L. W., and Sawyer, D. T. (1972) Proton nuclear magnetic resonance studies of several polyaminocarboxylic acid complexes of vanadium(V). *Inorg. Chem.* **11**, 2692–2697.
- (39) Crans, D. C., and Shin, P. K. (1994) Characterization of vanadium(V) complexes in aqueous solutions: ethanolamine- and glycine-derived complexes. *J. Am. Chem. Soc.* **116**, 1305–1315.

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Indium-111 Labeling of Low Density Lipoproteins with the DTPA–Bis(stearylamide): Evaluation as a Potential Radiopharmaceutical for Tumor Localization

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In order to use the LDL receptor pathway to target radionuclides to cancer sites for imaging and diagnostic purposes, a labeling procedure of LDL with ¹¹¹In using the DTPA–bis(stearylamide) (L) has been developed. This bifunctional ligand is intended to be incorporated into the phospholipid monolayer of LDL and to specifically chelate the In³⁺ cation at the surface. The ligand was incorporated into LDL in buffered medium with a 65–80% yield. The L–LDL samples are stable over a 24 h period when examined by dialysis, allowing their storage before indium-111 radiolabeling. *In vitro* studies of In–L–LDL particles show that indium labeling is rapidly achieved (1 h). More than 85% of the indium atoms are bound to the chelating functions of the incorporated DTPA derivatives and less than 10% to the nonspecific complexation sites of LDL (e.g., protein residues). After incubation in human serum, the indium activity recovered in the LDL fraction of In–L–LDL samples (95%) is much higher than in In–LDL samples (35%), pointing out the strong stabilizing chelating effect of the ligand. Competitive binding studies show that In–L–LDL are recognized by LDL receptors of A549 cells like native LDL when the In–L/LDL ratio varies from 5 to 30. All these *in vitro* experiments demonstrate that the In–L–LDL conjugates possess properties suitable for further work with *in vivo* experiments.

INTRODUCTION

Since the recognition of receptor-mediated LDL¹-uptake in tumors cells (1–8), drug targeting *via* LDL has been envisaged for improving the chemotherapy of neoplastic diseases (9–16). The use of LDL to target radionuclides to cancer sites is also of considerable interest for diagnostic or therapy purposes. Different radioisotopes have been used during the last years for labeling LDL, and their suitability for a particular application is strongly dependent upon the availability and the nuclear and chemical properties of each radiometal. In the past years several labeling procedures have been proposed for LDL. The most commonly used method has been to directly affix the radionuclide to the surface of LDL using ¹²⁵I (17, 18) and ^{99m}Tc (19–23). Another procedure for labeling LDL has been to link ⁶⁸Ga or ¹¹¹In on protein residues *via* the bicyclic anhydride of DTPA (24–26) following the protocol described by Hnatowich et al. (27). These [⁶⁸Ga]- or [¹¹¹In]DTPA–LDL conjugates permitted catabolism studies *in vivo*. All these procedures have shown the great interest of non-invasive techniques for studying LDL metabolism and biodistribution *in vivo*.

The present work aims to develop a new labeling method of LDL available for external imaging of tumors with γ cameras and diagnostic exploration in cancer patients. Among the radiometals available for such a purpose, ¹¹¹In was selected over ^{99m}Tc because it is a γ -emitter with emission energies (173 and 247 keV) perfectly fitted for scintigraphies and because compared to technetium it has a longer half-life (2.83 days instead of 6.02 h for ^{99m}Tc). Also, γ scintigraphies can be improved 24–48 h after radiopharmaceutical injection, and knowing that the LDL plasma half-life is about 3 days, studies of radiolabeled LDL uptake by tumors can be performed during this period without the need for a high amount of radioelements. Indium also presents the advantage that it exists in only one oxidation state (In³⁺) in aqueous solution in contrast to technetium. One important aspect of radiometal labeling is the method used to attach the radioelement to LDL. Among chelators which are available, DTPA was selected because it presents a high thermodynamic equilibrium constant with the indium trivalent cation ($K_f = 10^{29}$ (28)). The most common method used to conjugate DTPA to LDL has been *via* its bicyclic anhydride form (24–26). However, this bifunctional agent presents some problems; for example, it can cause *intra*- or *intermolecular* cross-linking (29–30). Therefore, the number of DTPA molecules and the amount of radioelement added have to be severely limited in order to preserve the three-dimensional structure of the recognition protein of LDL (Apo-B). To minimize structural modifications of this protein which could be deleterious for the specific LDL cell-receptor recognition (R-LDL), we have chosen to incorporate a lipophilic derivative of DTPA into the phospholipid monolayer of LDL, as has been proposed for lipid vesicles (31–34). The DTPA–bis(stearylamide) (35–36) has been selected because it possesses two long hydrocarbon chains which confer a high lipophilic character

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¹ Abbreviations: LDL, low density lipoproteins; R-LDL, specific LDL cell-receptor; Apo-B, recognition protein of LDL; DTPA, diethylenetriaminepentaacetic acid; EDTA, ethylenediaminetetraacetic acid; L, DTPA–bis(stearylamide); In-cit, indium citrate; L–LDL, LDL carrying anchored L; In–L–LDL, L–LDL labeled with indium; Tris, tris(hydroxymethyl)aminomethane; LPDS, human lipoprotein deficient serum; PBS, phosphate-buffered saline; FCS, fetal calf serum; BSA, bovine serum albumine; A, absorbance.

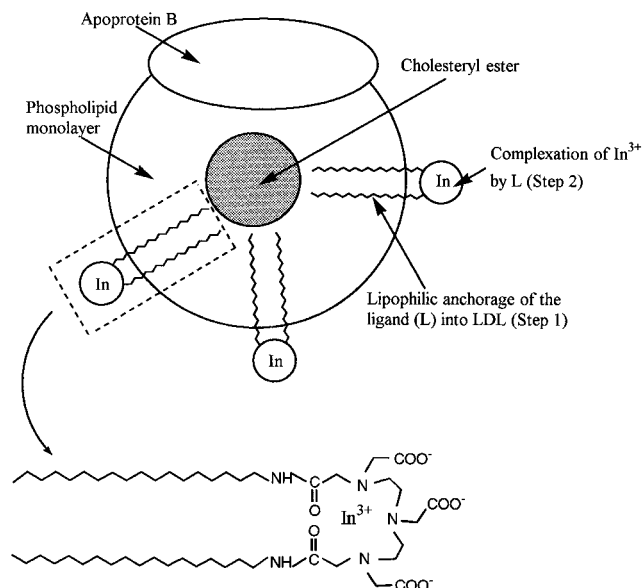


Figure 1. Schematic representation of the In-L-LDL particles and of the In-L complex. L is intended to be incorporated into the phospholipid monolayer of LDL and to chelate In^{3+} at the surface. The preparation includes two steps: (1) L are incorporated into LDL (L-LDL), and (2) L-LDL are labeled with In^{3+} (In-L-LDL).

to that ligand and because the three carboxylate functions lead to a neutral complex with In^{3+} .

The present work reports the labeling procedure of LDL with indium-111 using the DTPA-bis(stearylamine) (L) incorporated into the LDL and the evaluation of these modified LDL as potential radiopharmaceuticals for tumor localization. The labeling procedure was designed in two major steps: in step 1, the ligands L were incorporated into the LDL to give the L-LDL conjugates; in step 2, labeling of L-LDL with In^{3+} gave the In-L-LDL particles (Figure 1).

EXPERIMENTAL PROCEDURES

Reagents and Materials. All chemicals were of analytical grade, used without further purification unless otherwise indicated, and obtained from the following sources: bicyclic anhydride of DTPA, stearylamine, tropolone, tris(hydroxymethyl)aminomethane, BSA, and heparin from Sigma Chemical Co. (St. Louis, MO), InCl_3 from Aldrich Chemical Co. (St. Quentin Fallavier, France), ammonium nitrate from Merck (Darmstadt, Germany), Bio-Rad protein assay (Coomassie Blue) from Bio-Rad Laboratories (Ivry sur Seine, France), EDTA trisodium, diphenylthiocarbazone (dithizone), citric acid trisodium, solvents, acids, bases and others salts from Prolabo (Paris, France); sodium chloride solution 0.9% from Laboratoire Biosedra (Louviers, France). TLC plates (Kieselgel 60 GF 254) were purchased from Merck (Darmstadt, Germany), Sephadex G-25 columns from Pharmacia-LKB Biotechnology (Uppsala, Sweden), and membrane filters (Millipore MinisartR) from Sartorius (Göttingen, Germany). $^{111}\text{InCl}_3$ was obtained from Cis Bio International (Gif sur Yvette, France). Other materials are cited under specific sections. Deionized water was obtained from an ELGA-Filtro system, ELGASTAT UHQPS from Cofralab (Colomiers, France). Tris saline buffer pH 7.4 was prepared as follows: Tris 10 mM, NaCl 0.15 M, and pH fixed to 7.4 by adding an aqueous HCl solution.

Material for Cell Culture. A549 cells (ATCCCL 185) were obtained from the American Type Culture Collection (Rockville, MD). Sodium 125-iodide (100 mCi/mL) was obtained from Amersham (Les Ulis, France), FCS, gen-

tamycin, amphotericin, RPMI 1640 culture medium, and Dubelcco's PBS were purchased from Gibco (Les Ulis, France). Thrombin 500 was from Houdé Laboratories (Paris, France). Human plasma was collected by the Centre Inter régional de Transfusion Sanguine (Toulouse, France). Six-well multidishes (Nunc) were purchased from Poly Labo (Strasbourg, France).

Terminology. In the entire paper the term "ligand or L" refers to the bis(stearylamine) of DTPA, and the term "proteins" refers to the protein fraction of LDL, including the recognition protein, the Apo-B, which is the major constituent of this protein part.

Preparation and Assay of DTPA-Bis(stearylamine) (L). L was prepared as previously reported (36): stearylamine (0.539 g; 2 mmol) in 40 mL of chloroform at 40 °C was slowly added to a solution of DTPA bisanhydride (0.393 g; 1.1 mmol) in 50 mL of DMF. After 2 h under stirring at 40 °C, the colorless solution was cooled at 4 °C during 2 h. The white precipitate was collected by filtration, washed with acetone (3×100 mL), and dried at 80 °C overnight. The precipitate was crystallized in boiling ethanol (800 mL). After 24 h at room temperature, the small crystals were collected by filtration and washed with water (800 mL, 80 °C, 3 h) and with chloroform (800 mL, reflux, 3 h) to eliminate unreacted DTPA and stearylamine. L was obtained in 45% yield. Purity of the product was checked by TLC (chloroform/methanol/water/acetic acid, 50:30:8:4, v/v; $R_f = 0.37$). Covalent linkage of two stearyl chains per DTPA molecule and symmetry of the ligand were shown by ^1H , ^{13}C , and ^1H - ^1H COSY NMR studies (see ref 36).

Solutions of L can be prepared from basic aqueous medium (pH about 9.5) with vigorous stirring at 50 °C. L concentrations are determined by an indirect spectrophotometric method. Typically, an excess of zinc(II) sulfate is added to an aqueous solution of L (pH = 7–8). The chelating DTPA analogue combines with an equivalent amount of zinc (1:1), and the remaining "free" zinc is extracted by a chloroform solution containing dithizone. The decrease in the absorbance of the zinc dithizonate complex in the organic phase is directly proportional to the amount of L present in the aqueous phase. Absorbances are measured at 535 nm (zinc(II) dithizonate; $\epsilon = 80\,000 \text{ L mol}^{-1} \text{ cm}^{-1}$; free dithizone; $\epsilon = 12\,000 \text{ L mol}^{-1} \text{ cm}^{-1}$) and at 605 nm (free dithizone; $\epsilon = 40\,000 \text{ L mol}^{-1} \text{ cm}^{-1}$), and total absorbance at 535 nm is corrected from the contribution of the free ligand absorption with the following formula:

$$A_{\text{corrected}} = (A''_{535} - A_{535}) - A'_{605}/A'_{535} (A''_{605} - A_{605})$$

$A_{\text{corrected}}$ is proportional to the amount of L in the aqueous solution of an unknown sample; A_{535} and A_{605} are the measured absorbance of an unknown sample; A'_{535} and A'_{605} are the measured absorbance of the chloroform free dithizone solution; and A''_{535} and A''_{605} are the measured absorbance of a sample without L (blank). Calibration curves can be prepared by plotting the absorbance at 535 nm against the amount of chelating agent present in the aqueous phase. The calibration curves obey Beer-Lambert's law from 0 to 60 μM . Measurements have been carried out using a Kontron Uvikon 931 spectrophotometer (Kontron Instruments, Milan, Italy).

LDL Isolation and Lipoprotein Deficient Serum Preparation from Human Plasma. Human LDL ($d = 1.019\text{--}1.050 \text{ g/mL}$) and human lipoprotein deficient serum (LPDS) were isolated from fresh human plasma of healthy volunteers by sequential ultracentrifugation (1.5–7.5 ultracentrifuge, Beckmann Instruments, Inc.

Table 1. Incorporation Yield of L into LDL after Density Ultracentrifugation for Different L/LDL Ratios^a

	blank	0.48 mg/mL		1.5 mg/mL
initial L/LDL ratio:	no LDL	100 L/LDL	200 L/LDL	200 L/LDL
Fraction recovered after density ultracentrifugation in the density zone of LDL:				
% of L ^b	2.6 ± 0.2	65.7 ± 1.6	80.0 ± 1.1	63.7 ± 7.3
% of L/LDL ^b		66.6 ± 1.7	78.0 ± 1.1	55.2 ± 9.3
% of L/LDL ratio ^b		60.0 ± 2.5	68.5 ± 7.5	115.5 ± 20.5

^a Two L/LDL ratios were studied (100 and 200 L/LDL) at three different LDL concentrations (0, blank; 0.48 and 1.5 mg/mL, samples).

^b Final L, LDL, and L per LDL concentrations are expressed as percent of the initial values.

(Palo Alto, CA) 45 000 rpm, 10–15 °C) using a vertical rotor (VTi-50, Beckman) and KBr for density adjustment according to the method of Paumay and Ronveaux-Dupal (37). After the first 17 h of ultracentrifugation, the LPDS fraction was kept when necessary (*), and the lipoprotein fraction supernatant was centrifuged against the density gradient for 3 h. Following 48 h of dialysis against Tris saline buffer (1:100, v/v) to remove excess KBr and EDTA, LDL were immediately sterilized by filtration through a membrane filter (0.45 µm pore size). Purity of LDL was assayed as reported (23). LDL were stored at 4 °C for no longer than 2 weeks. After storage and before experimentations, LDL samples were filtered by 0.22 µm membrane filtration, and protein concentration was determined according to Bradford (38) using BSA as standard. For simplification reasons, mg/mL instead of mg protein/mL will be used as unity for LDL concentration in the text.

(*): after 48 h dialysis (1:100, v/v) against Tris saline buffer, human LPDS was treated with thrombase (250 u/100 mL) at 37 °C for 1 h, centrifuged at 1000g for 15 min, and dialyzed again. Human LPDS was sterilized by filtration and stored at –20 °C.

Variation of the Ratio L/LDL. The number of ligands L per LDL particle was varied in the range 0–200 (L/LDL ratio) in the whole reported experiments except in the sections *Binding Studies of the In-L–LDL Particles by A549 Cells* and *Stability of the In-L–LDL Particles in Plasma*. In these two last experiments, the L/LDL ratio was varied in the range 1–30 (see Discussion).

Incorporation of L into LDL. A 3.0 mM solution of L was obtained by dissolving the ligand in aqueous ammonia solution (NH₄OH/NH₄Cl, pH 9–9.5, 0.15 M) with vigorous stirring until a clear mixture was formed which was then diluted twice with water to give a 1.5 mM basic solution of L. This solution was added dropwise to a solution of LDL in Tris saline buffer under nitrogen and gentle magnetic stirring for 1 h at room temperature. The protein concentration of LDL samples was in the range 0.2 to 1.5 mg/mL. For all experiments concerning the addition of L to LDL, the volume of the added solution of L was approximately 100 times lower than the volume of the solution containing the LDL to keep constant the pH value of the final solution (7.4). The L/LDL ratio was calculated using the molecular mass of L (896.4 g/mol) and the molecular mass of the Apo-B protein (550 kDa) knowing that one LDL particle contains only one Apo-B (39–40). The L/LDL ratio and consequently the In-L/LDL ratio were varied in the following experiments to (i) validate the proteins, ligand, and indium assay methods and consequently the procedure of adding L to LDL, (ii) determine the capacity of LDL in incorporating different amounts of L, and (iii) determine the L/LDL ratio which does not lead to the precipitation of LDL.

Determination of the Average Number of L per LDL Particle. After L was added to LDL, the free ligands were separated by ultracentrifugation or dialysis (see below) and the sample was filtered by 0.22 µm

membrane filtration. After these experiments, both proteins and ligands were assayed. To validate an indirect colorimetric method (see above) for assaying L in the presence of LDL, several controls were carried out. Standard solutions of L (i.e., 0.50 mM) added to LDL samples at various concentrations (0.5–1.5 mg/mL) showed good reproducibility of the method (±7%). At this point, it was important to examine the results in two steps. In a first step, L and LDL concentrations recovered in a sample after one experiment were expressed as percentage of their initial values (L in moles and LDL in mg/mL). With this first expression of the results it was possible to display the negative effects of an experimental procedure on the L or LDL recovery from solution. In a second step, results were expressed as percentage of the initial L/LDL ratios. This global percentage could be higher than 100% when the loss of LDL was comparatively higher than the loss of L during the experimental procedure. LDL are subject to modifications mainly through oxidative reactions during incubation *in vitro*. Since lipid oxidation in LDL is generally held to precede, and to some extent to cause the modification of LDL's protein moiety, changing the net negative charge on LDL, these modifications change the LDL water solubility and cause their final precipitation. For these reasons it was important to decompose the expression of our results giving first L and LDL concentrations then L/LDL ratios.

Stability of the Lipophilic Anchorage between L and LDL. *Ultracentrifugation.* Samples of L–LDL conjugates were prepared and assayed according to the above procedures with a high and a low LDL concentration (0.48 and 1.50 mg/mL) and different L/LDL ratios (100 and 200 L/LDL). Blanks without LDL were prepared in the same way. After 1 h of incubation, the density was adjusted to 1.3 with KBr and the samples were centrifuged against the density gradient for 3 h (10–15 °C, 45 000 rpm). The density zone corresponding to LDL was collected (3 mL; 1.019 < *d* < 1.050), and KBr was removed by gel filtration on a Sephadex G-25 column using Tris saline buffer as eluant. After 0.22 µm membrane filtration, proteins and L were assayed. Triplicate experiments were performed. The initial concentrations before ultracentrifugation were the following (Table 1): blank, L: 54.6 nmol, LDL: 0 mg/mL; 100 L/LDL, L: 12.1 nmol, LDL: 0.48 mg/mL; 200 L/LDL, L: 24.2 nmol, LDL: 0.48 mg/mL; L: 27.3 nmol, LDL: 1.5 mg/mL.

Dialysis. Dialysis experiments were carried out as a function of time (96 h). Samples were prepared with three different L/LDL ratios at one LDL concentration (0.2 mg/mL). Blanks without LDL were prepared with the same L concentration as the samples containing the highest L/LDL ratio (140 L/LDL). After 0.22 µm membrane filtration, L and proteins were assayed and samples (15 mL) were introduced in the dialysis bags and dialyzed against Tris saline buffer (1:100, v/v) at 4 °C. After 1 h of dialysis, a precipitate was observed in the blanks whereas the L–LDL samples remained clear. This precipitate was collected, dried, and identified as L by IR spectroscopic analysis. Every 24 h, the dialysate was

changed, an aliquot (3 mL) of each sample was collected and filtered (0.22 μ m), and proteins and L were assayed. Two experiments were carried out, and for each one, assays were made in triplicate. The initial concentrations after filtration and before dialysis ($t = 0$ h) were as follows: (a) (blank) L, 51 nmol, LDL: 0 mg/mL; (b) L, 51 nmol, 140 L/LDL; (c) (blank) LDL, 0.2 mg/mL; (d) L, 25.5 nmol, 70 L/LDL; (e) L, 12.8 nmol, 35 L/LDL. Results for samples a and b are reported in Figure 2.

Indium Labeling and Determination of the Average Number of Indium Atoms per L-LDL Particle. The indium citrate (In-cit) solution was prepared as follows: InCl₃ (17.5 μ mol) in HCl solution (2 mL, pH 1–2) was added to a solution of sodium citrate (8 mL, 87.5 μ mol). The carrier sodium citrate was used as transfer agent to avoid the formation of indium hydroxides. The pH (about 5.5) was raised to pH 7.4 by dropwise addition of an aqueous ammonia solution (NH₄OH) to give the indium citrate solution (1.75 mM, pH 7.4). L-LDL particles were prepared as described above. The labeling was performed by slowly adding the indium citrate solution to L-LDL solutions in a metal to ligand ratio 1:1. After incubation for 1 h under nitrogen with gentle stirring at room temperature, a slight precipitate was formed and after filtration and drying identified as free In-L complexes by IR spectroscopic analysis. The insolubility of the free In-L complexes—when they are not incorporated into LDL—gave the possibility to eliminate them by filtration (0.22 μ m membrane). The insolubility of the free species In-L was also shown in the section *Indium Complexation by the Chelating Functions of L Incorporated into the LDL*. After In-L-LDL particles were prepared, protein and indium were determined to give the In/LDL ratios. Indium was assayed by electrothermic atomic absorption spectroscopy (Perkin-Elmer 110-B). Ammonium nitrate (1.5 M) was added to samples to suppress sodium interferences (final concentration 0.75 M) (41). After protein and indium assays, the average number of indium per LDL and the percentage of bound indium could be calculated. The reproducibility of this indium assay was good ($\pm 7\%$), being independent of the protein concentration in the sample (0.2–1 mg/mL).

Indium Complexation by the Chelating Functions of L Incorporated into the LDL. In the present work, the chelating functions of L (e.g., COO[−], =N−, −CONH−) incorporated into LDL are described as specific sites of indium complexation and other sites on L-LDL particles are described as nonspecific sites (e.g., protein residues).

Complexing Properties of Native LDL toward Indium: Exclusion Chromatography Experiments. In-LDL samples (2.5 mL) were prepared by slowly adding the indium citrate solution prepared as described above (818 nmol) to a sample of LDL (1.5 mg/mL in Tris saline buffer). In-L-LDL samples were prepared as described above (In³⁺, 818 nmol; L, 818 nmol; LDL, 1.5 mg/mL; final volume 2.5 mL). A blank was prepared by adding the indium citrate solution to Tris saline buffer. All samples were gently stirred under nitrogen at room temperature during 1 h and were eluted through a Sephadex G-25 column. After elution with Tris saline buffer, indium was assayed in the void and eluted volumes by electrothermic atomic absorption spectroscopy (Table 2).

Specific Complexation of Indium by L Incorporated into LDL: Stability of the Different Indium Species. Four species (In-cit; In-L, In-LDL; In-L-LDL), potentially formed in the preparations, were prepared by adding the indium citrate solution to solutions of Tris saline buffer, L, native LDL, and L-LDL. Experiments were carried out at three different indium and L concentrations with

Table 2. Percentage of Indium Recovered after Gel Exclusion Chromatography of In-cit, In-LDL,^a and In-L-LDL^{a,b} Samples

samples	indium concentration		
	In-citrate	In-LDL	In-L-LDL
initial concentration ^c (1.0 nmol)	6.82	6.82	6.82
fraction ^d (%)	100	100	100
void volume (1.0 nmol)	1.0 $\pm$ 0.9 ^e	6.1 $\pm$ 0.1	6.5 $\pm$ 0.5
fraction (%)	8 $\pm$ 3	89 $\pm$ 2	94.6 $\pm$ 0.10
eluted volume (1.0 nmol)	0.20 $\pm$ 0.04	0.55 $\pm$ 0.04	0.35 $\pm$ 0.03
fraction (%)	2.93 $\pm$ 0.01	8.06 $\pm$ 0.01	5.1 $\pm$ 0.4

^a LDL: 1.5 mg/mL. ^b 100 L/LDL. ^c Before gel filtration on Sephadex G-25 and elution with Tris-NaCl buffer. ^d After gel filtration. ^e Means $\pm$ SE of two independent experiments carried out in triplicate.

Table 3. Stability of the Four In³⁺ Species: Indium Citrate (In-cit), In-L, In-LDL and In-L-LDL in Tris Saline Buffered Solutions (A) and after Addition of Tropolone (B)

	LDL	In-cit	In-L	In-LDL	In-L-LDL
A. Fraction Recovered after Filtration and Before Addition of Tropolone ^a					
% of indium		105 $\pm$ 2.3	4.1 $\pm$ 0.5	84.8 $\pm$ 4.0	97 $\pm$ 7
% of LDL	91.3 $\pm$ 5.2			77.8 $\pm$ 7.7	95.6 $\pm$ 7.5
B. Fraction Recovered after Addition of Tropolone and after Filtration ^{a,b}					
% of indium		3.4 $\pm$ 0.05		6.6 $\pm$ 0.5	85.8 $\pm$ 5
% of LDL				82.1 $\pm$ 4.0	70.5 $\pm$ 2.0

^a Experiments were carried out at three different indium and L concentrations with a protein concentration varying from 1.07 to 1.33 mg/mL. Solutions were stirred for 1 h at 22 °C under nitrogen and for 1 h at 4 °C. Indium and proteins were assayed after filtration and expressed as percent of the initial concentration values. ^b Tropolone, the competitor ligand, was added to the three In³⁺ species, indium-citrate (In-cit), In-LDL, and In-L-LDL with a metal to competitor ligand ratio of 1:15 in Tris saline buffered solutions.

a protein concentration ranging from 1.08 to 1.33 mg/mL. For samples containing native LDL or L-LDL, the initial In/LDL or In-L/LDL ratios were equal successively to 50, 100, and 200. Each sample was gently stirred under nitrogen for 1 h at room temperature and cooled 1 h at 4 °C and then filtered (0.22 μ m) before indium and protein assays. Two experiments were carried out in triplicate, and the results are reported in Table 3.

Specific Complexation of Indium by L Incorporated into LDL: Competition with Tropolone. A competitor ligand (tropolone) was added to samples of the four species susceptible to be formed during the preparations. This ligand was chosen because it forms a stable and lipophilic complex with the In³⁺ ion, weakly soluble in water. An aqueous solution of tropolone (1.0 mmol·L^{−1}) was prepared and added in excess (indium to competitor ligand ratio 1:15) to the following samples: In-cit, native LDL, In-LDL, and In-L-LDL in Tris saline buffer. Experiments were carried out at three different indium and L concentrations with a protein concentration ranging from 0.69 to 0.81 mg/mL and initial In/LDL or In-L/LDL ratios ranging from 48 to 241. The samples were gently stirred for 1 h, during which time free indium and weakly bounded indium precipitated as tris(tropolonato)indium(III) (42) (Figure 3). After filtration, proteins and indium recovered in solution were assayed and the fraction of the initial indium concentration was determined. Two experiments were carried out in triplicate, and the results are reported in Table 3.

Table 4. Competitive Inhibition of ^{125}I -LDL Binding to LDL Receptors^a with In-L-LDL Particles and Native LDL^b

unlabeled LDL	bonded ^{125}I -LDL (fraction (%))	
	15 $\mu\text{g/mL}$	30 $\mu\text{g/mL}$
In-L/LDL ratio		
0	69 $\pm$ 4	49 $\pm$ 5
5	68 $\pm$ 4	52 $\pm$ 5
15	70 $\pm$ 3	50 $\pm$ 4
30	72 $\pm$ 3	52 $\pm$ 1

^a LDL receptors of A549 cells. ^b Means $\pm$ SE of two independent experiments carried out in triplicate.

Binding Studies of the In-L-LDL Particles by A549 Cells. LDL were iodinated with ^{125}I by the McFarlane monochloride procedure modified by Bilheimer (43). To 2 mL of an aqueous solution of LDL (4 mg proteins) were added 500 μL of glycine buffer (pH = 10), 10 μL of iodide-125 (1 mCi), and ICl (156 nmol, 6.24 μL). After gentle stirring for 1 min, samples were chromatographed on a size exclusion column (Sephadex G-25). Then iodinated LDL were collected and dialyzed during 24 h. The final specific activities varied between 150 and 250 cpm per ng of protein. The concentration of LDL and LPDS were expressed in terms of their protein content determined by the method of Bradford (38) with BSA as standard.

Cell Culture. Cells were grown in plastic tissue culture flasks (25 cm^2) with RPMI 1640 medium containing 0.02 M glucose, 0.24 M NaHCO_3 , and supplemented with gentamycin (10 mg/mL), amphotericin (200 $\mu\text{g/mL}$), and 10% (v/v) heat inactivated FCS (56 $^\circ\text{C}$, 30 min). The cell culture was kept at 37 $^\circ\text{C}$ in humidified incubators equilibrated with 5% CO_2 , 95% air. The growth medium was changed every 2 or 3 days unless confluence and cell transfer by treatment with trypsin occurred.

Competitive Binding of ^{125}I -LDL and In-L-LDL Particles by A549 Cells. Before each experiment cells were trypsinized in the exponential growth phase from stock flasks (250 mL) and seeded in 6-well multidishes (35 mm) at 50×10^3 cells/dish (day 0). At 24 h before the test (J5), cells were grown with 10% human LPDS supplemented medium. LDL-receptor binding was studied at 4 $^\circ\text{C}$ (44). The A549 cells were chilled to 4 $^\circ\text{C}$ 30 min prior the incubation for 2 h with 125 I-LDL (10 μg of protein/mL) and In-L-LDL particles (15 μg of protein/mL or 30 μg of protein/mL and 0, 5, 15, or 30 In-L complexes per LDL) or native LDL (15 μg of protein/mL or 30 μg of protein/mL). After the incubation, the dishes were chilled by placing them on ice, the medium was removed, and the cells were washed (45) with Tris buffer (50 mM, pH 7.4) containing BSA (2 mg/mL). The cells were then dissolved by incubation at room temperature for at least 30 min in 1 mL of 0.1 M NaOH. An aliquot was removed for protein determination, and the rest was counted for ^{125}I radioactivity (Table 4).

Radiochemical Labeling (Indium-111) of L-LDL Particles for *in Vitro* Stability Tests in Human Plasma (LPDS). A 200 μL aliquot of the carrier free $^{111}\text{InCl}_3$ (0.074 GBq, 2.0 mCi, 10 mCi/ μg indium atom in HCl 0.05 M) was added to 300 μL of an InCl_3 solution (1.74 μmol) in 0.05 M HCl. Two mL of sodium citrate in water (8.7 μmol) was added to this indium solution, and the pH was adjusted to 7–8 by adding 20 μL of NH_4OH (1%), leading to 669 nmol of indium atoms. ^{111}In -L-LDL were prepared by adding this solution of indium-111 citrate (830 μCi ; 830 μL ; 577 nmol) to a solution of L-LDL (L, 546 nmol; LDL, 15 mg proteins). Two blanks were prepared, the first one by adding indium-111 citrate (830 μCi ; 830 μL ; 54.6 nmol) to a solution of native LDL

(15 mg proteins, 7.5 mL) (In-LDL) and the second one by adding indium-111 citrate to Tris saline buffer (7.5 mL). Following the procedure described by Schultis et al. (46) aliquots of each sample (250 μL) were collected and added to plasma (250 μL , 10 mg of plasma-protein/mL) at room temperature. At different times, LDL fractions were precipitated by adding 500 μL of acetate buffer (sodium acetate 100 μM ; NaCl 200 μM ; pH 4.6) and 500 IU of heparin (2.5 μL). The samples were then centrifuged, and the activity was measured in the supernatant and in the pellet containing the LDL fraction. The percentage of free radiometal ions in the supernatant was taken as a measure of the instability of the indium complexation on the L-LDL particles (Figure 4).

RESULTS

Incorporation of the Lipophilic Chelator L into LDL. After isolation and purification, LDL were kept in Tris saline buffer free of EDTA to avoid competition reactions between EDTA and L in chelating the In^{3+} ion. The solubility of L under basic conditions (pH 9–9.5) and its amphiphilic character allow its incorporation into LDL in aqueous medium. To avoid possible LDL denaturation by pH variation, a small volume of a concentrated ligand solution was slowly added to the LDL solution (1:100, v/v).

To separate free ligands from L-LDL particles and to analyze the efficiency of the incorporation, density-gradient ultracentrifugation experiments were carried out. The results reported in Table 1 are expressed as percentages of the initial L and LDL concentrations in the samples. The percent of L incorporated into LDL increased from 65 to 80% when the initial L/LDL ratio increased from 100 to 200 (0.48 mg protein/mL). At the same L/LDL initial ratio (200) the ligand fraction recovered within the samples was 15% higher for the lower protein concentration than for the highest one (0.48 compared to 1.5 mg/mL). The final LDL concentration increased with initial values of L/LDL ratio, suggesting a protective effect of L on LDL. When the results were expressed as percentage of the average number of L/LDL with respect to the initial L/LDL ratio (Table 1), percentages higher than 100% were found. They are explained by a larger decrease of the LDL concentration than of the L concentration during the experimental procedure. Higher L/LDL ratios (200–400) were tested, and the results were comparable. These results have shown high and quantitative incorporation yields of L into LDL.

Stability of the L-LDL Particles. To test the stability of the lipophilic anchorage and to evaluate how long these L-LDL particles could be kept in the Tris saline buffer before labeling them with indium, dialysis experiments were performed. Before the dialysis experiments were started, control experiments were carried out to verify the effects of the two first steps, the incorporation of the ligand into LDL and the membrane filtration, on the decrease of the protein concentration. Assays revealed that 5–20% of proteins were lost during these two first steps (data not given). In spite of the precautions taken for the manipulation of the LDL (under nitrogen), the deleterious "oxidative modifications" could not be totally avoided leading to protein precipitation.

Dialysis studies (two experiments in triplicate) were carried out over a long period (96 h) against a Tris saline buffer (1:100, v/v) with different L/LDL ratios (35, 70, and 140). Curve a on Figure 2 shows the fast elimination of the free ligand in the blanks which is complete after a 24 h of dialysis. In samples containing L-LDL particles, the L/LDL ratio (140 L/LDL) is only slightly modified over

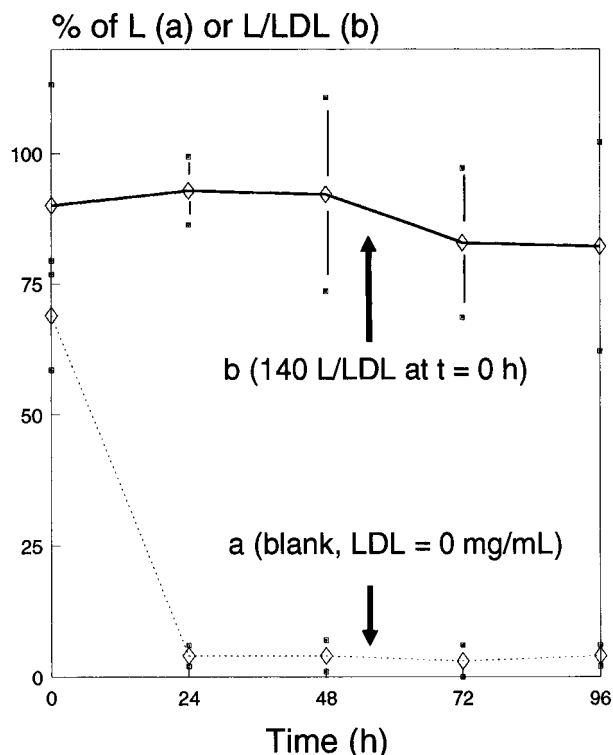


Figure 2. Stability of the lipophilic anchorage of L into LDL studied by dialysis as a function of time. At $t = 0$, blanks (a) contain L (51 nmol) and no LDL, and samples (b) contain L (51 nmol) and LDL (0.2 mg/mL) leading to 140 L/LDL. Final L (a) or L/LDL (b) concentrations are expressed as percent of the initial values.

a 96 h period (curve b on Figure 2). Percentages higher than 100% were found. They are explained by differences in loss of proteins by precipitation and loss of L. For simplification reasons, Figure 2 represents only a part of the results obtained from the studies performed by dialysis (one value for the L/LDL ratio). When the L/LDL ratios were changed (35, 70, 140 L/LDL) and when the L concentrations were analyzed, the percentage of L recovered in the samples showed little change after 24 h whatever the L/LDL ratio was. This result was very positive, taking into account the severe experimental conditions for the dialysis (v/v: 1/100). Sixty-five to 40% of the ligands remain in solution after 48 h and 40 to 20% after 96 h. In the blanks containing LDL without ligands, a decrease of 30% of the protein concentration was observed after the first 24 h, and this decrease reached 60% after 96 h. But in samples containing L and LDL the protein concentration decreased only 20% in the first 24 h and stayed nearly constant over the next 3 days, suggesting a protective effect of L on LDL, as observed after ultracentrifugation experiments.

Indium Complexation by the Chelating Functions of L Incorporated into the LDL. *Complexing Properties of Native LDL towards Indium: Exclusion Chromatography Experiments.* After labeling with In^{3+} , size exclusion chromatography was performed on In-L-LDL samples and blanks containing either In-LDL particles or indium citrate. After elution, indium was assayed in the void and eluted volumes, and the results are presented in Table 2. For the three samples, In-cit, In-LDL, and In-L-LDL, $8.1 \pm 3\%$, $89 \pm 2\%$, and $94.6 \pm 0.1\%$ of the initial amount of indium was found in the void volumes, respectively. For In-L and In-L-LDL samples, less than 8% of the initial amount of indium was found in the eluted volumes. Comparing these results, retention of the metal ion in the chromatographic

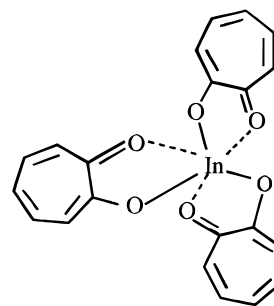


Figure 3. Schematic representation of the tris(tropolonato)-indium(III) (42).

support has been effective for the blank (indium citrate sample), but no difference was found between In-LDL and In-L-LDL samples. Both particles, LDL and L-LDL, were able to drag the In^{3+} ions along the column by elution. This chromatographic separation has failed, but it has shown the presence of nonspecific and weak complexation sites for In^{3+} on LDL in aqueous buffered solutions.

Specific Complexation of Indium by L Incorporated into LDL: Stability of the Different Indium Species. The presence of nonspecific indium complexation sites on LDL and the successive steps in the preparation suggested that four predominant species could be formed in solution: In-cit, In-L, In-LDL, and the desired species, In-L-LDL. In order to test the solubility of these four species in our experimental conditions and to test the homogeneity of the In-L-LDL samples, different preparations were made by adding indium citrate to solutions of Tris saline buffer, L, native LDL, and L-LDL. A constant protein concentration was used for three different indium and L concentrations leading to three In-L/LDL ratios (50, 100, 200). After being stirred for 1 h under nitrogen and filtration, proteins and indium were assayed, and the results are given in Table 3. In samples containing only In-cit, 100% of In^{3+} ions are recovered in solution after filtration contrary to the In-L samples where less than 5% of the initial amount of indium is found. These results have shown the insolubility of the free In-L complex in the buffered solution giving the possibility to eliminate this species simply by filtration. For the In-LDL samples, the percentage of indium recovered in solution is in the range 80–89%, and for the In-L-LDL samples, this percentage is higher and in the range 90–105%. These results are independent of the initial In/L or In-L/LDL ratios varying from 50 to 200. Protein assays on In-LDL and In-L-LDL samples (Table 3) have shown protein concentration decreasing about 10–25%. This decrease is observed for each sample independent of the In/LDL ratio and of the presence of L in the sample or not.

Specific Complexation of Indium by L Incorporated into LDL: Competition with Tropolone. To evaluate the number of indium atoms bound nonspecifically to LDL, out of the chelating groups of L, a competitor ligand, tropolone, was added to solutions containing In-cit, native LDL, In-LDL, and In-L-LDL particles in a metal to competitor ligand ratio of 1:15. This competitor ligand was selected because it forms a stable and highly lipophilic complex with indium(III) (42). This lipophilicity causes the immediate precipitation of the tris(tropolonato)indium(III) complex (Figure 3). After 1 h incubation and filtration to remove the precipitate of tris(tropolonato)indium(III), the indium fraction recovered in solution was found to be about 3.5% and 7% in samples In-cit and In-LDL, respectively, and about 86% in the sample containing In-L-LDL particles (Table 3). These experi-

ments show a large difference between LDL and L-LDL particles. Native LDL are able to retain less than 10% of the initial indium amount while L-LDL retain more than 85%. These results suggested that the amount of indium atoms bound to nonspecific sites (i.e., protein residues) could be estimated to be <10%. Table 3 showed that 70–82% of the proteins were recovered in the samples after tropolone was added.

Binding Studies of the In-L-LDL Particles by A549 Cells. The lung adenocarcinoma cell line A549 was used because the regulation of the LDL-receptor and the metabolism of LDL have been well characterized in these cells (47–48). Competitive binding of native LDL and In-L-LDL particles on LDL receptors of A549 cells were achieved with 5, 15, or 30 In-L complexes per LDL. The A549 cells were incubated with [^{125} I]LDL and unlabeled LDL after 2 h at 4 °C to avoid the receptor internalization (44) and determined rapidly the receptor bound [^{125} I]LDL. The competitive binding of [^{125} I]LDL (10 μ g/mL) and In-L-LDL particles on the LDL-receptor has been achieved at concentrations 15 μ g/mL and 30 μ g/mL corresponding to a nonsaturable specific competition (48). The [^{125} I]LDL decreased from 100% to 70% when In-L-LDL particles were at 15 μ M/mL (Table 4). No difference was observed between the native LDL and those containing 5, 15, or 30 In-L complexes. A similar result was obtained for particles at 30 μ g/mL, decreasing [^{125} I]LDL bound to 50%. These results have shown that In-L-LDL are recognized by the Apo-B receptor like the native LDL in the studied range (1–30 In-L/LDL).

Stability of the In-L-LDL Particles in Plasma. Native LDL or L-LDL particles, both labeled with indium-111, were incubated in plasma (1:1 v/v; 1 mg of LDL-protein/mL for 10 mg of plasma-protein/mL). Addition of heparin in acetate buffer (pH 4.6) as a function of time on different samples allowed the precipitation of the LDL fraction as described by Schultis and al. (46). Results are expressed as the percentage of activity in the LDL pellets divided by the total initial activity of the sample as a function of time (Figure 4). For the In-LDL samples, the indium atoms were very quickly transchelated by other plasma components (within 1 h), and after 5 h an equilibrium was reached with only 35% of the initial activity in the LDL fraction. These results showed the poor stability of the complexation of the In^{3+} cation by protein residues of the native LDL. For the In-L-LDL samples only 5% of the indium was lost within 1 h, and from 2–48 h an equilibrium was reached with 95% of the initial amount of indium recovered in the LDL fraction. These experiments revealed the high stability of the indium complexation, presumably through the DTPA chelating functions.

DISCUSSION

The objective of this study was to develop a labeling procedure of LDL with ^{111}In using a lipophilic anchorage of the metal chelator into their phospholipid monolayer which was sufficiently stable for further *in vivo* investigations. The DTPA-bis(stearylamide) was chosen because it possesses two long hydrocarbon chains which confer a high lipophilic character to the ligand with the appropriate geometry for the lipophilic anchorage. However, two carboxylate groups of the original DTPA are sacrificed for linking the two long chains. To evaluate the effects of such loss of chelating functions on the stability of the In^{3+} -DTPA-bis(stearylamide) complex compared to that of the In^{3+} -DTPA complex, structural studies by NMR spectroscopy and thermodynamic constant determination by potentiometry have been previ-

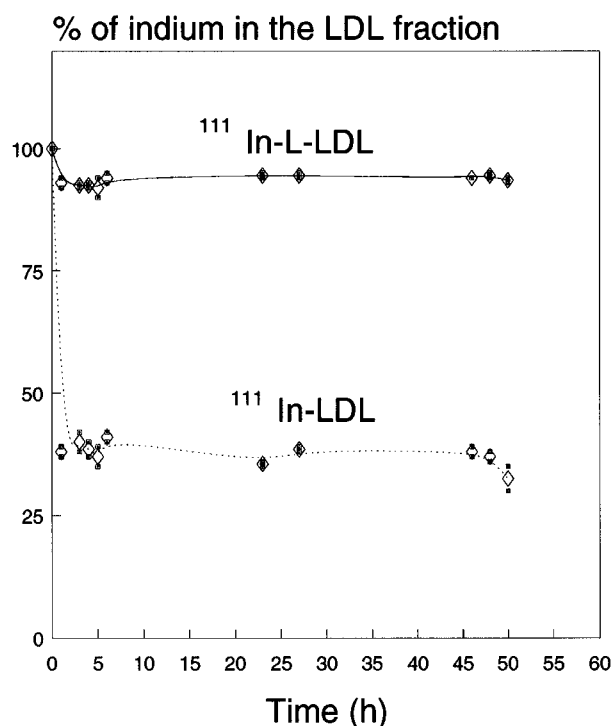


Figure 4. Stability of the ^{111}In labeling of ^{111}In -L-LDL and ^{111}In -LDL in plasma as a function of time. Results are expressed as percent of ^{111}In remaining in the final LDL fraction after precipitation of LDL with heparin.

ously carried out in solution (49). For those preliminary studies we have used a derivative with shorter hydrocarbon chains, the bis(butylamide) of DTPA for solubility reasons. NMR studies in solution at pH 7.4 showed that the In^{3+} -DTPA-bis(butylamide) complex forms a single structural isomer with an eight-coordination through the three carboxylate groups, the three amine functions, and the two amide groups (see Figure 1). Although the stability constant of the In^{3+} complex of DTPA-bis(butylamide) was 6.3 log units lower than that of DTPA ($\log K_{\text{ML}} = 22.7$ for DTPA-bis(butylamide) (49) and 29.0 for DTPA (28)), the comparison of pM values of the In^{3+} complex at pH 7.4 showed that from the standpoint of conditional stability, the In^{3+} bis(amide) complexes were promising for *in vivo* studies (pM = 20.74 and 24.39 for DTPA-bis(butylamide) and DTPA, respectively (49)). Those preliminary results were very encouraging to develop this labeling procedure for tumor imaging.

Some examples of drug incorporation into LDL have been reported by means of two principal procedures: "the dry film technique" for compounds insoluble or poorly soluble in water (14, 50) and "the direct addition" of an aqueous solution for molecules presenting both the hydrophilic and the lipophilic characters (51–52). It was possible to use the second method for the bis(stearylamide) of DTPA as it possesses amphiphilic character. The facility by which the L/LDL ratio can be modulated is one of the biggest advantages of this direct method as seen in the numerous experiments described above.

Ultracentrifugation experiments have demonstrated that the lipophilic chelator agent and the LDL interact strongly together independently of the protein concentration in the sample. Results have shown that the percentage of the average number of L per LDL with respect to the initial ratio (L/LDL) can be higher than 100%, which is explained by a comparatively higher loss in protein concentration than in ligand concentration during the manipulations. These first experiments have demon-

strated the capacity of LDL to incorporate an important number of lipophilic chelators in a quantitative way (molar ratio: 1 L/LDL to 200 L/LDL).

The first results of the dialysis experiments have shown that the free lipophilic chelating agent was eliminated mostly by precipitation in the dialysis bag after a 24 h dialysis showing its insolubility in Tris saline buffer when it was not anchored to LDL. When examined by dialysis experiments over longer periods (96 h), the L/LDL ratio is not modified after the first 24 h in spite of the harsh experimental conditions. These results demonstrate the strong affinity of the lipophilic part of the chelator for the LDL. The excellent stability of this lipophilic anchorage is the most important parameter for preparing, purifying, and storing L-LDL particles in Tris saline buffer some hours before the radiochemical labeling. An interesting result was also evidenced by the smaller decrease in the protein concentration of the L-LDL samples compared to native LDL samples. This may indicate that the ligand can chelate traces of metallic ions which can protect the LDL from oxidative processes. It has been shown that the kinetics of LDL modifications during incubation *in vitro* depend also on the composition of the incubation medium and particularly the metal ion content (53–54). The DTPA-bis(stearylamide) seems to be able to decrease the traces of metal ions in the medium. The radiolabeling with indium-111 has to be carefully performed to avoid the precipitation of indium hydroxides which are responsible for low radiochemical labeling yields. This can be achieved by using sodium citrate as transfer chelating agent and by slowly adding the ligand solubilized in an aqueous ammonia solution to the LDL solution. Studies on In-L-LDL particles have shown that indium labeling is rapidly achieved within 1 h. Size exclusion chromatography experiments showed a comparable behavior of the In-L and In-L-LDL particles after assays of indium in both the void and eluted volumes. Size exclusion chromatography is a nondestructive method which is currently employed to separate LDL from salts in solution (as we used it in different experiments). In this experiment, we would have expected that, in the case of In-LDL, the In^{3+} ions would not have been excluded at the same time as LDL. This chromatographic separation has failed, but it has shown the presence of nonspecific and weak complexation sites for In^{3+} on LDL in aqueous buffered solutions. Tropolone competition studies have shown a high affinity of indium atoms for L incorporated into LDL: more than 85% of the indium atoms are chelated by L.

The capacity of LDL to incorporate a large number of lipophilic chelating ligands (0–200) and the creation of specific sites of metal ion complexation on the L-LDL conjugates give the possibility to carry an important number of metal ions per LDL which could be of various types for therapy or diagnostic purposes. This possibility could be of great value to transport, for example, gadolinium(III) ions to tumor cells for improving the detection of tumors by magnetic resonance imaging (MRI). However, the present work was devoted to the radiolabeling of LDL with indium-111 for external imaging of tumors with γ scintigraphy, which, in terms of metal ion concentration necessary to the quality of the image contrast, is a more sensitive technique than MRI. Moreover, the radioactivity dose which has to be injected *in vivo* has to be limited to avoid deleterious effects, and a large enough quantity of LDL has to be handled for one injection. For these reasons, the L/LDL ratio was varied in a smaller range (0–30 L/LDL) in the biological tests on the ^{111}In -L-LDL particles (stability in plasma and binding studies).

After incubation for 48 h in human serum, the indium activity (95%) recovered in the LDL fraction of In-L-LDL samples is much higher than that recovered in In-LDL samples (35%), pointing out the strong stabilizing chelating effect of the ligand. In the case of the In-LDL samples, the 35% value of ^{111}In remaining on the LDL after 48 h could reflect a fair stability of the In-LDL in serum. But, in fact, the In^{3+} ions, which are weakly bound to the native LDL at the beginning of the experiment, distribute themselves on all possible binding sites of the whole proteins present in the plasma (transferrin, albumin, apoprotein B, ...). This 35% value is representative of the percentage of protein residues on LDL compared to the total protein residues present in the closed volume of the experiment. When examined by competitive binding studies, the In-L-LDL particles are recognized by LDL receptors of A549 cells like native LDL when the In-L/LDL molecular ratio was varied from 5 to 30 In-L/LDL. All these *in vitro* experiments demonstrate that the In-L-LDL conjugates possess the suitable properties for proceeding with *in vivo* experiments.

In conclusion, this work has shown that LDL may be labeled with indium-111 using the lipophilic chelator DTPA-bis(stearylamide). The lipophilic anchorage of the chelator allowed In-L-LDL particles to bind LDL receptors of A549 cells *in vitro* like native LDL for an In-L to LDL ratio of 30. Moreover, this radiolabeling procedure is rapid and can be stoichiometrically modulated. This new procedure appears to be of very great value for diagnostic or therapeutic purposes with various metal ions. Cellular uptakes of In-L-LDL and *in vivo* studies on tumor bearing mice are under study. Pharmacological studies (toxicity and clearance of In-L) will be performed in the future.

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LITERATURE CITED

- (1) Ho, Y. K., Smith, G. S., Brown, M. S., and Goldstein, J. L. (1978) Low-density lipoprotein (LDL) receptor activity in human acute myelogenous leukemia cells. *Blood* 52, 1099–1104.
- (2) Simpson, E. R., Bilheimer, D. W., MacDonald, P. C., and Porter, J. C. (1979) Uptake and degradation of plasma lipoproteins by human choriocarcinoma cells in culture. *Endocrinology* 104, 8–16.
- (3) Gal, D., MacDonald, P. S., Porter, J. C., Smith, J. W., and Simpson, E. R. (1981) Effect of cell density and confluency on cholesterol metabolism in cancer cells in monolayer culture. *Cancer Res.* 41, 473–477.
- (4) Vitols, S., Gahrton, G., Ost, A., and Peterson, C. (1984) Elevated low density lipoprotein receptor activity in leukemic cells with monocytic differentiation. *Blood* 63, 1186–1193.
- (5) Hynds, S. A., Welsh, J., Stewart, J. M., Jack, A., Soukop, M., McArdle, C. S., Calman, K. C., Packard, C. J., and Shepherd, J. (1984) Low-density lipoprotein metabolism in mice with soft tissue tumors. *Biochim. Biophys. Acta* 795, 589–595.
- (6) Rudling, M. J., Stahle, L., Peterson, C., and Skoof, L. (1986) Content of low density lipoprotein receptors in breast cancer tissue related to survival of patients. *Brit. Med. J.* 292, 580–582.

- (7) Lombardi, P., Norata, G., Maggi, F. M., Canti, G., Franco, P., Nicolin, A., and Catapano, A. L. (1989) Assimilation of LDL by experimental tumors in mice. *Biochim. Biophys. Acta* 1003, 301–306.
- (8) Rudling, M. J., Angelin, B., Peterson, C. O., and Collins, V. P. (1990) Low density lipoprotein receptor activity in human intracranial tumors and its relation to the cholesterol requirement. *Cancer Res.* 50, 483–487.
- (9) Gal, D., Ohashi, M., MacDonald, P. C., Buchsbaum, H. J., and Simpson, E. R. (1981) Low density lipoprotein as a potential vehicle for chemotherapeutic agents and radionuclides in the management of gynecologic neoplasms. *Am. J. Obstet. Gynecol.* 139, 877–885.
- (10) Iwanik, M. J., Shaw, K. V., Ledwith, B. J., Xanovich, S., and Shaw, J. M. (1984) Preparation and interaction of a low density lipoprotein–daunomycin complex with P388 leukemic cells. *Cancer Res.* 44, 1206–1215.
- (11) Masquelier, M., Vitols, S., and Peterson, C. (1986) Low density lipoprotein (LDL) as a carrier of antitumoral drugs: in vivo fate of drug–LDL complexes in mice. *Cancer Res.* 46, 3842–3847.
- (12) Lundberg, B. (1987) Preparation of drug-low density lipoprotein complexes for delivery of antitumoral drugs via the low density lipoprotein pathway. *Cancer Res.* 47, 4105–4108.
- (13) Samadi-Baboli, M., Favre, G., Blancy, E., and Soula, G. (1989) Preparation of low density lipoprotein-9-methoxy-ellipticin complex and its cytotoxic effect against L1210 and P388 leukemic cells in vitro. *Eur. J. Clin. Oncol.* 25, 233–241.
- (14) Samadi-Baboli, M., Favre, G., Bernadou, J., Berg, D., and Soula, G. (1990) Comparative study of the incorporation of ellipticine-esters into low-density lipoprotein (LDL) and selective cell uptake of drug–LDL complex via the LDL receptor pathway in vitro. *Biochem. Pharmacol.* 40, 203–212.
- (15) Lestavel-Delattre, S., Martin-Nizard, F., Clavey, V., Testard, P., Favre, G., Doualin, G., Houssaini, S. H., Bard, J. M., Duriez, P., Delbart, C., Soula, G., Lesieur, D., Lesieur, I., Cazin, J.-C. and Fruchart, J.-C. (1992) Low-density lipoprotein for delivery of an acrylophenone antineoplastic molecule into malignant cells. *Cancer Res.* 52, 3629–3635.
- (16) Chris de Smidt, P., and van Berkel, T. J. C. (1992) Characteristics of association of oleoyl derivatives of 5-fluorodeoxyuridine and methotrexate with low-density lipoproteins (LDL). *Pharm. Res.* 9, 565–569.
- (17) Moerlein, S. M., Dalal, K. B., Ebbe, S. N., Yano, Y., and Budinger, T. F. (1988) Residualizing and non residualizing analogues of low density lipoprotein as iodine-123 radiopharmaceuticals for imaging LDL catabolism. *Nucl. Med. Biol.* 15, 141–149.
- (18) Hay, R. V., Flemming, R. M., Ryan, J. W., Williams, K. A., Stark, V. J., Lathrop, K. A., and Harper, P. V. (1991) Nuclear imaging analysis of human low density lipoprotein biodistribution in rabbits and monkeys. *J. Nucl. Med.* 32, 1239–1245.
- (19) Lees, R. S., Garabedian, H. D., Lees, A. M., Schumacher, D. J., Miller, A., Isaacsohn, J. L., Derksen, A., and Strauss, H. W. (1985) Technetium-99m low density lipoproteins: preparation and biodistribution. *J. Nucl. Med.* 26, 1056–1062.
- (20) Isaacsohn, J. L., Lees, A. M., Lees, R. S., Strauss, H. W., Barlai-kovach, M., and Moore, T. J. (1986) Adrenal imaging with technetium-99m-labelled low density lipoproteins. *Metabolism* 35, 364–366.
- (21) Vallabhajosula, S., Paidi, M., Badimon, J. J., Le, N. A., Goldsmith, S. J., Fuster, V., and Ginsberg, H. N. (1988) Radiotracers for low density lipoprotein biodistribution studies in vivo: technetium-99m low density lipoprotein versus radioiodinated low density lipoprotein preparations. *J. Nucl. Med.* 29, 1237–1245.
- (22) Lees, A. M., Lees, R. S., Schoen, F. J., Isaacsohn, J. L., Fischman, A. J., McKusick, K. A., and Strauss, H. W. (1988) Imaging human atherosclerosis with 99mTc-labelled low density lipoproteins. *Arteriosclerosis* 8, 461–470.
- (23) Ponty, E., Favre, G., Benaniba, R., Boneu, A., Lucot, H., Carton, M., and Soula, G. (1993) Biodistribution study of 99mTc-labeled LDL in B16-Melanoma-bearing mice. Visualization of a preferential uptake by the tumor. *Int. J. Cancer* 54, 1–7.
- (24) Virgolini, I., Angelberger, P., Li, S. R., Koller, F., Koller, E., Pidlich, J., Lupattelli, G., and Sinzinger, H. (1991) Indium-111-labeled low-density lipoprotein binds with higher affinity to the human liver as compared to iodine-123-low-density-labeled lipoprotein. *J. Nucl. Med.* 32, 2132–2138.
- (25) Moerlein, S. M., Daugherty, A., Sobel, B. E., and Welch, M. J. (1991) Metabolic imaging with gallium-68- and indium-111-labeled low-density lipoprotein. *J. Nucl. Med.* 32, 300–307.
- (26) Rosen, J. M., Butler, S. P., Meinken, G. E., Wang, T. S. T., Ramakrishnan, R., Srivastava, S. C., Alderson, P. O., and Ginsberg, H. N. (1990) Indium-111-labeled LDL: a potential agent for imaging atherosclerotic disease and lipoprotein biodistribution. *J. Nucl. Med.* 31, 343–349.
- (27) Hnatowitch, D. J., Childs, R. L., Lantegne, D., and Najafi, A. (1983) The preparation of DTPA-coupled antibodies radiolabeled with metallic radionuclides: an improved method. *J. Immun. Methods* 65, 147–157.
- (28) Smith, R. M., and Martell, A. E. (1974) *Critical Stability Constants*, Vol. 1; (1982) Vol. 5; (1989) Vol. 6, Plenum Press, New York.
- (29) Hnatowitch, D. J., and McGann, J. (1987) DTPA-coupled proteins—Procedures and precautions. *Nucl. Med. Biol. Int. J. Radiat. Appl. Instrum. B* 14, 563–568.
- (30) Maisano, F., Gozzini, L., and de Haën, C. (1992) Coupling of DTPA to proteins: a critical analysis of the cyclic dianhydride method in the case of insulin modification lipidic vesicles. *Bioconjugate Chem.* 3, 212–217.
- (31) Kabalka, G. W., Davis, M. A., Moss, T. H., Buonocore, E., Hubner, K., Holmberg, E., Maruyama, K., and Huang, L. (1991) Gadolinium-labeled liposomes containing various amphiphilic Gd-DTPA derivatives: targeted MRI contrast enhancement agents for the liver. *Magn. Reson. Med.* 19, 406–415.
- (32) Bard, D. R., Knight, C. G., and Page Thomas, D. P. (1983) The retention and distribution in the rabbit knee of a radionuclide complexed with a lipophilic chelator in liposomes. *Clin. Exp. Rheumatol.* 1, 113–117.
- (33) Grant, C. W. M., Karlik, S., and Florio, E. (1989) A liposomal MRI contrast agent: phosphatidylethanolamine–DTPA. *Magn. Reson. Med.* 11, 236–243.
- (34) Ahkong, Q. F., and Tilcock, C. (1992) Attachment of 99mTc to lipid vesicles containing the lipophilic chelate dipalmitoylphosphatidylethanolamine–DTPA. *Nucl. Med. Biol. Int. J. Radiat. Appl. Instrum. Part B* 19, 831–840.
- (35) Hnatowitch, D. J., Friedman, B., Clancy, B., and Novak, M. (1981) Labeling of preformed liposomes with Ga-67 and Tc-99m by chelation. *J. Nucl. Med.* 22, 810–814.
- (36) Jasanada, F., and Nepveu, F. (1992) Synthesis of amphiphilic chelating agents: bis(hexadecylamide) and bis(octadecylamide) of diethylenetriaminepentaacetic acid. *Tetrahedron Lett.* 33, 5745–5748.
- (37) Paumay, Y., and Ronveaux-Dupal, M. F. (1985) Rapid preparative isolation of concentrated low density lipoprotein and of lipoprotein-deficient serum using vertical rotor gradient ultracentrifugation. *J. Lipid Res.* 26, 1476–1480.
- (38) Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72, 248–254.
- (39) Kostner, G. M., and Laggner, P. (1989) Chemical and physical properties of lipoproteins. *Human Plasma Lipoproteins* (J. C. Fruchary, and J. Shepherd, Eds.) pp 23–51, Walter de Gruyter, Berlin.
- (40) Yang, C. Y., Chen, S. H., Gianturco, S. H., Bradley, W. A., Sparrow, J. T., Tanimura, M., Li, W. H., Sparrow, D. A., De Loof, H., Rosseneu, M., Lee, F. S., Gu, Z. W., Gotto, A. M., and Chan, L. (1986) Sequence, structure, receptor-binding domains and internal repeats of human Apo B 100. *Nature (London)* 323, 738–742.
- (41) Price, W. J. (1979) *Spectrochemical Analysis by Atomic Absorption*, pp 194–195, J. Wiley & Sons, New York.
- (42) Nepveu, F., Jasanada, F., and Walz, L. (1993) Structural characterization of the two lipophilic tris(tropolonato) Gallium(III) and Indium(III) complexes of radiopharmaceutical interest. *Inorg. Chim. Acta* 211, 141–147.

- (43) Bilheimer, D. W., Eisenberg, S., and Levy, R. I. (1972) The metabolism of very low density lipoprotein proteins I. Preliminary in vitro and in vivo observations. *Biochim. Biophys. Acta* 260, 212–221.
- (44) Oram, J. F., Albers, J. J., and Bierman, E. L. (1980) Rapid regulation of the activity of the low density lipoprotein receptor of cultured human fibroblasts. *J. Biol. Chem.* 255, 475–485.
- (45) Golstein, J. L., and Brown, M. S. (1974) Binding and degradation of low density lipoprotein by cultured human fibroblasts: comparison of cells from a normal subject and from a patient with homozygous familial hypercholesterolemia. *J. Biol. Chem.* 249, 5153–5162.
- (46) Schultis, H. W., von Baeyer, H., Neitzel, H., and Riedel, E. (1991) Preparation of nucleoside-LDL-conjugates for the study of cell-selective internalization: stability characteristics and receptor affinity. *Eur. J. Clin. Chem. Clin. Biochem.* 29, 665–674.
- (47) Shiroeda, O., Yamaguchi, N., and Kawai, K. (1987) Stimulation of low Density lipoprotein receptor activity by conditioned medium from a human cancer cell line. *Cancer Res.* 47, 4630–4633.
- (48) Gueddari, N., Favre, G., Hachem, H., Marek, E., Le Gaillard, F., and Soula, G. (1993) Evidence for up-regulated low density lipoprotein receptor in human lung adenocarcinoma cell line A549. *Biochimie* 75, 811–819.
- (49) Geraldès, C. F. G. C., Delgado, R., Costa, J., Urbano, A. M., Jasanada, F., and Nepveu, F. (1995) Complexes of Ga³⁺ and In³⁺ with the N,N'-Bis(butylamide) Derivative of Diethylene triaminepentaacetic Acid: Stability Constants and Nuclear Magnetic Resonance Studies in Aqueous Solution. *J. Chem. Soc., Dalton Trans* 3, 327–335.
- (50) Shaw, J. M., Shaw, K. V., Yanovich, S., Iwanik, M., Futch, W. S., Rosowsky, A., and Schook, L. B. (1987) Delivery of lipophilic drugs using lipoproteins. *Ann. N.Y. Acad. Sci.* 507, 252–271.
- (51) Candice, C., Morliere, P., Maziere, J. C., Goldstein, S., Santus, R., Dubertret, L., Reyftmann, J. P., and Polonovski, J. (1986) In vitro interaction of the photoactive anticancer porphyrin derivative photofrin II with low density lipoprotein, and its delivery to cultured human fibroblasts. *FEBS Lett.* 207, 133–138.
- (52) Rudling, M. J., Collins, V. P., and Peterson, C. O. (1983) Delivery of aclacinomycin A to human glioma cells in vitro by the low-density lipoprotein pathway. *Cancer Res.* 43, 4600–4605.
- (53) Steinberg, D., Parthasarathy, S., Carew, T. E., Khoo, J. C., and Witztum, J. L. (1989) Beyond cholesterol: modification of low-density lipoprotein that increase its atherogenicity. *N. Engl. J. Med.* 320, 915–924 and references therein.
- (54) Pech, M. A., Myara, I., Moatti, N., and Caprani, A. (1993) Influence of culture medium characteristics on the ability to oxidize low-density lipoproteins. *Bioelectrochem. Bioenerg.* 29, 277–288 and references therein.

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***N*^ε-Cyclosuccinyllysine: Synthesis and Formation from Hemisuccinylated Polylysine and Its Hapten Conjugates with Sulfamethoxazole during Acid Hydrolysis**

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The novel amino acid *N*^ε-cyclosuccinyllysine (**4**) forms as a side reaction during acid hydrolysis of *N*^ε-hemisuccinylated succinylsulfamethoxazole–polylysine conjugates. The presence of **4** in hydrolysates can be obscured in amino acid analysis. Identification of **4** was based on chromatographic and electrophoretic comparisons with authentic *N*^ε-cyclosuccinyllysine, which was synthesized in high yield by acid-catalyzed ring closure, under anhydrous conditions, of *N*^ε-hemisuccinyl-L-lysine. The latter was prepared by an improved route, starting from *N*-α-(*tert*-butoxycarbonyl)-L-lysine. The amount of **4** formed is influenced by the extent of succinylation and the conditions of hydrolysis and has reached 30 mol % of lysine. Both the hemisuccinyllysyl and the haptenized succinyllysyl residues participate in cyclization. This was confirmed by the synthesis and hydrolytic behavior of hemisuccinylated polylysine (*n* = 16) and *N*⁴,*N*^ε-succinyl(sulfamethoxazole)(lysine). Formation of **4** can result in error in the estimation of the degree of ligand substitution based on lysine content in synthetic hemisuccinylated skin test antigens and peptide immunogens, especially when the degree of epitope substitution is low. **4** appears to be sufficiently stable for trial as an amino acid surrogate.

INTRODUCTION

Hemisuccinylation has long been in use as a stratagem to confer water solubility to drugs such as steroids. Hemisuccinylation of certain peptide derivatives yields solubilized substrates useful for assaying a variety of enzymatic activities. Skin test antigens consisting of polylysines substituted with drug moieties derived from penicillin G or sulfamethoxazole also are frequently hemisuccinylated as a final solubilizing step. Treatment of foods and feeds with succinic anhydride seems to confer desirable physical but undesirable nutritional qualities (1). Recently, albumin and transferrin hemisuccinylates have appeared promising as novel iron-carrying drugs for treatment of iron deficiency (2). The succinyl group also has long been in use in the preparation of immunogenic conjugates, serving as a carrier attaching small molecules to polylysine (3) or proteins (4). In view of the continuing interest in succinylated polypeptides and proteins, we wish to report a little known side reaction we recently encountered in analyzing a series of synthetic succinylated polylysine conjugates.

In connection with a drug allergy program whose goal is development of candidate skin test antigens, four polylysine conjugates substituted to varying degrees with sulfamethoxazole via a succinyl bridge (**1b–e**, Figure 1) had been synthesized (5). Each conjugate employed PL (*n* = 16, **1a**) as starting material and a final step in which the unsubstituted amino groups were hemisuccinylated. The conjugates were subjected to conventional quantitative ion-exchange amino acid analysis for lysine (**2a**) for use in calculation of the percent substitution by the bridged sulfonamide. Unexpectedly, a significant peak near the position of valine (**3**) was present in addition to

lysine in each hydrolysate. This paper concerns the identification of this material as *N*^ε-cyclosuccinyllysine (**4**) and presents evidence that it is a byproduct of hydrolysis of both the *N*^ε-hemisuccinylated polylysine residues and the haptenized succinyl bridges. For identification purposes, a synthetic route to *N*^ε-cyclosuccinyllysine was developed. In addition, an improved route to the *N*^ε-hemisuccinyllysine (**5**) that served as a precursor is given. As a model for conjugates and proteins containing hemisuccinylated lysine, hemisuccinylated polylysine (*n* = 16, **1f**) was synthesized. As a model for the drug-substituted succinyl bridge in conjugates **1b–e**, the new *N*⁴,*N*^ε-succinyl(sulfamethoxazole)(L-lysine) (**7**) was also synthesized. The hydrolytic behavior of both model compounds in acid was examined quantitatively and compared with that of the conjugates. To examine its suitability for use as an amino acid surrogate, the stability profile of the new amino acid **4** was determined in aqueous solution under various conditions of pH.

EXPERIMENTAL PROCEDURES

Materials. *N*-α-Boc-L-lysine,¹ *N*^ε-DNP-L-lysine, and succinic anhydride were obtained from Sigma, St. Louis, MO. Poly(L-lysine) hydrobromide (*n* = 16) was from Miles-Yeda LTD, Miles Scientific, Naperville, IL. THF and HBr/30% acetic acid were from Aldrich Chemical Co., Milwaukee, WI. Amberlite CG-50, 100–200 mesh, was from Mallinkrodt Chemical Works, St. Louis, MO. Triethylamine was from Eastman Kodak Co., Rochester, NY. TFA was from Matheson, Coleman & Bell, Norwood, OH.

General Chemical Procedures. Melting points, evaporations, and elemental analyses were performed as described previously (5). Proton magnetic resonance

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¹ Abbreviations: Boc, *tert*-butoxycarbonyl; Cbz, benzyloxycarbonyl; TFA, trifluoroacetic acid; THF, tetrahydrofuran; DNP, 2,4-dinitrophenyl; PL, poly(L-lysine); SMX, sulfamethoxazole; AAA, amino acid analysis; PITC, phenyl isothiocyanate; PITC-AAA, amino acid analysis with precolumn derivatization with PITC; *t*_R, retention time.

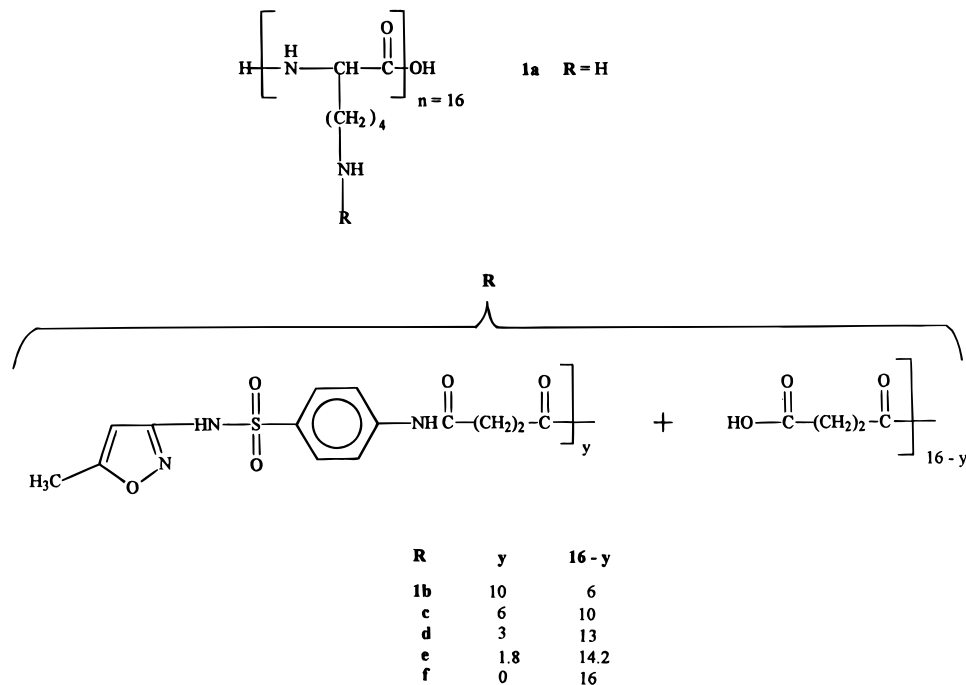


Figure 1. Structures of polylysine (1a), hemisuccinylated polylysine-sulfamethoxazole conjugates (1b–e), and hemisuccinylated polylysine (1f).

spectra were recorded on a Bruker AC-270 or a Varian EM 360 spectrometer at concentrations near 0.2 M. Chemical shifts are given as δ values in parts per million (ppm) with the HDO peak at 4.63 used as internal reference in aqueous solution and in ppm downfield from tetramethylsilane in nonaqueous solution. Multiplicities of resonances are described as singlet (s), triplet (t), or multiplet (m).

Hydrolyses and Amino Acid Analyses. Sulfa drug-polylysine conjugates (50 μg) were hydrolyzed in 0.6 mL of 6 N HCl/2% phenol for 1 h at 150 $^{\circ}\text{C}$ *in vacuo*. After suitable dilution, hydrolysates were analyzed quantitatively on a Beckman 7300 ion-exchange amino acid analyzer with ninhydrin detection. A 12 cm column was used with sodium citrate/HCl buffers (2%, pH 3; 1.7%, pH 4) and then 1.9%/5% sodium chloride (pH 6). The flow rate was 21 mL/h (6, 7). AAA was also carried out by precolumn derivatization of hydrolysates (150 $^{\circ}\text{C}$) with PITC and the resultant compound analyzed by high-performance liquid chromatography (HPLC) (8). Other hydrolyses were carried out in 6 N HCl under nitrogen in sealed tubes at 120 $^{\circ}\text{C}$ for 18 h and the compounds analyzed by paper electrophoresis and/or analytical thin layer chromatography (TLC). Bound SMX was determined by HPLC after alkaline hydrolysis (5).

Hemisuccinylation of 1f was evaluated indirectly by application of the DNP end group method (9). The colorless extracted reaction mixture was hydrolyzed and analyzed colorimetrically for N^ε-DNP-Lys at 360 nm.

HPLC was carried out on a Waters 30 cm $\mu\text{Bondapak C-18}$ (10 μm) column using two Waters M 6000 pumps and a 660 solvent controller. The gradient was linear from 5 to 60% CH₃OH in 0.025% AcOH (pH 4) over 20 min at 1 mL/min (system E). PITC derivatives were analyzed on a Rainin 15 cm Microsorb C-18 (5 μm) column. The gradient was concave from 0 to 100% of 60% acetonitrile in 12.5 mM sodium phosphate buffer (pH 6.8) over 24 min at 1 mL/min at room temperature, with 50% mixing attained at 17 min (system F). A similar gradient went from 0 to 50% of 60% acetonitrile in the buffer over 24 min (system G).

Paper Electrophoresis was carried out on Whatman #1 strips in 0.1 M sodium acetate buffer (pH 5.6) at 11 V/cm for 2.5 h at room temperature and detected with 0.1% ninhydrin in acetone.

Analytical TLC was carried out on Eastman Chromagram, polymer-backed, fluorescent indicator cellulose 13254 strips or Merck cellulose 5503 plates with butanol/pyridine/acetic acid/water, 15:10:3:12 (system A) or 15:8.3:2.5:7.5 (system B), or on fluorescent indicator silica gel 13181 strips with CHCl₃/CH₃OH, 1:1 (system C) or 3:7 (system D), and detected with ninhydrin or quenching of fluorescence. For stability and other studies requiring quantitation, the ninhydrin response on TLC was photocopied onto an overhead projection transparency (Labelon Corp., Canadaigua, NY). The transparency was scanned on a densitometer, model PD, with Imagequant software (Molecular Dynamics, Sunnyvale, CA). Standards and samples were in duplicate over an appropriate linear range.

Chemical Syntheses. Conjugates of Sulfamethoxazole and Polylysine. (SucSMX)₁₀-PL₁₆-suc₆ (1b) as well as the conjugate before hemisuccinylation were available from a previous study (5). Conjugates 1c–e were prepared similarly from PL·HBr ($n = 16$) and sucSMX-NSu (6) (5) with ratios of active ester/PL NH₂ of 1.1, 0.44, and 0.22, respectively.

Hemisuccinylated Polylysine (1f). A solution of PL·HBr ($n = 16$, 30 mg, 0.155 molar equiv of NH₂) in 1 mL of water was adjusted with 1 N NaOH to pH 8. Succinic anhydride (154 mg, 1.54 mmol) was added in portions with magnetic stirring over 40 min along with 5 N NaOH to maintain pH 8. The mixture was stirred for an additional 45 min and then dialyzed (tubing cutoff, 1000 Da) against water (3 $\times$ 2 L changes) at 4 $^{\circ}\text{C}$ over 2 days. Lyophilization gave 29.2 mg. The DNP end group method indicated that less than 0.1% of the amino groups remained unsuccinylated.

N^ε-Hemisuccinyl-L-lysine, [N^ε-(3-Carboxy-1-oxopropyl)-L-lysine, 5]. To a slurry of N- α -Boc-L-lysine (2b, 4 g, 16.2 mmol) and triethylamine (8.8 mL, 63.2 mmol) in 120 mL of dry THF was added a solution of succinic anhydride

(1.7 g, 17.8 mmol) in 40 mL of THF, and the mixture was stirred at 24 °C. The progress of the reaction was monitored by TLC in system C with ninhydrin. After 22 h, the slightly turbid solution was filtered quickly under anhydrous conditions. The solvent was then evaporated. The residual gum was dissolved in 30 mL of cold water. The solution was immersed in an ice bath and was carefully adjusted to pH 3 with 6 N HCl followed by 0.25 N HCl. The solution was then quickly extracted with 4 × 50 mL portions of EtOAc. The extract was washed with 3 × 5 mL portions of cold saturated NaCl, dried (MgSO₄), and concentrated to dryness. The residue was dried over P₂O₅ (5.78 g). TFA, 25 mL, was added, and the solution was maintained at 12–15 °C for 30 min. TFA was then removed by evaporation. The syrup was dissolved in 30 mL of EtOH, and the solution was chilled in an ice bath and adjusted to pH 4 with concentrated NH₃. After 4 h at –20 °C, the white solid was collected on the filter and washed with cold 30% EtOH followed by EtOH (3.05 g) (77%); mp 218–221 °C. The material was homogeneous on TLC in system B (*R_f* = 0.19) as well as on paper electrophoresis at pH 5.6, traveling 3.3 cm from the origin toward the anode. The product was recrystallized from 0.5% AcOH: mp 221–222 °C [lit. 227–230 °C (1)]. It eluted as a single peak on AAA, with *t_R* close to that of Glu: ¹H NMR (D₂O) δ 1.29 (m) + 1.31 (m) + 1.63 (m), 6 (C-4, C-5, C-3 CH₂); 2.3 (t, 2) + 2.4 (t, 2, succinyl CH₂); 2.96 (t, 2, C-6 CH₂); 3.51 (t, 1, CH); 4.63 (s, HDO).

N^ε-Cyclosuccinyllysine [6-*N*-(Pyrrolidine-2,5-dione)-2-aminohexanoic acid, **4**]. A solution of crude **5** (1.5 g, 6.1 mmol) in 25 mL of 30% HBr/AcOH in a 180 mL dry glass pressure bottle was kept in an oil bath held at 120 °C for 65 h. The mixture was concentrated under reduced pressure at 80–100 °C to a dark thick syrup that was dried further over P₂O₅ and KOH. The syrup was dissolved in 3 mL of water, and the solution was adjusted to pH 4.5 with concentrated NH₃ and then applied to a column (2.5 × 48 cm) of Amberlite CG-50 (H⁺ cycle, 100–200 mesh). The column was treated with 0.5% AcOH at a flow rate of 40 mL/h, and 1.6–2 mL fractions were collected. Ninhydrin-positive fractions were examined by TLC on Merck cellulose plates in system B, and appropriate fractions were pooled and lyophilized. Combined eluates (121–227 mL) (472 mg) contained halide and small amounts of **2a**, **4**, and **5** and were not processed further. Combined eluates (227–327 mL) yielded 406 mg (28%) of **4** containing 3% of **5**. Combined eluates (328–530 mL) gave 765 mg of **4** (55%) that was homogeneous on TLC (*R_f* = 0.28). After recrystallization from water, **4** melted at 222–225 °C dec: ¹H NMR (D₂O) δ 1.12 (m, 2) + 1.36 (m, 2) + 1.63 (m, 2) (C-4, C-5, C-3 CH₂); 2.53 (s, 4, succinimide CH₂); 3.26 (t, 2, C-6 CH₂); 3.47 (t, 1, CH); 4.63 (s, HDO). Anal. Calcd for C₁₀H₁₆N₂O₄: C, 52.62; H, 7.07; N, 12.27. Found: C, 52.41; H, 7.09; N, 12.17.

N⁴,N^ε-Succinyl(sulfamethoxazole)(L-lysine) [*L*-Lysine, N⁶-[4-[[4-[(5-Methyl-3-isoxazolyl)amino]sulfonyl]phenyl]amino]-1,4-dioxobutyl]-, **7**]. To a solution of **2b** (792 mg, 3.21 mmol) and NaHCO₃ (406 mg, 4.83 mmol) in 4.1 mL of water was added portionwise with magnetic stirring a solution of sucSMX-NSu (**5**) (**6**, 724 mg, 1.61 mmol) in 3.7 mL of DMF. Stirring was continued for 3 h at room temperature. The mixture was then diluted with an equal volume of water, cooled in an ice bath and adjusted to pH 2 with 6 N HCl, and then extracted with 4 × 15 mL of EtOAc. The extract was washed with 2 × 3 mL of cold saturated NaCl, dried (MgSO₄), and then concentrated to incipient precipitation (10–15 mL). Cooling furnished two crops of 60 and 153 mg, both melting at

237–239 °C, identified as N⁴-cyclosuccinylSMX (**8**) (**5**) by melting point, TLC, and HPLC (single peak, *t_R* = 11.2 min, linear gradient in system E). Further concentration of the EtOAc mother liquor left a gum that was taken up in *i*-PrOH. Hexane was added to incipient crystallization. After several hours at 25 °C, the crude product was collected by filtration (365 mg) (39%); mp 132–135 °C. HPLC in system E showed 93% sucSMX-α-Boc-Lys (*t_R* = 20.2 min), 6% sucSMX (**5**) (*t_R* = 12.6 min), and 1% **8** (*t_R* = 10.8 min).

The crude product (350 mg, 0.6 mmol) was treated with 2 mL of TFA at 8–12 °C for 40 min. The solution was then concentrated to a syrup that was triturated with ether. The resulting solid was freed of Et₂O *in vacuo* and treated with 1 mL of water. It was then extracted with 2 × 1.5 mL of EtOAc. The organic extract was washed with 1 mL of 10% TFA. The combined aqueous layer was extracted with EtOAc. The aqueous layer that started to deposit a semisolid was chilled in ice and adjusted to pH 5 with concentrated NH₃. The white crystalline product that separated was collected on the filter, washed well with cold water, and dried: 180 mg (62%), mp 227 °C dec. The crude product showed a single ninhydrin-positive spot (*R_f* = 0.3) on TLC in system D. For analysis, it was recrystallized from a solution of 32% *N,N*-dimethylacetamide: mp 230 °C dec; ¹H NMR (TFA-*d*₁) δ 1.97–2.4 (m, Lys C-3, C-4, C-5 CH₂) + 2.62 (s, CH₃), 9; 3.37 (s, br, 4, succinyl CH₂); 3.9 (m, 2, Lys C-6 CH₂); 4.77 (m, 1, α-CH); 6.77 (s, 1, vinyl); 8.43 (m, 4, aromatic); 12.4 (s, 5.5, exchangeable H). Anal. Calcd for C₂₀H₂₇N₅O₇S: C, 49.88; H, 5.65; N, 14.55. Found: C, 49.65; H, 5.82; N, 14.32.

An acid hydrolysate (150 °C) of **7** on PITC-AAA in system F showed Lys (*t_R* = 19.8 min) and **4** (*t_R* = 16.8 min). The ratio of SMX:Lys:**4** was 1.0:0.76:0.16, with 96% recovery of SMX and 88% recovery of Lys + **4**.

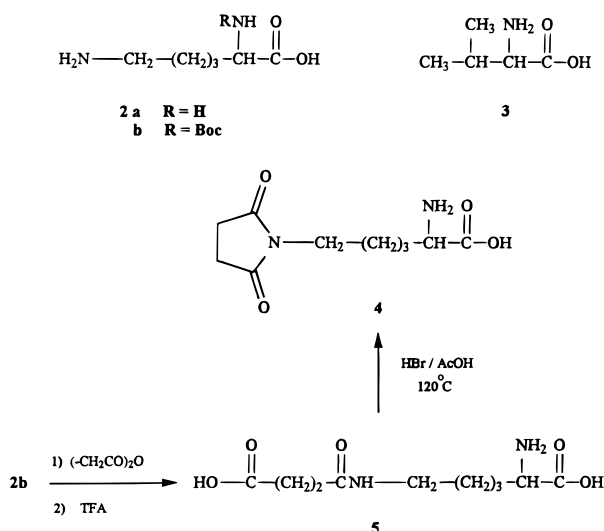
Identification of N^ε-Cyclosuccinyllysine in Hydrolysates of Conjugates 1c and 1e. On ion-exchange AAA, **4** had *t_R* = 27.6 min close to those of Val (28.3 min) and cystine (26.0 min); the buffer change effect is 31 min. A hydrolysate (150 °C) of **1e** had an unidentified peak (*t_R* = 27.9 min) labeled as Val and Lys (*t_R* = 54.4 min) in the ratio 0.31:1.0. Admixture of the hydrolysate with **4** led to a single peak (*t_R* = 27.9 min) and Lys (*t_R* = 54.3 min), both with the expected recovery. On paper, electrophoresis at pH 5.6 of a hydrolysate (120 °C) of **1c** showed ninhydrin-positive material located near the origin in the position of **4**, as well as Lys. TLC indicated material with the mobility of **4** (*R_f* = 0.49, system A; and *R_f* = 0.31, system B) in a yield of 11%, in addition to Lys and a trace of **5**.

Identification of N^ε-Cyclosuccinyllysine in the Presence of Valine and Cystine. A nearly base line separation of **4** and **3** was attained by Pico-Tag analysis in system G; *t_R* values for **4**, **3**, and Nle are 20.8, 21.2, and 24 min, respectively. A mixture of **4**, **3**, and cystine separated on TLC on 20 cm strips of Eastman Chromagram cellulose in system A; *R_f* = 0.49, 0.55, and 0.13.

RESULTS AND DISCUSSION

Synthesis of N^ε-Cyclosuccinyllysine. The synthetic route to **4** is shown in Scheme 1. N^ε-Hemisuccinyl-L-lysine (**5**) needed as starting material had been prepared before without detail via the copper chelate of Lys (**1**). It had also been synthesized in unspecified yield by succinylation of α-Cbz-L-Lys followed by hydrogenolysis over Pd/C (**10**) and in 70% yield by hydrogenolysis over Pd/C/cyclohexene followed by desalting on Dowex 50 resin (**11**). By substituting α-Boc-L-Lys (**2b**) in this route,

Scheme 1



followed by succinylation and deprotection in TFA, we isolated **5** directly in crystalline form and 77% overall yield.

Treatment of **5** in 6 N HCl at 120 °C for 18 h yielded a mixture containing 13% **4**, 1% **5**, and 86% **2a**. In an attempt to decrease the extensive hydrolysis to Lys found in aqueous acid, cyclization of **5** was examined under nonaqueous conditions using 30% HBr/AcOH. The reaction course was followed by paper electrophoresis at pH 5.6 which readily separated **4** from **2a** and **5**. After 24 h at 60 °C, the cyclization reaction mixture showed no detectable **2a** and contained approximately 25% **4** and 75% **5**. After 24 h at 120 °C, the mixture contained 60% **4**, 30% **5**, and 10% **2a**. After 60 h at 120 °C, the mixture contained 93% **4**, 2% **5**, and 5% **2a**. These conditions were adopted for the synthesis of **4**. The product was readily purified on a weak anion-exchange column. The yield of chromatographically satisfactory **4** from **5** was 83%. It was then crystallized from water. The product **4** showed the expected ¹H NMR and elemental analysis.

Properties of *N*^ε-Cyclosuccinyllysine. In 13 mM solution in 0.1 M sodium hydroxide, **4** was converted completely to **5** within 1 h at 22 °C, as determined by TLC on Eastman cellulose in systems A and B with a 6% limit of detection of **4**. Such behavior is consistent with the known tendency of a succinimide in alkali to form a succinamic acid (12). In 0.1 M sodium phosphate buffer (pH 8) at 22 and 37 °C, **4** formed only **3** and 12% **5**, respectively, after 6 days. In water at pH 6 and in 0.1 M sodium citrate buffer (pH 3) under these conditions, *N*^ε-cyclosuccinyllysine was stable as judged from the estimation of **5**. The kinetics are shown in Figure 2.

N^ε-Cyclosuccinyllysine can be considered to be a surrogate of lysine, where the positively charged NH₃⁺ group has been replaced by the large uncharged 2,5-dioxypyridinyl group. Possible biological effects of this change remain to be studied. The stability characteristics of this new amino acid should make it satisfactory for most biological studies. Incorporation of **4** into peptides should also be possible using protecting groups susceptible to cleavage in mild anhydrous acid.

***N*^ε-Cyclosuccinyllysine in Hydrolysates of Succinylated Sulfa Drug Conjugates.** The peak near the position of valine found in hydrolysates of the skin test antigens **1b–e** on AAA was unexpected since in none of these conjugates had valine been incorporated. For conjugates **1b** and **1e**, the molar ratio of the “valine” material to Lys was 0.10:1.0 and 0.30:1.0, respectively.

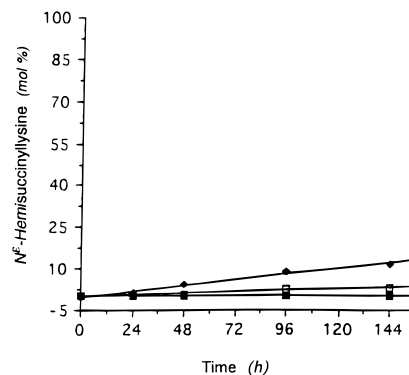


Figure 2. Stability of *N*^ε-cyclosuccinyllysine to hydrolysis to *N*^ε-hemisuccinyllysine in 0.04 M aqueous solution under various conditions: □, 0.1 M sodium phosphate buffer (pH 8) at 22 °C; ♦, 0.1 M sodium phosphate buffer (pH 8) at 37 °C; and ■, water or 0.1 M sodium citrate buffer (pH 3) at 22 and 37 °C. SucLys was determined by analytical TLC using densitometry on Merck cellulose plates in system B after treatment with ninhydrin.

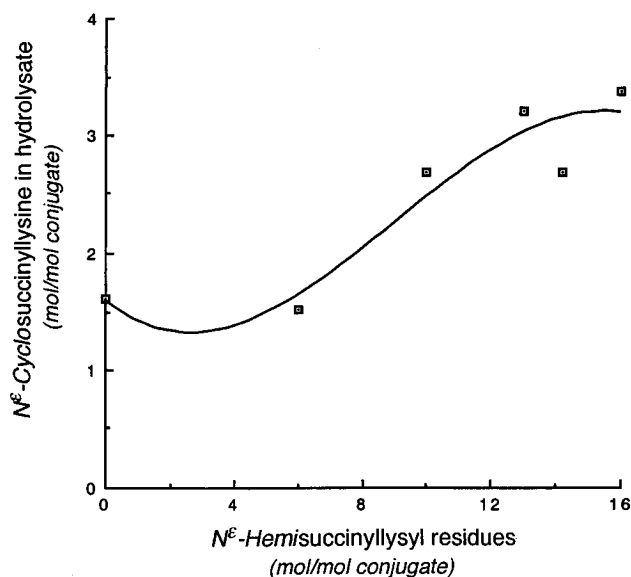
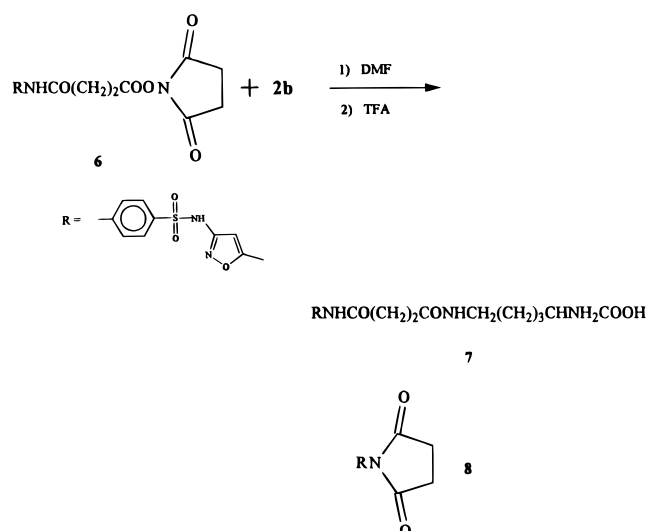


Figure 3. Dependence of formation of *N*^ε-cyclosuccinyllysine on hemisuccinyllysyl residues in hemisuccinylated drug conjugates. Dialyzed hemisuccinylated PL₁₆ conjugates with varying degrees of substitution by sucSMX, a conjugate not hemisuccinylated, and hemisuccinyl-PL₁₆ were hydrolyzed in 6 N HCl/2% phenol for 1 h at 150 °C and analyzed by AAA. Cyclosuccinyllysine in hydrolysates was calculated using the ninhydrin color constant of valine. The number of *N*^ε-hemisuccinyllysyl residues per conjugate was calculated by subtraction of the number of residues of the ligand from 16 and are designated as ·suc_n following PL₁₆. Regression analysis showed the fit of the curve as a polynomial (*r* = 0.94) to be superior to a straight line (*r* = 0.89). The conjugates in sequence were (sucSMX)₁₀PL₁₆, (sucSMX)₁₀PL₁₆·suc₆, (sucSMX)₆PL₁₆·suc₁₀, (sucSMX)₃PL₁₆·suc₁₃, (sucSMX)_{1.8}PL₁₆·suc_{14.2}, and PL₁₆·suc₁₆.

These conjugates were 62.5 and 11% substituted by the branched SMX ligand, suggesting that the formation of such material was related to the degree of hemisuccinylation of *N*^ε-lysyl residues unsubstituted by the ligand. Figure 3 examines the formation of the “valine” material of all the available conjugates **1b–e** in terms of the degree of hemisuccinylation. For comparison, the results of hydrolysis of the conjugate of **1b** before it had been hemisuccinylated and hemisuccinylated polylysine **1f** have been included as the initial and final points.

A search of the literature then revealed observations of small amounts of unusual material in acid hydrolysates of succinylated pepsinogen and subtilopectidase and their DNP derivatives. Since the material also

Scheme 2



formed from hemisuccinylated tryllysine and lysine, it was thought to be the ϵ -aminosuccinimide of Lys, i.e., N^ϵ -cyclosuccinyllysine, but was not further identified (13, 14). Synthesis of authentic N^ϵ -cyclosuccinyllysine (**4**), undertaken for this reason, allowed the material in the acid hydrolysates of the hemisuccinylated PL sulfa drug conjugates to be identified as **4**. This was based on comparison of TLC on cellulose, electrophoresis on paper, and ion-exchange chromatography and cochromatography on AAA.

Synthesis and Hydrolytic Behavior of N^4,N^6 -Succinyl(sulfamethoxazole)(lysine) (7**).** The contribution to the formation of **4** of the internal disubstituted succinyl bridge in conjugates **1b–e** was examined by the synthesis and acid hydrolysis of new **7**. The synthetic route to **7** is shown in Scheme 2. SucSMX-NSu (**6**) was coupled with α -Boc-L-Lys (**2b**) in aqueous DMF essentially as described for the condensation of **6** with PL₁₆ (5) except that the amine component was in excess. Yield of crude sucSMX- α -Boc-L-Lys was 39% adversely affected by partial cyclization of **6** to N^4 -cyclosuccinylSMX (**8**) (5). The crude product was deprotected smoothly in TFA to give **7** in 62% yield and crystalline form with the expected chemical properties. Hydrolysis (6 N HCl, 150 °C) followed by PITC-AAA showed lysine and **4** in the ratio 1.0:0.21 or formation of 0.17 mol of **4** per mole of **7**. This agrees reasonably with the hydrolytic behavior of unhemisuccinylated (sucSMX)₁₀PL₁₆ that formed 1.61 mol of **4** per mole of conjugate or 0.16 mol of **4** per succinylSMX bridge (data from Figure 3).

Apparently, both the N^ϵ -hemisuccinyllysyl residue and the haptenized disubstituted succinyl bridge in conjugates contribute to the formation of **4** during acid hydrolysis, the former somewhat more than the latter. PL₁₆·suc₁₆ (**1f**), the hemisuccinyllysyl model, yielded 0.21 mol of **4** per residue, whereas succinyl(sulfamethoxazole)(lysine) (**7**), the model for the disubstituted succinyl bridge, yielded 0.17 mol of **4** per mole. Probably in **7** and in conjugates **1b–e**, the disubstituted succinyl bridge cyclizes in part to cyclosuccinylSMX (5) (not analyzed) as well as to **4**, resulting in a lower yield of **4** from the succinyl bridge as compared to the yield from the hemisuccinyllysyl residue. The increasing yield of **4** with decreasing ligand substitution and concomitant increasing hemisuccinylation seen in Figure 3 is in general consistent with the relative yields of **4** from the two types of succinyl residues.

Consequences of Cyclization during Acid Hydrolysis. It is clear that the formation of **4** in acid

hydrolysates of N^ϵ -hemisuccinylated peptides or proteins or those containing haptenized succinyl bridges could lead to error in determination of the degree of ligand substitution when based on the content of lysine. With ligands hydrolyzable from lysine, percent error may be calculated as follows:

$$\% \text{ error} = [mg/(1 - mg)] \times 100$$

where m = fraction of succinylated lysyl ϵ -NH₂ undergoing cyclization and g = fraction of lysyl ϵ -NH₂ unsubstituted by ligand. For hemisuccinylated proteins with ligands hydrolyzable from lysine, $m = 0.21$; for haptenized succinyl bridges, $m = 0.17$ for succinylSMX but may vary with the hapten.

An example is PL containing 16 lysyl ϵ -amino groups that are 5% substituted with a hydrolyzable ligand and then hemisuccinylated. During subsequent hydrolysis, unrecognized cyclization of the N^ϵ -hemisuccinyllysyl residues to **4** in a molar yield of 21% would lead to a calculated value for ligand substitution that is 25% high. For 60% substitution with the ligand, the error would be 9%. More direct calculation of the error due to cyclization could be obtained by determining **4** in hydrolysates by subjecting the valine and possibly cystine peaks to Pico-Tag amino acid analysis, by TLC, and probably by mass spectrometry.

To our knowledge, cyclization of succinyl derivatives was first observed over fifty years ago during hemisuccinylation of several heterocyclic amines at elevated temperatures (15). We described cyclization as a side reaction occurring during the synthesis of the N -hydroxy-succinimide active ester of N^4 -hemisuccinylSMX with a carbodiimide in DMF and when this active ester was allowed to stand in DMF, both at room temperature (5). Subsequent similar attempts to prepare the corresponding active esters of N^4 -hemisuccinyl-3-hydroxySMX and N^4 -hemisuccinylsulfapyridine yielded only the corresponding cyclosuccinyl derivatives.² Recently, similar cyclization was encountered with N -hemisuccinyl chlorinated 4-aminophenyl and biphenyl derivatives (16). The problem of cyclization during synthesis has been surmounted by extending the succinyl to a succinylglycyl (5) or succinyl- ϵ -aminohexyl carrier (5) or by replacing the succinyl with a glutaramyl- β -alanyl (16), malonyl², or malonylglycyl² carrier. The present work demonstrates that acid catalysis at elevated temperature, as commonly used for the hydrolysis of proteins and haptenized protein conjugates for amino acid analysis, also tends to promote cyclization of an N -succinamide. Moreover, the amine component may be alkyl as well as aromatic. The cyclization side reaction occurring during hydrolysis does not detract from the viability of hemisuccinylation as a technique used to alter the solubility or ionic charge of proteins, peptides, and drugs or the use of succinyl as a carrier of ligands, although suitable correction in the analyses for lysine and valine or possibly cystine should be made for cyclization when indicated.

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² J. G. Tatake, G. A. Phillips, N. Phelan, and C. Ressler, unpublished results.

LITERATURE CITED

- (1) Groninger, H. S., and Miller, R. (1979) Some Chemical and Nutritional Properties of Acylated Fish Protein. *J. Agric. Food Chem.* 27, 949–955.
- (2) Cremonesi, P., and Caramazza, I. (1993) Chemical and biological characterization of iron-protein succinylate (ITF282). *Int. J. Clin. Pharmacol., Ther. Toxicol.* 31, 40–51.
- (3) Stason, W. B., Vallotton, M., and Haber, E. (1967) Synthesis of an antigenic copolymer of angiotensin and succinylated poly-L-lysine. *Biochem. Biophys. Acta* 133, 582–584.
- (4) Erlanger, B. F. (1980) The Preparation of Antigenic and Hapten-Carrier Conjugates: A Survey. *Methods Enzymol.* 70, 85–104.
- (5) Tatake, J. G., Knapp, M. M., and Ressler, C. (1991) Synthesis and Characterization of Protein and Polylysine Conjugates of Sulfamethoxazole and Sulfanilic Acid for Investigation of Sulfonamide Drug Allergy. *Bioconjugate Chem.* 2, 124–132.
- (6) Slocum, R., and Lee, P. (1987) *Beckman System 6300 Application Notes A6300-00AN-002A*, Spinco Division, Beckman Instruments, Inc., Palo Alto, CA 94304-0803.
- (7) *Publication A6300-IM* (1985) Spinco Division, Beckman Instruments, Inc., Palo Alto, CA 94304-0803.
- (8) Furst, P., Pollack, L., Grasser, T. A., Godel, H., and Stehle, P. (1990) Appraisal of Four Pre-column Derivatization Methods for the High-Performance Liquid Chromatographic Determination of Free Amino Acids in Biological Materials. *J. Chromatogr.* 499, 557–569.
- (9) Fraenkel-Conrat, H., Harris, J. I., and Levy, A. L. (1955) Terminal and Sequence Studies in Peptides and Proteins. *Methods Biochem.* 2, 358–425.
- (10) Matoba, T., and Doi, E. (1979) In vitro Digestibility of Succinylated Protein by Pepsin and Pancreatic Protease. *J. Food Sci.* 44, 537–539.
- (11) Lin, Y., Myhrman, R., Schrag, M. L., and Gelb, M. H. (1988) Bacterial N-Succinyl-L-diaminopimelic Acid Desuccinylase. *J. Biol. Chem.* 263, 1622–1627.
- (12) Wheeler, O. H., and Rosado, O. (1970) Chemistry of imidic compounds. In *The chemistry of amides* (J. Zabicky, Ed.) pp 362–363, Interscience, New York.
- (13) Gounaris, A., and Ottesen, M. (1965) Some Properties of Succinylated Subtilopeptidase. *C. R. Trav. Lab. Carlsberg* 35, 35–62.
- (14) Gounaris, A. D., and Perlman, G. E. (1967) Succinylation of Pepsinogen. *J. Biol. Chem.* 242, 2739–2745.
- (15) Shapiro, D., and Bergmann, F. (1941) Condensation of Heterocyclic Amines with Dicarboxylic Acid Anhydrides. *J. Org. Chem.* 6, 774–779.
- (16) Goon, D. J. W., Nagasawa, H. T., Keyler, D. E., Ross, C. A., and Pentel, P. R. (1994) Glutaramyl- β -alanyl Spacer Group for Haptenic Coupling to Proteins. Preparation of Immunogens for Antibody Production against Polychlorinated Biphenyls. *Bioconjugate Chem.* 5, 418–422.

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Extended Length Heterobifunctional Coupling Agents for Protein Conjugations

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A series of extended length heterobifunctional coupling agents is described. The successive aminocaproic acid homologation of succinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate, a known 9-atom long maleimide active ester linker, yielded 16-, 23-, and 30-atom long maleimide active ester homologues. The performance study of these coupling agents in automated microparticle enzyme immunoassays showed that, in the α fetoprotein assay, in which the linkers were employed in the construction of the alkaline phosphatase–antibody conjugates, the signal increased 64% when the length of the linker was incremented from 9 atoms to 23 atoms and 82% for the 30-atom long linker as compared with the 9-atom homologue. Similar improvements were observed in the performance of carbohydrate antigen, marker of ovarian cancer (CA-125), immunoassay where the linkers were used for conjugation of the capture antibody anti-CA-125 to the microparticle. Thus, a 300% signal improvement resulted when a 30-atom linker was used instead of the 9-atom homologue. The observed differences in the performance of the conjugates are interpreted as resulting from improved antibody binding and lowering of the steric hindrance of the complementarity-determined region of the antibody when longer coupling agents were used.

INTRODUCTION

The cross-linking agents currently in use for bioconjugations exploit a wide variety of functionalities and reactivities. The chemistry of the bioconjugation reagents has been extensively reviewed (1–5). Heterobifunctional reagents, especially maleimide active ester linkers, are particularly attractive, in that they may be reacted chemoselectively with amines on the first molecule and then conjugated to a thiol-containing second molecule in a highly controlled manner, at physiological pH (6–8), and they are quite more selective than iodoacetyl groups in that they do not react with histidine and methionine or thionucleotides (10, 11). Steric effects are among the most important factors influencing the reactivity of organic molecules. In particular, steric hindrance exerts very large influence over the rates of reactions in organic and aqueous media (12). An essential aspect in the construction of a bioconjugate is the preservation of the optimal biological and chemical characteristics of the elements in the conjugate. Although it is generally assumed that the length of the spacer arm is important, with longer spacer arms presumably more effective in coupling large proteins, there is a relative dearth of studies relating to the effects of the length of cross-linking agents on the performance of the conjugates.

We have synthesized a series of heterobifunctional coupling reagents of various lengths useful for covalent coupling of proteins through active ester carbonyl–amine and maleimide–thiol bonds (13–16). Successive elongation of the commercially available 9-atom linker SMCC¹

(8), compound **1** of Figure 1, with aminocaproic acid yielded a series of 16-, 23-, and 30-atom long heterobifunctional maleimide active esters, compounds **2–4** shown in Figure 2. In the work presented here, these linkers were then used to construct two types of conjugates: (a) alkaline phosphatase–monoclonal antibody (mAb) conjugates and (b) microparticle–polyclonal antibody conjugates. We studied the effect of these linkers on the performance of the microparticle capture enzyme immunoassay (MEIA) (17). In this work, we show that the length of the cross-linking reagent in the conjugate is directly related to the performance of these conjugates in the MEIA format. We observed this effect both for antibody–enzyme label conjugates as in the case of alkaline phosphatase–anti-AFP mIgG (a) and where the capture antibody anti-CA-125 IgG was immobilized through a series of increasing length linkers to aminated microparticles (b).

EXPERIMENTAL SECTION

Materials. Except as noted, reagents were obtained commercially and used without further purification. All solvents were high-performance liquid chromatography (HPLC) grade. Anhydrous DMF, *N*-hydroxysuccinimide, dicyclohexylcarbodiimide, Sephadex G-25 chromatographic packing, and silica gel 60 Merck 70–230 mesh were purchased from Aldrich Chemical Co. SMCC and EDC were purchased from Pierce, Rockford, IL. 5,5'-Dithiobis(2-nitrobenzoic acid), (2-mercaptoethyl)amine, 4-methyl umbelliferyl phosphate, and *p*-nitrophenyl phosphate were from Sigma Chemical Co. Bovine intestinal alkaline phosphatase (EC 3.2.1.23, immunoassay grade) was purchased from Boehringer Mannheim Co. as a 10

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¹ Abbreviations: SMCC, succinimidyl 4-(*N*-maleimidomethyl)-cyclohexane-1-carboxylate; AFP, α fetoprotein; CA-125, carbohydrate antigen, marker of ovarian cancer; DMF, dimethylformamide; EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride; EDTA, ethylenediaminetetraacetic acid; TRIS, tris(hydroxymethyl)aminomethane; DTT, dithiothreitol; SPDP, *N*-succinimidyl 3-(2-pyridyldithio)propionate.

mg/mL solution in triethanolamine, NaCl, MgCl₂, and ZnCl₂. Monoclonal anti-AFP IgG was produced at Abbott Laboratories, North Chicago, IL. Monoclonal anti-CA-125 IgG was from Centocor Co. Polyclonal IgGs were produced at Abbott Laboratories. Amine and carboxyl microparticles were from Seradyn or Interfacial Dynamics Corp., Portland, OR. All detergents and buffer components were from Sigma and Aldrich.

General Methods. Immunoassays were performed on an Abbott Laboratory automated fluorescence analyzer IMx. Electronic spectra were recorded on a Hewlett-Packard 8452A diode array spectrophotometer. Nuclear magnetic resonance spectra were obtained on a Varian Unity 300 MHz instrument. HPLC analyses of the conjugates were done on a Perkin-Elmer Series 4 analytical HPLC equipped with a 7125-075 Rheodyne injector or Waters 600 equipped with a U6K injector and a Waters 484 tunable absorbance detector. Elemental analyses were by Oneida Research Services Inc., Whitesboro, NY.

Synthesis of the Extended Heterobifunctional Linker Arms. (a) 4-[(2,5-Dihydro-2,5-dioxo-1H-pyrrol-1-yl)methyl]-N-[6-[(2,5-dioxo-1-pyrrolidinyl)oxy]-6-oxohexyl]cyclohexanecarboxamide (**2**). To a 1.0 mL dry DMF stirred solution of 1-[[4-[(2,5-dioxo-1-pyrrolidinyl)oxy]carbonyl]cyclohexyl]methyl]-1H-pyrrole-2,5-dione (SMCC, 9-atom linker **1**) (3.34 g, 0.01 mol) was added 6-aminocaproic acid (2.51 g, 0.01 mol) in 1.0 mL of DMF, and stirring under a nitrogen atmosphere was continued at room temperature for 12 h. A solution of dicyclohexylcarbodiimide (2.06 g, 0.01 mol) in 1.0 mL of DMF was added, followed by *N*-hydroxysuccinimide (1.15 g, 0.01 mol), and the solution was stirred at room temperature for an additional 6 h. Precipitated dicyclohexylurea was removed by filtration, and the filtrate was evaporated under reduced pressure to give a tacky solid, which was purified by flash chromatography on silica gel using as eluent 5% CH₃OH/95% CHCl₃ to give 2.46 g of the 16-atom linker, compound **2**. Yield: 2.46 g, 55%. Mp: 147–149 °C. Analytical HPLC on Waters Associates system using a μ Porosil silica gel column, 4% CH₃OH/CHCl₃, a 20 μ L injection, a flow of 20 mL/min, and a Waters tunable detector at 300 nm showed a single peak (peak area > 98%) at 1.61 min. ¹H NMR (300 MHz, 1:1 CD₃OD/CDCl₃): δ 6.78 (s, 2H), 3.35 (d, 2H, *J* = 7.2 Hz), 3.33 (m, 1H), 3.18 (m, 2H, *J* = 7.3 Hz), 2.88 (s, 4H), 2.63 (t, 2H, *J* = 7.2 Hz), 2.09 (tt, 1H, *J* = 14.4, 4.3 Hz), 1.92–0.93 (m, 16H). FAB *m/z* 448 (M + H)⁺. Anal. Calcd for C₂₂H₂₉O₇N₃·0.5H₂O: C, 57.88; H, 6.62; N, 9.20. Found: C, 58.09; H, 6.63; N, 9.54.

(b) 4-[(2,5-Dihydro-2,5-dioxo-1H-pyrrol-1-yl)methyl]-N-[6-[[6-[(2,5-dioxo-1-pyrrolidinyl)oxy]-6-oxohexyl]amino]-6-oxohexyl]cyclohexanecarboxamide (**3**). This compound was prepared in a manner analogous to the preparation of **2**. With 2.00 g of **2** as starting material, 1.25 g or 50% of **3** was obtained after flash chromatography using 10% CH₃OH/90% CHCl₃ as eluent. Mp: 144–145 °C. Analytical HPLC performed under conditions identical to those for compound **2** showed a single peak (peak area > 98%) at 2.16 min. ¹H NMR (300 MHz, 1:1 CD₃OD/CDCl₃): δ 6.78 (s, 2H), 3.47 (tt, 1H, *J* = 12.0, 3.6 Hz), 3.35 (d, 2H, *J* = 7.2 Hz), 3.33 (m, 2H), 3.19 (t, 2H, *J* = 7.2 Hz), 3.16 (t, 2H, *J* = 7.2 Hz), 2.88 (s, 4H), 2.64 (t, 2H, *J* = 7.2 Hz), 2.18 (t, 2H, *J* = 8.4 Hz), 2.09 (tt, 1H, *J* = 13.2, 4.3 Hz), 1.93–0.92 (m, 20H). FAB *m/z* 561 (M + H)⁺. Anal. Calcd for C₂₈H₄₀O₈N₄·0.5H₂O: C, 59.02; H, 7.26; N, 9.84. Found: C, 58.92; H, 7.54; N, 10.00.

(c) 4-[(2,5-Dihydro-2,5-dioxo-1H-pyrrol-1-yl)methyl]-N-[6-[[6-[[6-[(2,5-dioxo-1-pyrrolidinyl)oxy]-6-oxohexyl]amino]-6-oxohexyl]amino]-6-oxohexyl]cyclohexanecarboxamide (**4**).

This compound was prepared as **2** and **3** above. With 1.00 g of **3** as starting material, 0.60 g or 50% of **4** was obtained after flash chromatography using 10% CH₃OH/90% CHCl₃ as eluent. Mp: 170–173 °C. Analytical HPLC performed under conditions identical to those for compounds **2** and **3** showed a single peak (peak area > 98%) at 4.01 min. ¹H NMR (300 MHz, 1:1 CD₃OD/CDCl₃): δ 6.78 (s, 2H), 3.55 (bm, 1H), 3.37 (d, 2H, *J* = 7.2 Hz), 3.35 (m, 3H), 3.17 (m, 6H, *J* = 7.2 Hz), 2.88 (s, 4H), 2.63 (t, 2H, *J* = 7.2 Hz), 2.18 (t, 2H, *J* = 8.4 Hz), 2.16 (t, 2H, *J* = 8.4 Hz), 2.09 (tt, 1H, *J* = 13.2, 4.3 Hz), 1.98–0.92 (m, 26H). FAB *m/z* 674 (M + H)⁺. Anal. Calcd for C₃₄H₅₀O₉N₅: C, 60.70; H, 7.49; N, 10.41. Found: C, 60.43; H, 7.46; N, 10.24.

Conjugation of Monoclonal Anti-AFP IgG to Calf Intestinal Alkaline Phosphatase Using **4.** (a) *Derivatization of Alkaline Phosphatase.*

A 250 μ L sample of a 10 mg/mL, 7.4×10^{-5} M, original solution of calf intestinal alkaline phosphatase was placed in a vial. A 100 μ L volume of a 5.0×10^{-3} M DMF solution of compound **4** was added (27 equiv of **4**/enzyme), and the solution was agitated on a rotary agitator for 30 min at room temperature. The crude reaction solution was chromatographed on a pre-equilibrated Sephadex G-25 (coarse) column with 0.1 M sodium phosphate buffer (pH 7.0), 0.1 M NaCl, 1.0 mM MgCl₂, and 0.1 mM ZnCl₂ as eluent. Enzyme-containing fractions were detected by examination of their catalytic activity of inducing color in the *p*-nitrophenyl phosphate solutions (18). Enzyme fractions were pooled, and protein concentration was estimated by measuring absorbance at 280 nm using the equation protein concentration (mg/mL) = $A_{280}/1 \text{ cm} \times 1.05 \text{ mL mg}^{-1} \text{ cm}^{-1}$.

(b) *Monoclonal Anti-AFP IgG Derivatization.* A 6.4 mg/mL, 4.7×10^{-5} M, solution of monoclonal anti-AFP was incubated for 20 min in a pH 7.0 sodium phosphate buffer at room temperature with 25 mM dithiothreitol (DTT), stirring on a rotary agitator. The solution of partially reduced antibody was then chromatographed on a pre-equilibrated Sephadex G-25 (coarse) column with 0.1 M sodium phosphate buffer (pH 7.0), 0.1 M NaCl, and 5 mM EDTA. Fractions from the column were collected, protein-containing fractions were pooled, and protein concentration of the pooled solutions was estimated by measuring absorbance at 280 nm using the equation protein concentration (mg/mL) = $A_{280}/1 \text{ cm} \times 1.39 \text{ mL mg}^{-1} \text{ cm}^{-1}$.

(c) *Conjugation of Partially Reduced Antibody with Derivatized Alkaline Phosphatase.* A 1.00 mL solution of the derivatized alkaline phosphatase (0.48 mg/mL, pH 7.0) from (a) was combined with an equal volume of a 0.40 mg/mL solution of the partially reduced antibody from (b) (molar ratio of 1.2:1 enzyme to antibody known through preceding experimentation to yield the best conjugates). This solution was made 0.1 M in NaCl and 0.1 M in TRIS, 3% Triton X-100, and 1 mM MgCl₂ and 0.1 mM ZnCl₂ and 10% mannitol, yielding the final conjugation solution (pH 7.0). The solution was stirred for 10 h at 2–8 °C on a rotary agitator. After that, unreacted thiols were capped with 100 μ L of 5 mM DMF solution of *N*-ethylmaleimide. It was determined earlier that up to 30% DMF in the buffered conjugation solutions was not detrimental either to the enzyme or to the antibody activities (13, 19). The conjugate concentrate thus obtained could be diluted as necessary for use in sandwich-automated IMx assays or stored in a refrigerator at 2–5 °C for extended periods of time. HPLC gel filtration analysis of this conjugate was performed as described before (19, 20) and revealed mostly a monomeric 1:1 conjugate of enzyme to antibody, although a significant amount of material, 20–25%, had a higher

molecular weight. Conjugations using shorter heterobifunctional linkers **1–3** were done in a manner analogous to the procedure described above.

Conjugation of the Polyclonal Anti-AFP IgG to the Carboxylated Microparticle. The capture antibody in the AFP immunoassay was attached to carboxylated microparticles by incubation of a 1.00 mL volume of microparticles (0.39 μm , 1.25% solids) with 0.18 M 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride (EDC) and 0.76 mg, 5.2×10^{-9} mol, of polyclonal anti-AFP IgG in 3.9 mM 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer (pH 6.5). After being stirred at room temperature for 2 h, the microparticles were filtered through a medium sintered glass filter and washed with a 0.05 M TRIS, 0.1 M NaCl, and 0.1% Tween detergent (pH 8.0) buffer. The derivatized microparticles were centrifuged at 15 000 rpm and diluted in storage buffer 0.05 M TRIS, 0.1 M NaCl, 14% sucrose, and 0.1% sodium azide (pH 8.0).

Conjugation of Polyclonal Anti-CA-125 IgG to Amine Microparticle Using 4. (a) *Pretreatment of Amine Microparticles.* A 1.00 mL volume of amine microparticles (0.164 μm , 2.5% solids) was mixed with 0.5 g of Biorex ion exchange resin (20–50 mesh, Bio-Rad catalog no. 142–7425). The mixture was rotated end-over-end for 1 h at room temperature and then vacuum-filtered through a coarse sintered glass funnel with washing. The filtrate was collected and centrifuged at 15 000 rpm for 30 min. The supernatant was discarded, and the microparticle pellet was resuspended in distilled water with vortexing and adjusted to 2.5% solids by addition of distilled water.

(b) *Derivatization of the Microparticles.* Resuspended, pretreated microparticles from part (a) were mixed with an equal volume of a 3 mM DMF solution of 30-atom linker compound **4** and allowed to react at room temperature for 1 h with end-over-end rotation. The reaction mixture was then diluted 10-fold with phosphate-buffered saline (PBS) (pH 7.2) and centrifuged at 15 000 rpm for 30 min. The resulting supernatant was discarded, and the microparticle pellet was resuspended with PBS. The centrifugation–resuspension sequence was repeated twice, the solution was again centrifuged, the supernatant was discarded, and the pellet was resuspended to a concentration of 2.5% solids with 0.05 M TRIS buffer (pH) (8.0) and 0.1 M NaCl.

(c) *Reduction of the Anti-CA-125 IgG.* A 7.4 mg/mL, 4.9×10^{-5} M, solution of sheep anti-CA-125 IgG in PBS buffer was incubated with 25 mM DTT for 20 min at room temperature with stirring on a rotary agitator. The solution of partially reduced antibody was then chromatographed on a pre-equilibrated Sephadex G-25 (coarse) column with pH 7.0 phosphate buffer, 0.1 M phosphate, 0.1 M NaCl, and 5 mM EDTA as eluent. Fractions were collected, protein-containing fractions were pooled, and the protein concentration of the pooled solution was estimated by measuring absorbance at 280 nm.

(d) *Conjugation of Partially Reduced Antibody to Derivatized Microparticles.* A 1 mL volume of a 1.0 mg/mL, 6.7×10^{-6} M, solution of the partially reduced antibody from part (c) was combined with 1.0 mL of the 2.5% solids maleimide-derivatized microparticles from part (b). The mixture was rotated end-over-end at room temperature for 12 h. The reaction mixture was diluted 10-fold with wash buffer (pH 7.2), 0.01 M sodium phosphate, and 1% Tween detergent and then centrifuged at 15 000 rpm for 30 min. The resulting supernatant was discarded, and the microparticle pellet was washed twice, by vortexing in 1.0 mL of wash buffer, diluting 10-fold with wash buffer, and then centrifuging at 15 000 rpm

for 30 min followed by discarding of the supernatant. The washed pellet was resuspended to a final concentration of 0.125% solids in storage buffer (pH 8.1), 0.01 M TRIS, 0.1 M NaCl, 0.1% sodium azide, and 13.6% sucrose. The final microparticle suspension was first passed through a 23- and then a 25-gauge needle. These microparticles were stored in storage buffer until further use. Amine microparticle derivatizations with shorter heterobifunctional linkers **1–3** were done in a manner analogous to the procedure described above.

Conjugation of the Monoclonal Anti-CA-125 IgG to Bovine Alkaline Phosphatase. The other half of the sandwich in the CA-125 immunoassay, alkaline phosphatase-labeled monoclonal anti-CA-125, was prepared by a method entirely analogous to the one employed in the conjugation of monoclonal anti-AFP IgG to calf intestinal alkaline phosphatase described above. This conjugate was made using compound **4**.

Immunoassay Performance of the Conjugates. The assay performance of the conjugates was evaluated with an Abbott IMx automated immunoassay analyzer on the basis of the microparticle capture enzyme immunoassay technology (MEIA) (17). In MEIA technology, antibody-derivatized microparticles capture the analyte and are then reacted with an alkaline phosphate-labeled antibody conjugate, which completes the sandwich. The nonfluorescent substrate, 4-methylumbelliferyl phosphate, is added, and the rate of the appearance of the fluorescent product, 4-methylumbelliferone, is measured (21). A 500 ng/mL AFP standard was used to compare the performance of the conjugates by examination of the rate of fluorescence increase expressed in arbitrary units of counts per second per second at the given concentrations. AFP (α fetoprotein) is an oncofetal antigen expressed in hepatic carcinoma cells (22), and CA-125 is an epithelial ovarian cancer marker used in detection and quantitation of the ovarian tumor-associated antigens (23).

Control Experiments. *Derivatization of Alkaline Phosphatase and Amine Microparticles with Heterobifunctional Linkers.* Aliquots of alkaline phosphatase were treated with 27 equiv of SMCC and heterobifunctional linkers **2–4** in 1 mL of 0.1 M sodium phosphate, 0.1 M NaCl, 1 mM MgCl_2 , and 0.1 mM ZnCl_2 as described before for the preparation of anti-AFP–alkaline phosphatase conjugates.

Microparticles used in the following experiments did not contain any detergent and therefore did not require any pretreatment. Aliquots (1 mL) of the particles (0.18 μm , 1.2% solids) containing 2.16 μ equiv of amine were washed twice by mixing with an excess of 0.1 M sodium phosphate buffer (pH 7.0) containing 0.1 M NaCl, vortexing the mixture vigorously, and then recovering the particles by centrifugation at 15 000 rpm for 30 min. The washed particles were suspended in 1 mL of 0.1 M sodium phosphate and 0.1 M NaCl (pH 7.0) and allowed to react with the linkers at linker to amine molar ratios of 1:1 and 3:1. The linkers were dissolved in 0.5 mL of DMF and slowly added to the particles. After incubation for 1 h at ambient temperature on a rotator, the particles were washed three times with 0.1 M sodium phosphate and 0.1 M NaCl (pH 7.0) to remove unreacted linkers. The derivatized particles were suspended in a known volume of 0.1 M sodium phosphate and 0.1 M NaCl (pH 7.0) and stored on ice until quantitation of maleimide groups, usually within 30–60 min.

Quantitation of Maleimide Groups Introduced into Alkaline Phosphatase and Amine Microparticles. The determinations of maleimide groups incorporated into alkaline phosphatase upon linker derivatization and the

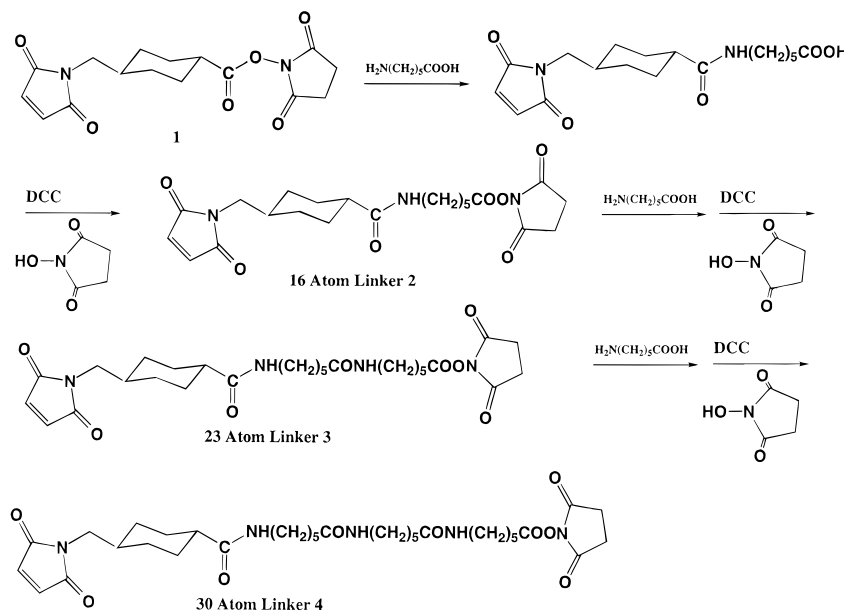


Figure 1. Syntheses of the extended heterobifunctional linkers.

phosphatase activity were carried out as already published (19). This method was also used with minor modifications to quantitate maleimide groups introduced into amine microparticles upon linker derivatization. Samples of derivatized microparticles were incubated with 100 μM (2-mercaptoethyl)amine in a final volume of 1 mL of 0.1 M sodium phosphate buffer (pH 7.0) containing 0.1 M NaCl and 2 mM EDTA. After incubation for 15 min at ambient temperature, particles were removed by filtration through a 0.2 μm filter, and the remaining thiol content in the filtrate was quantitated by the method of Ellman (24). As a control, amine microparticles treated with just DMF were also reacted with (2-mercaptoethyl)amine to determine if there was any nonspecific consumption of thiols. In another control, carboxyl microparticles (1 mL, 1.2% solids) were treated with an excess of SMCC (7 μmol) for 1 h at ambient temperature, washed as amine microparticles, and then reacted with (2-mercaptoethyl)amine.

RESULTS

The syntheses of the extended heterobifunctional linkers 2–4 are shown in Figure 1. Successive homologation of SMCC with aminocaproic acid yielded the indicated coupling agents. These reagents remain stable for periods of several years when stored as solids in the desiccator at 2–8 $^{\circ}\text{C}$. Millimolar solutions of these reagents in dry DMF (25) in brown glass ampules were stable for several months at 2–8 $^{\circ}\text{C}$. We examined the rates of aqueous hydrolysis of the active ester functionality in these reagents by UV spectroscopy by following the time-based increase of the absorbance at 259 nm ($\epsilon = 8600 \text{ M}^{-1} \text{ cm}^{-1}$) which is due to the release of the *N*-hydroxysuccinimidyl anion at pH 7.0, with 0.1 M sodium phosphate buffer, at 25 $^{\circ}\text{C}$, and with 15% DMF (26). Under those conditions, half-life times for hydrolysis of SMCC, 2, 3, and 4 are 3.0, 2.2, 2.5, and 2.5 h, respectively. We studied the effect of the length of the coupling agents on the performance of the conjugates in the automated immunoassays in two different settings. The conjugates of bovine intestinal alkaline phosphatase and anti-AFP mIgG were constructed as depicted in Figure 2a, while derivatization of the aminated microparticle with a polyclonal anti-CA-125 was prepared as shown in Figure 2b.

Anti-AFP mIgG–Alkaline Phosphatase Conjugates.

Figure 3 shows the rate of appearance of the MEIA fluorescence signal as the function of the length of the linker arms used in the antibody–enzyme conjugates. The signal of the 500 ng/mL calibrator increased as the function of the length of the heterobifunctional linker. The increase in signal paralleled the increase of the linker arm length at all concentrations of the AFP standards (data not shown). The solid bars show the values of the blank background signal (noise) corresponding to 0 ng/mL of AFP antigen. The signal of the high standard increased 64% when the length of the spacer arm was increased from 9-atom SMCC to 23-atom linker 3 and increased 82% when the spacer arm was increased to 30-atom linker 4. The performance of the conjugates was optimized by three-dimensional plots correlating signal/noise as the function of the equivalents of linker used and Ab/enzyme ratios. All conjugates were compared under identical values of these last two independent variables. In order to keep constant the distance between the capture antibody and the microparticle, in all the runs, the covalent conjugation of the capture antibody to the carboxylated microparticle was done using a soluble carbodiimide EDC (27), a zero-length conjugation reagent. Therefore, it appears that the enhancement of the performance of the assay is directly related to the length of the linker arm in the antibody–enzyme conjugate. The conjugates made with increasing lengths of spacer arms also showed increased thermal stabilities. Thus, conjugates made with 23-atom linker showed 65% less degradation than those made with SMCC when stressed at 45 $^{\circ}\text{C}$ for 7 days. Although the background signal was identical, 2.9 counts per second per second for the shortest and the longest linkers, signal/noise almost doubled when the 30-atom linker was compared with 9-atom SMCC, 250 and 138, respectively, although these ratios for the two intermediate length linkers were markedly lower, 65 and 92 for the 16- and 23-atom linkers, respectively.

Anti-CA-125–Aminated Microparticle Conjugates.

In order to examine the effect of the different length spacers between the capture antibody and the microparticles, polyclonal anti-CA-125 IgG was covalently conjugated to the aminated microparticles using 9-, 16-, 23-, and 30-atom heterobifunctional coupling agents as shown

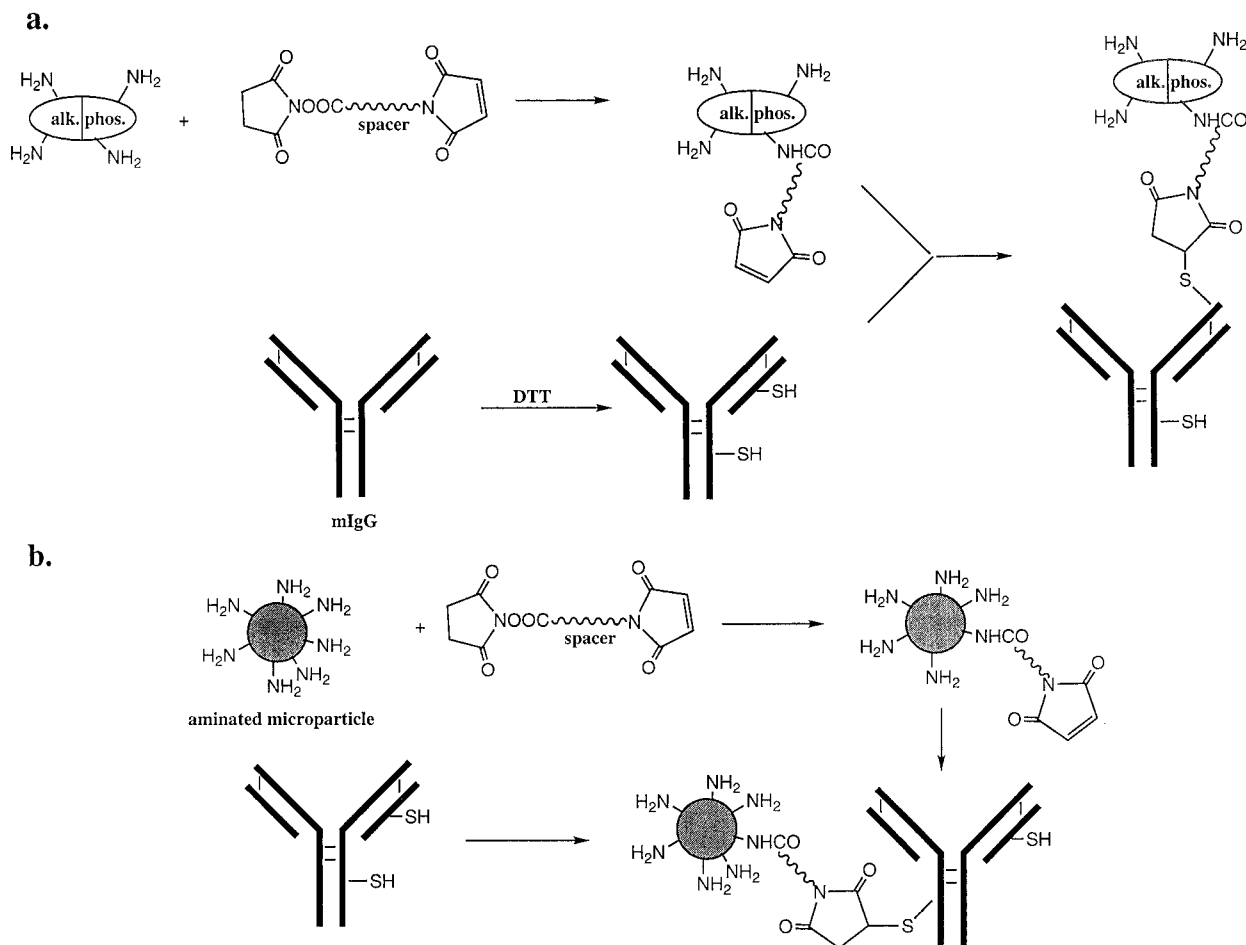


Figure 2. (a) Conjugation of alkaline phosphatase to antibodies using extended length heterobifunctional linkers. (b) Derivatization of the aminated microparticle with capture antibody using extended linkers.

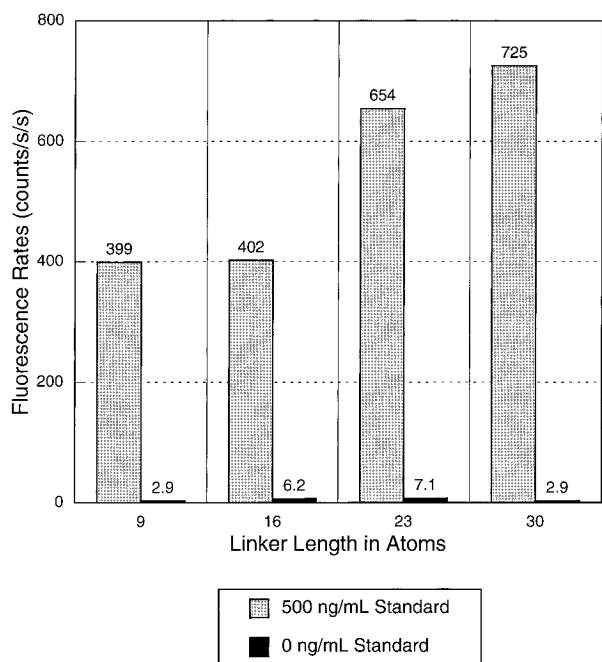


Figure 3. Rate of AFP MEIA fluorescence signal (counts per second per second) as a function of the length of the linker arm used in the antibody–enzyme conjugate. Dark bars represent the background signal at 0 ng/mL AFP, and light bars show the signal at 500 ng/mL AFP.

in Figure 2b. Incubation with 35, 65, 165, 325, and 650 units/mL CA-125 standards followed by addition of the anti-CA-125 mIgG–alkaline phosphatase conjugate and

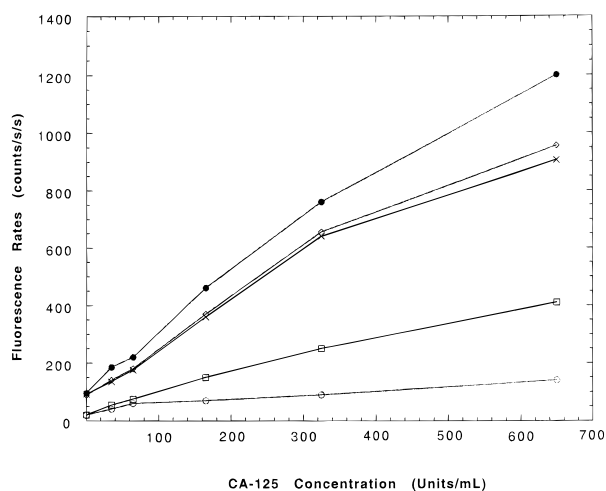


Figure 4. Rates of appearance of the CA-125 MEIA fluorescence signal (counts per second per second) as a function of CA-125 concentration for conjugates of varying lengths: (○) EDC, (□) linker 1, (◇) linker 2, × linker 3, and (●) linker 4.

MUP fluorogenic enzyme substrate yielded graphs shown in Figure 4. In these experiments, the antibody–enzyme conjugates were all prepared using the 9-atom linker SMCC. The optimized ratios of linkers per microparticle, molarity of the capture polyclonal IgG, and antibody–enzyme conjugates were kept constant throughout all the experiments. Therefore, the observed effects may be confidently attributed to the effect of the varying length of the spacer between the microparticle and the capture antibody. As shown in Figure 4, there is a 3-fold increase

Table 1. Number of Maleimide Groups Introduced into Calf Intestinal Alkaline Phosphatase upon Activation with SMCC and Heterobifunctional Linkers 2–4

linker used	maleimide groups introduced	% starting activity retained
SMCC	4.0	92
linker 2	4.3	94
linker 3	4.6	90
linker 4	4.2	92

of the signal at the 650 and 325 units/mL standards going from the 9- to the 30-atom linker in capture antibody–microparticle conjugates. Interestingly, at the reagent stoichiometries used in these experiments, the 16- and 23-atom linkers yielded conjugates with very similar performance, with the 16-atom linker yielding slightly better conjugates than the 23-atom homologue. Signal/noise ratios of 650 units/0 unit standards were 20.5, 10.6, 10.4, and 12.6 for SMCC, 16-, 23-, and 30-atom linkers, respectively, showing relative invariance at higher linker lengths. The higher value of signal/noise for the SMCC conjugate is due to the lowest value of the blank, 20 counts per second per second, while the blank values at 0 unit/mL for microparticles derivatized with 16-, 23-, and 30-atom linkers were 88, 92, and 95 counts per second per second, respectively. Figure 4 also shows that the performance of the best optimized conjugate based on EDC-mediated coupling of the antibody to a carboxylated microparticle was markedly inferior. The stability of the aminated microparticles derivatized with the polyclonal anti-CA-125 IgG was studied by comparison of the performance of the capture antibody–microparticle conjugates incubated at 4 and 45 °C for 7 days. The standard curves of the 30-atom conjugates incubated at those two temperatures were virtually superimposable and identical to the standard curve at time zero. Unlike the anti-AFP–alkaline phosphatase conjugates, little difference in thermal stabilities was observed between the microparticle conjugates made with different length linkers.

The Effect of Introduction of Maleimide Groups into Alkaline Phosphatase and Amine Microparticles. The relative reactivity of the linkers with alkaline phosphatase was determined by quantitation of maleimide groups introduced by treatment with the linkers under identical conditions. The number of maleimide groups introduced was determined by treatment of the derivatized enzyme with excess of (2-mercaptoethyl)amine, followed by titration of the unreacted thiol groups with DTNB (19). The effect of linker derivatization on the enzyme activity was also examined. It can be seen from the results presented in Table 1 that the extent of the modification of alkaline phosphatase upon treatment with the linkers, as judged by the number of maleimide groups incorporated, was essentially constant for all the linkers. Treatment of the enzyme with a 27-fold molar excess of the linkers resulted in the incorporation of four to five maleimide groups per enzyme molecule. The derivatized enzyme retained 90–94% of the starting activity. These results are consistent with those reported earlier (19).

The relative reactivity of the linkers with amine microparticles was also determined by quantitation of maleimide groups incorporated upon treatment with the linkers at linker to amine molar ratios of 1:1 and 3:1. As seen from the data presented in Table 2, the extent of derivatization by the various linkers was essentially constant for all the linkers, ranging from 2.3–3.0% for 1:1 and 25.0–31.9% for 3:1 linker to amine molar ratios. Carboxyl microparticles treated with excess SMCC as

Table 2. Maleimide Modification of Amine Microparticles by Treatment with SMCC and Heterobifunctional Linkers 2–4

linker	% fraction of amine sites derivatized with linker maleimide	
	1:1 linker to amine	3:1 linker to amine
SMCC	2.7	25.0
linker 2	2.3	29.6
linker 3	2.7	26.9
linker 4	3.0	31.9

well as amine microparticles treated only with DMF showed no incorporation of maleimide groups.

DISCUSSION

Although it is generally accepted that the length and the flexibility of the coupling agent are important in the design of a bioconjugate, few systematic studies on this subject appeared in the literature. Some literature reported enhanced binding of the antibody covalently conjugated to the solid phase through an extended, flexible coupling agent (28, 29). The enhancement in the antibody ability to recognize antigens attached through linkers when compared to passive adsorptions has also been described (30). The present work shows that the length of a heterobifunctional coupling agent in an antibody–enzyme conjugate is a crucial factor in the performance of the latter in an automated MEIA. Similarly, the performance of the conjugates of the capture antibodies and the microparticles was enhanced by employing successively longer and more flexible coupling agents. Since the performance of the bioconjugate in the MEIA setting is the composite of the performances of the antibody and the signal-generating enzyme, the question arises whether the observed enhancement of the signal with longer coupling agents is the consequence of improved immunoglobulin binding of the antigen or improved enzyme activity. Our data in Figure 3 show that the blank signal at the 0 ng/mL AFP standard, which originates from nonspecific interaction of the anti-AFP IgG–alkaline phosphatase conjugate with the matrix, and incomplete removal of the unbound conjugate, is relatively constant for all the conjugates, regardless of the length of the linker. Thus, the alkaline phosphatase activity in the unbound conjugates is constant, regardless of the length of the linker. The data in Table 1 show that the activity of the alkaline phosphatase retained after the modification with different length linkers is essentially the same for all the linkers. Moreover, the data show a virtually identical number of maleimides introduced into the alkaline phosphatase, regardless of the length of the linker used. Thus, the differences in performance of the conjugates may be confidently attributed to the length of the linkers. Spurious factors, i.e., differential reactivity of the linkers and differences in the remaining enzymatic activity, may be discounted. Similarly, Table 2 shows that the degree of amine microparticle derivatization is essentially the same for all the linkers, regardless of their length. As expected, lower ratios of the linker/amine in the microparticle resulted in lower levels of microparticle derivatization.

We have shown previously that the functionalization of the alkaline phosphatase with a 30-atom heterobifunctional reagent only minimally affects the enzymatic activity (31). We have also found that, in many antibody–alkaline phosphatase conjugations, enzyme activity can be uncompromised in conjugates that have lost most of their antibody activity. Moreover, conjugation, even with lower linker arm concentrations, can cause considerable

loss (>95%) in antibody binding (32). Our data in Figure 4 show that successive elongation of the distance between the microparticle and the capture antibody in the CA-125 assay also results in increases in signal. Since the antibody-alkaline phosphatase conjugate was held constant throughout these experiments, the signal increase may be attributed to the successively longer distance between the capture antibody and the microparticle. We therefore conclude that the signal enhancement is the result of the alleviation of the steric crowding between the antibody and the entity conjugated to it, i.e., enzyme or solid phase in this work. The complementarity-defined region (CDR) of the immunoglobulins is most severely affected in the case of shorter linkers, i.e., SMCC, and the least when longer linkers are employed. Thus, the effect of the longer linkers on the enzyme activity appears to be smaller than the effect of alleviating the steric hindrance on the antibody. We have recently shown that the construction of the Fc site-specific conjugates based on the extended linkers resulted in 200–400% improvement in signal as compared to the best non-Fc conjugates (19). Apparently, factors relieving steric crowding of an antibody CDR may lead to conjugates of enhanced activity. Figure 4 patently illustrates the last point by showing the zero-length EDC-mediated conjugates' markedly inferior performance as compared with that of even the shortest linker SMCC. Although the chemistries of these two conjugates are different, we believe that the steric hindrance of the immunoglobulin's CDR is the principal reason for the difference in the conjugates' performance.

The findings of the enhancement of signal as a result of the increasing distance between the two entities in a bioconjugate are general. We confirmed the above in many different conjugates of monoclonal and polyclonal antibodies modified not only by DTT disulfide reduction but also by Traut iminothiolane derivatization (33) or SPDP-mediated thiolation (34). The design of the coupling agents providing appropriate chemical and geometric environment for the conjugates will no doubt continue to be a crucially important endeavor in bioconjugate chemistry. Thus, the design of coupling agents possessing several advantageous characteristics, i.e., length, flexibility, hydrophilicity, and ability to introduce selective functionalities in site-specific regions of the biomolecules, will be a challenging objective of bioconjugate chemistry.

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LITERATURE CITED

- Means, G. E., and Feeney, R. E. (1990) Chemical Modification of Proteins: History and Applications. *Bioconjugate Chem.* 1, 2.
- Maggio, E. T. (1985) *Enzyme Immunoassay*, CRC Press, Boca Raton, FL.
- Wong, S. H. (1991) *Chemistry of Protein Conjugation and Cross-linking*, CRC Press, Boca Raton, FL.
- Brinkley, M. (1992) A Brief Survey of Methods for Preparing Protein Conjugates with Dyes, Haptens, and Cross-Linking Reagents. *Bioconjugate Chem.* 3, 2.
- Feeney, R. E. (1987) Chemical Modification of Proteins: Comments and Perspectives. *Int. J. Pept. Protein Res.* 29, 145.
- Kitagawa, T., and Aikawa, T. (1976) Enzyme Coupled Immunoassay of Insulin Using a Novel Coupling Reagent. *J. Biochem. (Tokyo)* 79, 233.
- Youle, R. J., and Neville, D. M. (1980) Anti-thy 1.2 Monoclonal Antibody Linked to Ricin is a Potent Cell-Type-Specific Toxin. *Proc. Natl. Acad. Sci. U.S.A.* 77, 5483.
- Yoshitake, S., Yamada, Y., Ishikawa, E., and Masseyeff, R. (1979) Conjugation of Glucose Oxidase from *Aspergillus niger* and Rabbit Antibodies using *N*-Hydroxysuccinimide ester of *N*-(4-carboxycyclohexyl-methyl) maleimide. *Eur. J. Biochem.* 101, 395.
- Gregory, J. D. (1955) The Stability of *N*-Ethylmaleimide and Its Reaction with Sulfhydryl Groups. *J. Am. Chem. Soc.* 77, 3922.
- Smyth, D. G., Blumenfeld, O. O., and Konigsberg, W. (1964) Reaction of *N*-Ethylmaleimide with Peptides and Aminoacids. *Biochem. J.* 91, 589.
- Brown, R. D., and Matthews, K. S. (1979) Chemical Modification of Lactose Repressor Proteins Using *N*-Substituted Maleimides. *J. Biol. Chem.* 254, 5128.
- March, J. (1992) *Advanced Organic Chemistry*, pp 275–278, John Wiley & Sons, Inc., New York.
- Bieniarz, C., Welch, C. J., and Barnes, G. (1991) Heterobifunctional Couplings Agents. U.S. Patent 4,994,385.
- Bieniarz, C., Welch, C. J., Barnes, G., and Schlesinger, C. A. (1991) Covalent Attachment of Antibodies and Antigens to Solid Phases Using Extended Length Heterobifunctional Coupling Agents. U.S. Patent 5,002,883.
- Bieniarz, C., Welch, C. J., and Barnes, G. (1991) Heterobifunctional Maleimide Containing Coupling Agents. U.S. Patent 5,053,520.
- Bieniarz, C., Welch, C. J., Barnes, G., and Schlesinger, C. A. (1991) Covalent Attachment of Antibodies and Antigens to Solid Phases Using Extended Length Heterobifunctional Coupling Agents. U.S. Patent 5,063,109.
- Fiore, M., Mitchell, J., Doan, T., Nelson, R., Winter, G., Grandone, C., Zeng, K., Haraden, R., Smith, J., Harris, K., Leszczynski, J., Berry, D., Stafford, S., Barnes, G., Scholnick, A., and Ludington, K. (1988) The Abbott IMx Automated Benchtop Immunochemistry Analyzer System. *Clin. Chem. (Washington, D.C.)* 34, 1726.
- Boehringer Mannheim Biochemicals (1993/4) *Biochemicals for the Diagnostic Industry Clinical Chemistry*, Indianapolis, IN.
- Husain, M., and Bieniarz, C. (1994) Fc Site-Specific Labeling of Immunoglobulins with Calf Intestinal Alkaline Phosphatase. *Bioconjugate Chem.* 5, 482.
- Yoshitake, S., Imagawa, M., and Ishikawa, E. (1982) Efficient Preparation of Rabbit Fab'-Horseradish Peroxidase Conjugates Using Maleimide Compounds and its Use for Enzyme Immunoassay. *Anal. Lett.* 15 (B2), 147.
- McComb, R. B., Bowers, G. N., Jr., and Posen, S. (1979) *Alkaline Phosphatase*, Plenum Press, New York and London.
- Roitt, I. (1991) *Essential Immunology*, p 297, Blackwell Scientific Publication, Oxford.
- Bast, R. C., Jr. (1993) Perspective on the Future of Cancer Markers. *Clin. Chem. (Washington, D.C.)* 39, 2444.
- Ellman, G. L. (1959) Tissue Sulphydryl Groups. *Arch. Biochem. Biophys.* 82, 70–77.
- Perrin, D. D., Armarego, W. L. F., and Perrin, D. R. (1980) *Purification of Laboratory Chemicals*, 2nd ed., Pergamon Press, Oxford.
- Pollak, A., Blumenfeld, H., Wax, M., Baughn, R. L., and Whitesides, G. M. (1980) Enzyme Immobilization by Condensation Copolymerization into Cross-Linked Polyacrylamide Gels. *J. Am. Chem. Soc.* 102, 6324.
- Hoare, D. G., and Koshland, D. E. (1967) A Method for the Quantitative Modification and Estimation of Carboxylic Acid Groups in Proteins. *J. Biol. Chem.* 242, 2447.
- Suter, M., and Butler, J. E. (1986) The Immunoassay of Sandwich ELISAs. II. A Novel System Prevents the Denaturation of Capture Antibodies. *Immunol. Lett.* 13, 313.
- Peterman, J. H., Tarcha, P. J., Chu, V. P., and Butler, J. E. (1988) The Immunochemistry of Sandwich ELISAs. IV. The Antigen Capture Capacity of Antibody Covalently Attached to Bromoacetyl Surface-Functionalized Polystyrene. *J. Immunol. Methods* 55, 271.
- Kennel, S. J. (1982) Binding of Monoclonal Antibody to Protein Antigen in Fluid Phase or Bound to Solid Supports. *J. Immunol. Methods* 55, 1.

- (31) Bieniarz, C., Young, D. F., and Cornwell, M. J. (1994) Thiolate and Phosphorothioate Functionalized Fluoresceins and Their Use as Fluorescent Labels. *Bioconjugate Chem.* 5, 31.
- (32) Rohr, T., Young, D. F., and Bieniarz, C. Unpublished results.
- (33) Jue, R., Lambert, J. M., Pierce, L. R., and Traut, R. R. (1978) Addition of Sulfhydryl Groups to *Escherichia coli* Ribosomes by Protein Modification with 2-Iminoethiolane-(methyl 4-mercaptobutyrimidate). *Biochemistry* 17, 5399.
- (34) Carlsson, J., Drevin, H., and Axen, R. (1978). Protein Thiolation and Reversible Protein-Protein Conjugation. *N-Succinimidyl 3-(2-pyridyldithio)propionate*, a New Heterobifunctional Reagent. *Biochemistry J.* 173, 723.

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Effect of Molecular Architecture of Poly(*N*-isopropylacrylamide)–Trypsin Conjugates on Their Solution and Enzymatic Properties

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Polymer–enzyme hybrid conjugates modified by a temperature-responsive polymer, poly(*N*-isopropylacrylamide) (PIPAAm), have been synthesized. We have investigated the molecular architecture of PIPAAm–enzyme conjugates by preparing two types of PIPAAm–trypsin conjugates, wherein PIPAAm chains are attached by either single-end or multipoint chemistry. A semitelechelic co-oligomer (IDc) was attached to trypsin by single-point conjugation (IDc–trypsin). A copolymer (PIDAAc) consisting of acrylic acid and IPAAm randomly linked in polymer chains was attached to trypsin using multipoint conjugation (PIDAAc–trypsin). Both conjugates exhibited reversible temperature-responsive phase separation. The IDc–trypsin conjugate exhibited phase separation at the same temperature as pure IDc, due to the highly mobile free polymer end group which remains sensitive to small temperature changes. The PIDAAc–trypsin conjugate precipitated at higher temperatures than pure PIDAAc, whose movement was restricted by multiple binding points. Enzyme stability in solution was improved after introduction of PIPAAm chains, which prevented autolysis attributed to conjugate steric hindrance. Stability under repeated temperature cycling was also dependent on the architecture of conjugates; the IDc–trypsin conjugate was more stable than the PIDAAc–trypsin. As a consequence, single-end conjugation of polymer to enzyme provides novel bioconjugate with novel functionality attributed to attached polymer while retaining native biological function with high stability.

INTRODUCTION

Chemical modification of biomolecules with synthetic and natural polymers has allowed new applications of protein conjugates in the fields of medicine, bioengineering, and bioreactors. Poly(ethylene glycol) modification of proteins, for example, has broadened the applications of many proteins. Decreased immunoreactivity and/or immunogeneity (1), increased stability *in vivo* (2), and use in organic synthesis (3) have been described for PEG-modified proteins. Using these techniques, functional polymers are capable of integrating new functions into biomolecules without destroying the original protein function.

We have been studying temperature-responsive bioconjugates using poly(*N*-isopropylacrylamide) (PIPAAm)¹ and proteins connected with a single-end attachment (4–7). PIPAAm is water soluble at room temperature and exhibits a lower critical solution temperature (LCST), at which the aqueous solution demonstrates phase separa-

tion and polymer precipitation (8). This unique hydration-dehydration change occurs reversibly in 1 ms. Application of PIPAAm in the biotechnology fields has been studied in the forms of hydrogel and surface modification, including protein modification (9–13). PIPAAm hydrogels exhibit swelling–deswelling behaviors modulated by temperature changes, and the release of solute can be regulated by temperature, producing a drug delivery system (9–11). With the introduction of hydrophobic or hydrophilic comonomers, the shrinking–swelling temperature is changeable (12). Okano *et al.* have reported that the architecture of PIPAAm hydrogels can regulate the speed of shrinking, particularly by introducing free mobile chains within the polymer network (13). With increasing temperature, strong hydrophobic interactions between grafted chains enhance the entire gel-shrinking process. Cultured cells were recovered from PIPAAm-grafted cell culture dishes by reduction of the temperature. This cell recovery process reduces cell damage over detachment of cells by enzymatic treatments (14, 15). Previously, we reported preparation of PIPAAm–lipase in which PIPAAm chains were attached to the enzyme by single-chain ends (6). The bioconjugate exhibited an LCST and retained its native activity. Recovery of conjugates was possible by changing temperature, with multiple cycles achieved without denaturation. Although PIPAAm has been introduced to various proteins to date (16, 17), there was no report which discussed its molecular architecture comparing single-end attachment chemistry with multiple attachment chemistry in the same system.

In this paper, we discuss the molecular architecture of a PIPAAm–trypsin conjugate prepared with two types of PIPAAm; one is a semitelechelic co-oligomer of IPAAm with *N,N*-dimethylacrylamide (DMAAm) bearing a single

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¹ Abbreviations: AAc, acrylic acid; AIBN, *N,N'*-azobisisobutyronitrile; BAPNA, *N*- α -benzoyl-D,L-arginyl-*p*-nitroanilide hydrochloride; DCC, *N,N'*-dicyclohexylcarbodiimide; DMAAm, *N,N*-dimethylacrylamide; DMF, *N,N*-dimethylformamide; IDc, co-oligo(IPAAm–DMAAm) with a carboxyl end group; IDc–trypsin, IDc–trypsin conjugate; IPAAm, *N*-isopropylacrylamide; MPA, 3-mercaptopropionic acid; NHSI, *N*-hydroxysuccinimide; PIDAAc, copoly(IPAAm–DMAAm–AAc); PIDAAc–trypsin, PIDAAc–trypsin conjugate; PEG, poly(ethylene glycol); PIPAAm, poly(IPAAm).

carboxyl group at one end, and the other is a copolymer of IPAAm, DMAAm, and acrylic acid (AAc). These conjugates were characterized and their properties, including temperature response, enzymatic activity, and stability in solution under oscillating temperature changes, were discussed to demonstrate a new concept of protein modification by functional polymers. These temperature-responsive bioconjugates are capable of heat-induced drug delivery and bioreactor, where the advantages of their functions have to be considered.

EXPERIMENTAL PROCEDURES

Materials. *N*-Isopropylacrylamide (IPAAm) and *N*,*N*-dimethylacrylamide (DMAAm) were kindly provided by Kojin (Tokyo, Japan). IPAAm was purified by recrystallization from toluene/petroleum ether and dried at room temperature *in vacuo* (8). DMAAm was distilled under reduced pressure, and the fraction of bp 60 °C/9 mmHg was used. 3-Mercaptopropionic acid (MPA; Aldrich Chemical Co., Milwaukee, WI) was distilled under reduced pressure, and the fraction of bp 95 °C/5 mmHg was used. Acrylic acid (AAc) was obtained from Wako Pure Chemicals (Osaka, Japan) and distilled under reduced pressure, and the fraction of bp 44.5 °C/14 mmHg was used. *N*,*N'*-Azobisisobutyronitrile (AIBN) and *N*,*N*-dimethylformamide (DMF) were obtained from Wako Pure Chemicals and purified by conventional methods. Tetrahydrofuran (THF) was obtained from Kanto Chemicals (Tokyo, Japan) and dried with Molecular Sieves 4A 1/8 (Nacalai Tesque, Kyoto, Japan). *N*-Hydroxysuccinimide (NHSI; Tokyo Kasei, Tokyo, Japan) and *N*,*N'*-dicyclohexylcarbodiimide (DCC; Kanto Chemicals) were used without purification. Trypsin from *Porcine* pancreas was purchased from Wako Pure Chemicals and purified by dialysis. Trypsin concentration was determined by the method of Lowry (18) using albumin standard (Pierce, Rockford, IL). *N*- α -Benzoyl-DL-argininyl-*p*-nitroanilide hydrochloride (BAPNA) was purchased from Wako Pure Chemicals and used without purification.

Polymerization Procedure. Semitelechelic IPAAm co-oligomer (IDc) was prepared by radical oligomerization of IPAAm with DMAAm in the presence of MPA as a chain transfer agent in DMF as previously reported (4). The mole fraction of DMAAm in feed was 10 mol % to obtain an IDc oligomer having its LCST higher than that of the PIPAAm homopolymer. AIBN was used as an initiator.

PIPAAm copolymer was synthesized using IPAAm, DMAAm, and AAc as comonomers. Molar ratio in feed was IPAAm:DMAAm:AAc = 48:5:1 and 51:1:1. These monomers were dissolved in distilled DMF (50 mL), and monomer solution was bubbled with N₂ gas for 30 min. AIBN was added, and then the mixture was degassed again for 30 min before the reaction was started. The reaction was carried out at 70 °C for 24 h under N₂ gas atmosphere. Methanol was added to the reaction mixture to quench the reaction, and then polymer was precipitated into diethyl ether. The precipitated polymer was redissolved in THF for a second reprecipitation. The recovered precipitate was dried *in vacuo* overnight.

Characterization of Polymers. Proton NMR spectra were recorded for CDCl₃ solutions of IDc and PIDAAc at ambient temperature on a spectrometer operating at a 270 MHz frequency (JEOL, NJ-270E, Tokyo, Japan). The molecular weights of the oligomers were determined by gel permeation chromatography (GPC; Shimadzu, C-R4AX, column; Shodex, KF-80Mx2) using THF [Wako Pure Chemicals high-performance liquid chromatography (HPLC) grade] as a mobile phase at 40 °C. Polystyrene standards were used (Polyscience Inc., Warrington, PA).

The molecular weights of the oligomers were also determined by nonaqueous titration with 0.01 M sodium methylate solution in dioxane using thymol blue as an indicator under N₂ gas atmosphere at room temperature.

Transmittance Measurements. Optical transmittance of oligomer solutions (10 mg/mL) at various temperatures was carried out by monitoring transmittance at 500 nm using a spectrophotometer (Jasco Ubest 30, Tokyo, Japan). The cell was thermostated by a Jasco temperature controller.

Activation of Polymer Carboxyl Groups. Activation of polymer acid groups was carried out in 50 mL of DMF in a molar ratio of polymer:NHSI:DCC = 1:10:10. IDc or PIDAAc (1.0 g) was dissolved in DMF and the mixture stirred at 4 °C, and NHSI (0.19 g) was added. After 1 h, DCC (0.34 g) was added to the reaction mixture and the mixture stirred for another 12 h. After removal of dicyclohexylurea by filtration, the reaction mixture was reprecipitated into diethyl ether (200 mL) three times and dried *in vacuo* overnight. Activated ester was quantified by the method of Miron *et al.* (19).

Modification of Trypsin. Trypsin (30 mg) was dissolved in 0.1 M phosphate buffer solution (pH 8.0, 10 mL) at 4 °C. Activated polymer was dissolved in 1.0 mL of DMF. To the trypsin solution was added every 30 min 200 μ L of polymer/DMF solution while the mixture was stirred at 4 °C. After all of the polymer/DMF solution was added, the coupling reaction was carried out for another 1 h at 4 °C. The solution was then dialyzed overnight and passed through a Sephacryl 100HR column (1.0 cm ϕ , 60 cm long; flow rate, 0.5 mL/min; eluent, PBS) to separate unreacted trypsin and polymer from the conjugate. The fraction absorbance was monitored at 280 nm. The conjugate fractions were collected and dialyzed to desalt and then lyophilized. The degree of modification was determined by fluorescamine assay (20). Protein concentration was determined by the method of Lowry.

Temperature Response of the PIPAAm–Trypsin Conjugate. Optical transmittance of conjugate aqueous solution (1 wt %) was measured at 500 nm by changing temperature. The cell temperature was controlled by a Jasco temperature controller.

Enzymatic Activity Assay. Enzymatic activity of IDc–trypsin and PIDAAc–trypsin was measured using BAPNA as a substrate according to the method of Erlanger *et al.* (21). The extent of BAPNA hydrolysis was determined by measurement of *p*-nitroanilide at 410 nm, where the extinction coefficient is 8800 and no contribution to the absorbance was made by other elements. To determine kinetic constants, trypsin concentration was normalized to be 450 μ g/mL. Trypsin solution (0.2 mL) was added to the reaction mixture of BAPNA/Tris-HCl buffer solution (pH 8.15). The inverse of hydrolysis velocity was plotted against the inverse of substrate concentration, as the well-known Lineweaver–Burke plot is unreliable with large errors at low substrate concentrations. The effect of temperature on enzymatic activity was also determined. Trypsin solution (100 μ g/mL) (0.2 mL) was added to 2.78 mL of Tris-HCl buffer solution (pH 8.15), followed by the addition of 20 μ L of BAPNA (6.63×10^{-4} M). Absorbance changes at a definite temperature were monitored for 10 min at 410 nm.

Solution Stability. Protein stability in solution was determined by incubation of native trypsin, IDc–trypsin (68%), and PIDAAc3–trypsin in Tris-HCl buffer solution (pH 8.15) without Ca²⁺ at 35 °C. Trypsin concentration was normalized to 100 μ g/mL. At several intervals, 100 μ L of each sample was taken out to determine retained

Table 1. Composition of IDc Co-oligomer and PIDAAc Copolymers

code	IPAAm ^a (mol %)	DMAAm ^a (mol %)	AAc ^b (mol %)	MW	LCST (°C)
IDc	89	11	—	7100 ^c	37.0
PIDAAc3	95	3	2	24000 ^d	33.5
PIDAAc14	84	14	2	24000 ^d	37.0

^a Determined by NMR. ^b Determined by titration. ^c Determined by GPC in THF. ^d Determined by light scattering in ethanol.

Table 2. Preparation of PIPAAm–Trypsin Conjugates

code	feed ratio of polymer: trypsin (mol)	degree of modifi- cation ^a (%)	amount of polymer per conjugate ^b (g/mmol)	LCST (°C)
IDc–trypsin (42%)	9:1	42.0	4.4	37.0
IDc–trypsin (68%)	23:1	68.0	7.1	37.0
PIDAAc3–trypsin	9:1	35.6	21.2	37.0
PIDAAc14–trypsin	9:1	29.7	18.2	40.0

^a Determined by fluorescamine assay. ^b Calculated from protein assay.

enzymatic activity in Tris-HCl buffer (1.325 mL), adding 7.5 μ L of BAPNA (9.93×10^{-2} M) at 25 °C as described above.

Thermostability after repeated incubation temperature changes was also measured. Samples were incubated at 50 °C for 10 min and then at 4 °C for 10 min, and retained activity was assayed.

RESULTS AND DISCUSSION

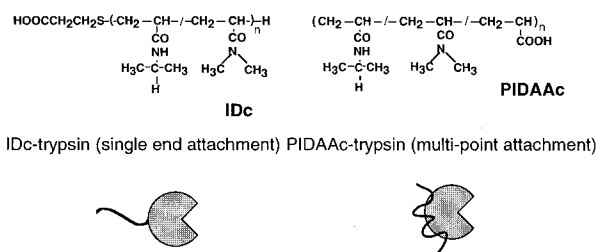
Synthesis of IDc Co-Oligomer and PIDAAc Copolymer. The IDc polymer obtained was characterized by ¹H-NMR, and nonaqueous titration was used for end group analysis. The molecular weight was calculated to be 7100, and the mole fraction of DMAAm was 11%. The molecular weight of PIDAAc was 24 000 by light scattering (Otsuka SLS-700), and the composition of copolymer was calculated from NMR data and titration as shown in Table 1. PIDAAc contained four units of carboxyl groups per polymer chain, which indicated that PIDAAc is consistent with four IDc co-oligomer chains.

Both activated IDc and PIDAAc exhibited absorption at 260 nm, and the introduction of active ester was confirmed.

Modification of Trypsin. Modification of trypsin was carried out at 4 °C while it was being stirred, as the polymer is well-dissolved at low temperatures. The reaction mixture was passed through a Sephacryl 100HR column for purification. The elution peak of both modified trypsins shifted to a larger molecular weight over native trypsin. PIDAAc–trypsin showed a larger apparent molecular weight due to the long polymer chain attached to each trypsin molecule by multipoint conjugation. Both IDc–trypsin and PIDAAc–trypsin were soluble in water at room temperature as well as in organic solvents such as methanol and chloroform.

The degree of trypsin modification was determined by fluorescamine assay. PIPAAm–trypsin conjugates with different modification degrees were obtained, and these results are summarized in Table 2.

Temperature Response of PIPAAm–Trypsin Conjugates. Optical transmittance of 1 wt % polymer solution or conjugate solution was measured at 500 nm as a function of temperature. The LCSTs of polymer solutions as well as trypsin conjugates were defined as temperature at which solution transmittance was 50% and are summarized in Tables 1 and 2. IDc co-oligomer exhibits a phase separation at 37 °C. IDc–trypsin also exhibited the phase separation at the same temperature

**Figure 1.** Structural formulae of used polymers and schematic illustration of temperature-responsive biomolecules.

with IDc itself. PIDAAc copolymers also exhibited an LCST which shifted to higher temperature with increasing mole fraction of DMAAm. This observation was already reported for IDc co-oligomers with increasing mole fractions of DMAAm (6). Increasing hydrophilic monomer content in a PIPAAm copolymer shifts its LCST to a higher temperature. Yoshida *et al.* also reported that, in PIPAAm hydrogels, increasing hydrophobic monomers decrease the transition temperature (12), demonstrating that the transition temperature can be controlled by changing the mole fraction of comonomers to a certain extent. PIDAAc–trypsin conjugates also exhibited phase separation. However, their LCSTs are shifted to much higher temperatures than those of PIDAAc copolymers. As shown in Figure 1, IDc is segregated from each trypsin molecule due to its single-end attachment, and the solubility of trypsin did not affect the solubility of the attached polymers. However, PIDAAc immiscibility in solution is restricted by its protein attachment points; the solubility of the polymer is dependent on the solubility of its attached trypsin. Hoffman *et al.* reported that PIPAAm–alkaline phosphatase conjugates showed a LCST higher than that of the modifying polymer. They concluded that unbound activated esters were hydrolyzed, and as a consequence, the conjugate became hydrophilic (16). However, in our case, PIDAAc contained only 2 mol % acrylic acid which would not be enough to affect the LCST of the conjugates. The PIDAAc–trypsin conjugate behaves as another different copolymer, with trypsin being another hydrophilic segment, shifting the LCST to a higher temperature. These results indicate that IDc–trypsin having free mobile polymer chains is capable of regulating the conjugate phase separation precisely and independently by the LCST of the IDc oligomer.

Two series of IDc–trypsin conjugates differing in their degree of modification were prepared by changing the IDc concentrations in the reaction mixture (60 mg/mL, 150 mg/mL). From the fluorescamine assay, degrees of trypsin modification were 42 and 68%, respectively. As trypsin has fourteen lysine residues, IDc–trypsin modified to 68 and 42% had nine and five polymer chains per trypsin molecule, respectively (Table 2). Degrees of modification for PIDAAc–trypsin conjugates were almost the same as that for IDc–trypsin (42%). However, they possessed 5 times more polymer per trypsin molecule.

In order to determine the kinetics of phase separation, conjugate concentration was normalized to a protein concentration of 250 μ g/mL. At this protein concentration, polymer concentrations necessarily differ from each other. The speed of response to temperature changes from 35 to 45 °C was measured. Each solution was equilibrated at 35 °C in a spectrophotometer, and the cell temperature was instantly raised to 45 °C. Figure 2 shows the optical transmittance changes at 500 nm as a function of time for aqueous solutions of IDc–trypsin and PIDAAc–trypsin. Transmittance of IDc–trypsin rapidly decreased, and the more polymer chains were attached

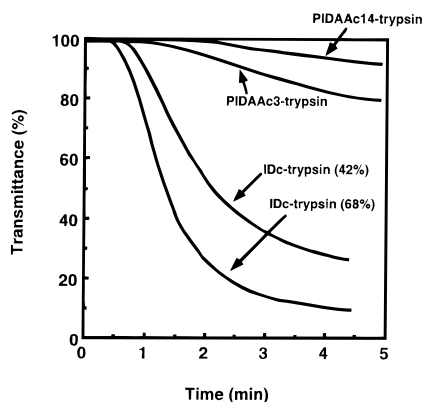


Figure 2. Speed of response toward temperature changes (35–45 °C) of IDc–trypsin solutions and PIDAAc–trypsin solutions (concentration normalized by protein concentration, 250 $\mu\text{g/mL}$).

Table 3. Kinetic Constants of PIPAAm–Trypsin Conjugates

code	$K_m \times 10^3$ (M)	$V_{\max} \times 10^4$ (M/s)
native	1.320	1.75
IDc–trypsin (42%)	0.461	1.75
IDc–trypsin (68%)	0.278	1.77
PIDAAc14–trypsin	0.524	1.59

to the trypsin molecule, the faster the transmittance changed. By contrast, response of PIDAAc–trypsin was less dramatic, even though they have much more polymer in solution. This demonstrates that free mobile polymer chains dehydrate quickly in response to temperature changes as they can segregate from the trypsin molecule and remain more temperature-sensitive, while the mobilities of polymers coupled by multipoints were restricted in their collapse capability. IDc–trypsins also showed that the conjugate formed aggregates and quickly precipitated due to the more rapid aggregation among conjugate molecules. This also demonstrated that aggregates are formed through hydrophobic interactions between polymers, which dehydrate instantly by temperature changes.

Enzymatic Activity of PIPAAm-Modified Trypsins.

Enzymatic activities of IDc–trypsin with 42 and 68% modification and PIDAAc14–trypsin were measured by the method of Erlanger *et al.* using a synthetic substrate, BAPNA (21). Since the speed of hydrolysis was proportional to the protein concentration in the range of 0–450 $\mu\text{g/mL}$ (data not shown), all enzymatic assays were carried out in this range. To determine kinetic constants at 25 °C, K_m and $V_{\max}$ were calculated from conventional methods (Table 3). The K_m value decreased after modification and also with increasing number of polymer chains attached to the trypsin molecule. This indicates that the PIPAAm–trypsin conjugate shows a greater affinity for the substrate than native trypsin. This phenomenon has already been discussed in poly(*N*-vinylpyrrolidone)–trypsin (PVP–trypsin) (22), PEG–trypsin (23), and PEG–chymotrypsin (24). These results indicated that, when amphiphilic polymers, such as PVP and PEG, were introduced to trypsin, the conjugate increased the local substrate concentration by interaction with hydrophobic substrate, resulting in a decrease in K_m value. This may be the same for the PIPAAm–trypsin conjugate because the number of polymer chains conjugated to trypsin effected a decrease in the K_m value. However, the microenvironment of PIPAAm–trypsin is unknown and will be discussed in a forthcoming study.

The temperature dependence of enzymatic activity was measured. The final protein concentration was normalized to 6.67 $\mu\text{g/mL}$, at which no transmittance changes

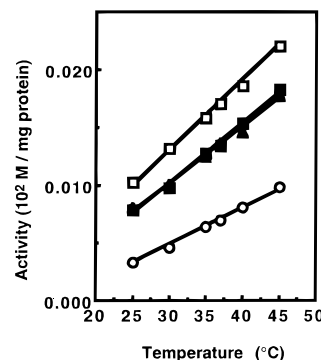


Figure 3. Effect of temperature on enzymatic activity of PIPAAm-conjugated trypsin: (○) native trypsin, (□) IDc–trypsin (42%), (■) IDc–trypsin (68%), and (▲) PIDAAc14–trypsin. Trypsin concentration = 6.67 $\mu\text{g/mL}$.

were observed above their LCSTs. To our surprise, no drastic change in enzymatic activity is observed with temperature (Figure 3). In the case of IDc–lipase, enzymatic activity was dependent on its LCST (6). Concentrated IDc–lipase solution was used to form precipitates in the reaction mixture, which prevented interaction with substrate. In the case of PIPAAm–trypsin conjugates, the reaction occurs in low concentrations and conjugate does not form intermolecular aggregation above its LCST. This may be one reason that there was no dramatic change in activity. The mixture of IDc or PIDAAc with native trypsin, both of which were prepared at the same concentration as IDc–trypsin (68%) or PIDAAc14–trypsin, showed the same activity as native trypsin at 25 and 45 °C (data not shown). This indicates that the viscosity did not affect the enzymatic activity and the increased activity was due to the conjugation in which the polymers were attached to a trypsin molecule far from the active site. Valuev *et al.* also modified PIPAAm to trypsin and reported that scarce temperature dependence of enzymatic activity was observed (25). They also modified peroxidase with the same polymer, whose activity dramatically dropped above its LCST. Hoffman *et al.* prepared PIPAAm– β -galactosidase conjugates and observed no change in enzymatic activity by changing temperature (17). All these results may suggest that some enzymes are not dependent on hydrophobic environment, while others are sensitive. In our study, the enzymatic activity increased with temperature, and increases in activity of modified trypsins were slightly larger than that of the native one, which indicated the possibility of enhancement of hydrophobic interaction with substrate above their LCSTs.

Stability in Aqueous Solution. The protein stability in solution was studied by incubation of conjugate solutions at 35 °C, which was below the LCST of the conjugates. IDc–trypsin and PIDAAc3–trypsin conjugates, both of which had their LCST at 37 °C, were used for experiments, with protein concentration normalized to 450 $\mu\text{g/mL}$. From incubated solutions, 100 μL of each sample was taken out to determine its retained activity at 25 °C. Results are shown in Figure 4. Native trypsin rapidly decreased its initial activity. However, IDc–trypsin and PIDAAc3–trypsin retained their activities to higher extents. IDc–trypsin maintained more than 90% of its original activity even after incubation for 120 h. Trypsin is well-known for autolysis; however, storage at pH 2–5 or with Ca^{2+} prevents its autolysis (26). We rationalize our results with several points. First, in this experiment, trypsin was incubated under severe conditions, at pH 8.15 without Ca^{2+} . In fact, when trypsin was incubated in Tris–HCl buffer (pH 8.15) including 0.01 M

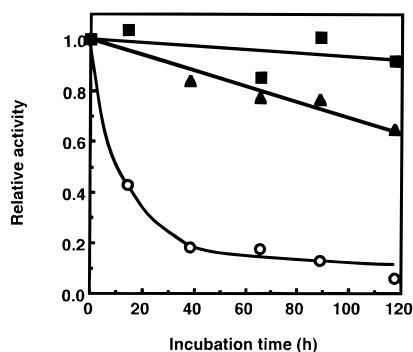


Figure 4. Stability of PIPAAm-trypsin/Tris-HCl buffer solutions (pH 8.15), incubating at 35 °C: (○) native trypsin, (■) IDC-trypsin (68%), and (▲) PIDAAc3-trypsin.

Ca²⁺, native trypsin showed no decrease in enzymatic activity. This indicated that attached polymer chains prevent trypsin from autolysis and act as a stabilizer. In this case, hydrated IPAAm polymer chains may slightly change trypsin conformation and stabilize it by steric hindrance. PIDAAc3-trypsin also maintained high activity, but it was lower than that of IDC-trypsin. As a PIDAAc3 molecule has an LCST at 33.5 °C, when being incubated at 35 °C, the polymer chain may exist in shrunken form, causing some damage in enzyme higher-order structure, resulting in denaturation.

In general, PEG is introduced to protein surfaces in the form of an activated PEG via triazine, in which monomethoxypoly(ethylene glycol) contains substituted chlorines via cyanuric chloride. PEG chains have been substituted for either one or two chlorines to be used as modifier (2, 27). 2,4-Bis[O-methoxypoly(ethylene glycol)]-6-chloro-s-triazine (PEG₂) made it possible to introduce two PEG chains per amino group. PEG₂-modified asparaginase retained higher enzymatic activity than PEG₁-modified asparaginase having the same extent of immunoreactivity. PEG-trypsin having two PEG chains also exhibited higher stability than that having one PEG chain (28). Recently, a copolymer of poly(ethylene glycol) derivative and maleic anhydride (PM) was used in comparison with PEG₁ and PEG₂ (29). PM-modified asparaginase retained higher enzymatic activity and decreased immunoreactivity more than PEG₂-asparaginase. When our results are added to theirs, it is concluded that the increase of free mobile end groups provides great advantages in modification of polymers as well as stabilization of the native biopolymer function.

Protein stability in repetitive temperature cycles between 4 and 50 °C was also tested with native trypsin and PIPAAm-trypsin conjugates. All these conjugates used showed a common LCST near 37 °C. The effect of changing incubation temperature across conjugate LCSTs on enzymatic activity was estimated from retained activity. As can be seen in Figure 5, PIDAAc3-trypsin lost its initial activity more than native trypsin, while IDC-trypsin kept its activity more than the native one under these conditions. This indicates that PIDAAc3-trypsin encountered changes in enzyme higher-order structure during the process of hydration-dehydration which the mobility of PIDAAc affected. Although trypsin itself is quite thermostable, single-point conjugation of polymer chains around trypsin molecules such as IDC-trypsin increases its thermostability. This result also supports the possibility of temperature-induced separation and recycle as reported for IDC-lipase. It is clear that the architecture of the polymer-protein attachment affects the properties of conjugates, especially when intelligent polymers, responsive to heat, ionic strength, or electric

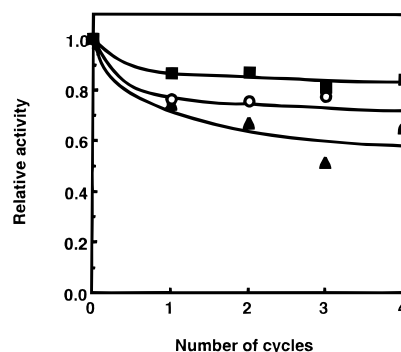


Figure 5. Effect of temperature cycling between 50 and 4 °C on enzymatic activity of PIPAAm-trypsin: (○) native trypsin, (■) IDC-trypsin (68%), (▲) PIDAAc3-trypsin.

field, are coupled to proteins. As the local surroundings and the conformations of the conjugates change, the molecular architecture of the conjugate must respond with new properties while retaining native biological function.

CONCLUSIONS

(1) IDC-trypsin was synthesized through the conjugation of semitelechelic carboxylate co-oligo(IPAAm-DMAAm) to trypsin by single-point attachment to investigate molecular architectural effect on enzyme function, comparing with PIDAAc-trypsin where polymer was attached to trypsin via multiple points.

(2) IDC-trypsin showed the same LCST with IDC oligomer due to the free mobile ends of polymers, which were segregated away from trypsin by attachment chemistry.

(3) The speed of response to temperature changes of IDC-trypsin rapidly increased with the number of attached polymer chains, whereas the speed of response of PIDAAc-trypsins was slow, due to the restriction of polymer shrinkage.

(4) *K_m* values decreased according to the number of polymer chains attached to trypsin, being interpreted by polymer chains which increased the local substrate concentration.

(5) When the incubation temperature was changed across the conjugate LCSTs in a cyclic manner, IDC-trypsin maintained its enzymatic activity, whereas PIDAAc-trypsin lost its activity with temperature cycles.

(6) The molecular architecture of PIPAAm-enzyme affects both new and native functions of the enzyme. Moreover, introduction of free mobile functional polymers to biomolecules can endow new functions such as rapid temperature response and high stability without destroying the native protein functions.

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LITERATURE CITED

- (1) Lee, W. Y., and Sehon, H. (1977) Abrogation of reaginic antibodies with modified allergens. *Nature* 267, 618-619.
- (2) Abchowski, A., van Es, T., Palczuk, N. C., and Davis, F. F. (1977) Alteration of immunological properties of bovine serum albumin by covalent attachment of polyethylene glycol. *J. Biol. Chem.* 252, 3578-3581.
- (3) Yoshimoto, T., Takahashi, K., Nishimura, H., Ajima, A., Tamaura, Y., and Inada, Y. (1984) Modified lipase having high stability and various enzymatic activities in benzene,

- and its re-use by recovering from benzene solution. *Biotechnol. Lett.* 6, 337–340.
- (4) Takei, Y. G., Aoki, T., Sanui, K., Ogata, N., Okano, T., and Sakurai, Y. (1993) Temperature-Responsive Bioconjugates. 1. Synthesis of Temperature-Responsive Oligomers with Reactive End Groups and Their Coupling to Biomolecules. *Bioconjugate Chem.* 4, 42–46.
- (5) Takei, Y. G., Aoki, T., Sanui, K., Ogata, N., Okano, T., and Sakurai, Y. (1993) Temperature-Responsive Bioconjugates. 2. Molecular Design for Temperature-Modulated Bioseparations. *Bioconjugate Chem.* 4, 341–346.
- (6) Matsukata, M., Takei, Y. G., Aoki, T., Sanui, K., Ogata, N., Sakurai, Y., and Okano, T. (1994) Temperature modulated solubility-activity alterations for poly(*N*-isopropylacrylamide)-lipase conjugates. *J. Biochem.* 116, 682–686.
- (7) Takei, Y. G., Matsukata, M., Aoki, T., Sanui, K., Ogata, N., Kikuchi, A., Sakurai, Y., and Okano, T. (1994) Temperature-Responsive Bioconjugates. 3. Antibody–Poly(*N*-isopropylacrylamide) Conjugates for Temperature-Modulated Precipitation and Affinity Bioseparations. *Bioconjugate Chem.* 5, 577–582.
- (8) Heskins, M., Guillet, J. E., and James, E. (1968) Solution properties of poly(*N*-isopropylacrylamide). *J. Macromol. Sci., Chem.* A2, 1441–1455.
- (9) Bae, Y. H., Okano, T., Hsu, R., and Kim, S. W. (1987) Thermosensitive polymers as on-off switches for drug release. *Makromol. Chem., Rapid Commun.* 8, 481–485.
- (10) Bae, Y. H., Okano, T., Hsu, R., and Kim, S. W. (1990) Temperature dependence of swelling of crosslinked poly(*N,N'*-alkylsubstituted acrylamides) in water. *J. Polym. Sci., Polym. Phys. Ed.* 28, 923–936.
- (11) Okano, T., Yui, N., Yokoyama, M., and Yoshida, R. (1994) *Advances in Polymeric Systems for Drug Delivery. Japanese technology Reviews Section E: Biotechnology*, Vol. 4, No. 1, Gordon and Breach Science Publishers SA, Yverdon, Switzerland.
- (12) Yoshida, R., Sakai, K., Okano, T., and Sakurai, Y. (1994) Modulating the phase transition temperature and thermosensitivity in *N*-isopropylacrylamide copolymer gels. *J. Biomater. Sci., Polym. Ed.* 6, 585–598.
- (13) Yoshida, R., Uchida, K., Kaneko, Y., Sakai, K., Kikuchi, A., Sakurai, Y., and Okano, T. (1995) Comb-type grafted hydrogels with rapid de-swelling response to temperature changes. *Nature* 374, 240–242.
- (14) Takezawa, T., Mori, Y., and Yoshizato, K. (1990) Cell culture on a thermo-responsive polymer surface. *Bio/Technology* 8, 854.
- (15) Yamada, N., Okano, T., Sakai, H., Karikusa, F., Sawasaki, Y., and Sakurai, Y. (1990) Thermo-responsive polymeric surfaces; control of attachment and detachment of cultured cells. *Makromol. Chem., Rapid Commun.* 11, 571–576.
- (16) Park, G. T., and Hoffman, A. S. (1993) Synthesis and characterization of a soluble temperature-sensitive polymer-conjugated enzyme. *J. Biomater. Sci., Polym. Ed.* 4, 483–504.
- (17) Chen, G., and Hoffman, A. (1993) Preparation and Properties of Thermoreversible, Phase-Separating Enzyme–Oligo- (*N*-isopropylacrylamide) Conjugates. *Bioconjugate Chem.* 4, 509–514.
- (18) Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193, 265–275.
- (19) Miron, T., and Wilchek, M. (1982) A spectrophotometric assay for soluble and immobilized *N*-hydroxysuccinimide. *Anal. Biochem.* 126, 433–435.
- (20) Stocks, S. J., Jones, A. J. M., Ramey, C. W., and Brooks, D. E. (1986) A fluorometric assay of the degree of modification of protein primary amines with polyethylene glycol. *Anal. Biochem.* 154, 232–234.
- (21) Erlanger, B. F., Kokowsky, N., and Cohen, W. (1961) The preparation and properties of two new chromogenic substrates of trypsin. *Arch. Biochem. Biophys.* 95, 271–278.
- (22) von Spech, B. U., and Brendel, W. (1977) Preparation and properties of trypsin and chymotrypsin coupled covalently to poly(*N*-vinylpyrrolidone). *Biochim. Biophys. Acta* 484, 109–114.
- (23) Abchowski, A., and Davis, F. F. (1979) Preparation and properties of polyethylene glycol-trypsin adducts. *Biochim. Biophys. Acta* 578, 41–46.
- (24) Chiu, H. C., Zalipsky, S., Kopeckova, P., and Kopecek, J. (1993) Enzymatic Activity of Chymotrypsin and Its Poly-(ethylene glycol) Conjugates toward Low and High Molecular Weight Substrates. *Bioconjugate Chem.* 4, 290–295.
- (25) Valuev, L. I., Zefirova, O. N., Obyednova, I. V., and Plate, N. A. (1994) Targeted delivery of drugs provided by water-soluble polymeric systems with low critical solution temperature (LCST). *J. Bioact. Compat. Polym.* 9, 55–65.
- (26) Lazdunski, M., and Delaage, M. (1965) Sur la morphologie des trypsines de porc et de boeuf etude des denaturations reversibles. *Biochim. Biophys. Acta* 105, 541–561.
- (27) Matsushima, A., Nishimura, H., Ashihara, Y., Yokota, Y., and Inada, Y. (1980) Modification of *E. coli* asparaginase with 2, 4-bis(*O*-methoxypolyethylene glycol)-6-chloro-*s*-triazine (activated PEG₂): disappearance of binding ability toward antiserum and retention of enzymic activity. *Chem. Lett.* 7, 773–776.
- (28) Monfardini, C., Schiavon, O., Caliceti, P., Morpurgo, M., Harris, J. M., and Veronese, F. M. (1995) A Branched Monomethoxypoly(ethylene glycol) for Protein Modification. *Bioconjugate Chem.* 6, 62–69.
- (29) Kodera, Y., Tanaka, H., Matsushima, A., Saito, Y., and Inada, Y. (1992) Chemical modification of L-asparaginase with a comb-shaped copolymer of polyethylene glycol derivative and maleic anhydride. *Biochem. Biophys. Res. Commun.* 184, 44–148.

No-Carrier-Added (4-Fluoro-3-[¹³¹I]iodobenzyl)guanidine and (3-[²¹¹At]Astatobenzyl)guanidine

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With 3-bromo-4-fluorotoluene as starting material, [4-fluoro-3-(trimethylsilyl)benzyl]guanidine was prepared in five steps in 1.5% overall yield. Radioiodination of this silicon precursor using *N*-chlorosuccinimide in trifluoroacetic acid at room temperature for 5 min gave (4-fluoro-3-[¹³¹I]iodobenzyl)guanidine ([¹³¹I]FIBG) in 50–60% radiochemical yield. A byproduct which had a retention time in two HPLC systems similar to that of (*m*-iodobenzyl)guanidine (MIBG) was formed in about 30% yield. [¹³¹I]FIBG was stable up to 3 h under these conditions of iodination, indicating that the byproduct is not generated as a result of [¹³¹I]FIBG degradation. Using hydrogen peroxide as the oxidant in aqueous medium and a reaction time of 30 min at 50 °C, yields of [¹³¹I]FIBG could be increased to 75–80%, with less than 7% of the byproduct formed under these conditions. Astatination of the silicon precursor using *N*-chlorosuccinimide in trifluoroacetic acid at 70 °C gave 65–70% radiochemical yield of (3-[²¹¹At]astato-4-fluorobenzyl)guanidine ([²¹¹At]AFBG) in 10–15 min; about 17% of the byproduct formation was seen. Astatination of the silicon precursor under aqueous conditions using hydrogen peroxide was not successful.

INTRODUCTION

Over the past decade (*m*-[¹³¹I]iodobenzyl)guanidine (MIBG,¹ Figure 1) has been used extensively for the localization and therapy of neuroendocrine tumors, such as neuroblastoma and pheochromocytoma (McEwan *et al.*, 1986), and for the scintigraphy of pathophysiological conditions of the myocardium (Glowniak *et al.*, 1989). Although [¹³¹I]MIBG has been used successfully for many applications, investigators have sought to enhance this type of tracer through the development of (halobenzyl)guanidines labeled with radionuclides with more favorable decay properties for either imaging or therapy. For example, the possibility of positron emission tomographic (PET) imaging has been explored using both (*m*-[¹²⁴I]iodobenzyl)guanidine ([¹²⁴I]MIBG; Ott *et al.*, 1992) and (*m*-[⁷⁶Br]bromobenzyl)guanidine ([⁷⁶Br]MBBG; Vallette *et al.*, 1993; Loc'h *et al.*, 1994). Because ¹⁸F is the longest-lived positron-emitting nuclide routinely available at most PET centers, an analog of MIBG labeled with ¹⁸F is attractive. Development of (*m*-[¹⁸F]fluorobenzyl)- and (*p*-[¹⁸F]fluorobenzyl)guanidines has been reported; however, compared with those of MIBG, their specific binding to SK-N-SH human neuroblastoma cells in vitro and their myocardial uptake in mice were significantly lower (Garg *et al.*, 1994). Our own efforts to develop an MIBG analog suitable for PET have focused on (4-[¹⁸F]fluoro-3-iodobenzyl)guanidine ([¹⁸F]FIBG; Vaidyanathan *et al.*, 1994a, 1995). This strategy has yielded encouraging results; compared with MIBG, [¹⁸F]FIBG had higher uptake in SK-N-SH human neuroblastoma cells in vitro and in innervated mouse tissues in vivo.

Our results suggest that [¹⁸F]FIBG could be a promising agent for PET; moreover, this tracer might be of

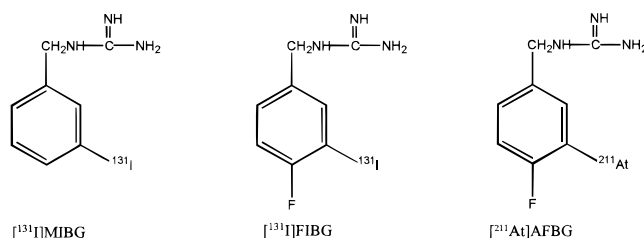


Figure 1. Structures of [¹³¹I]MIBG, [¹³¹I]FIBG, and [²¹¹At]AFBG.

particular value for prediction of the tumor and normal tissue dosimetry which would occur for a therapeutic administration of (4-fluoro-3-[¹³¹I]iodobenzyl)guanidine ([¹³¹I]FIBG). In addition, since we have recently demonstrated that the α -emitting analog of MIBG, (*m*-[²¹¹At]astatobenzyl)guanidine ([²¹¹At]MABG) is extremely cytotoxic to human neuroblastoma cells (Strickland *et al.*, 1994), the astatinated analog (3-[²¹¹At]astato-4-fluorobenzyl)guanidine ([²¹¹At]AFBG) also might have favorable properties for radiotherapy. In this paper, we report on the no-carrier-added synthesis of (4-fluoro-3-[¹³¹I]iodobenzyl)guanidine ([¹³¹I]FIBG) starting from a silicon precursor. Using this precursor, it was also possible to prepare [²¹¹At]AFBG.

MATERIALS AND METHODS

General. All chemicals were purchased from Aldrich Chemical Co. except as noted. A previously reported method (Vaidyanathan *et al.*, 1994a) was used to prepare macrolevels of (4-fluoro-3-iodobenzyl)guanidine. Sodium [¹³¹I]iodide in 0.1 N NaOH was supplied by DuPont-New England Nuclear (North Billerica, MA). Melting points were determined on a Fisher Johns apparatus and were uncorrected. High-performance liquid chromatography (HPLC) was conducted using one of the following systems: (1) two LKB Model 2150 pumps, an LKB Model 2152 control system, an LKB Model 2138 fixed-wavelength UV detector, and a Beckman Model 170 radioisotope detector (peak analysis was performed using a Nelson Analytical software package on an IBM computer); or (2) a Perkin-Elmer Series 4 Liquid Chromato-

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¹ Abbreviations: MIBG, (*m*-iodobenzyl)guanidine; FIBG, (4-fluoro-3-iodobenzyl)guanidine; [²¹¹At]AFBG, (3-[²¹¹At]astato-4-fluorobenzyl)guanidine; nca, no-carrier-added; TMSBG, [3-(trimethylsilyl)benzyl]guanidine; MBBG, (*m*-bromobenzyl)guanidine.

graph connected to a Perkin-Elmer LC-95 UV/visible spectrophotometer detector and a Perkin-Elmer LCI-100 Laboratory Computing Integrator (for programming the methods, a Perkin-Elmer 6312 display terminal was used). Reverse-phase chromatography was used to identify and to isolate the final product. A Waters μ Bondapak C18 (10 μ m, 3.9 $\times$ 300 mm) column was used and eluted with (A) 96.5/2/1/0.5 (v/v/v/v) water/tetrahydrofuran/triethylamine/phosphoric acid at a flow rate of 3 mL/min or with (B) 80/20 (v/v) 0.2 M $\text{NH}_4\text{H}_2\text{PO}_4$ /tetrahydrofuran at 0.8 mL/min. NMR spectra were obtained on a General Electric Midfield GN-300 spectrometer. Proton chemical shifts were reported in δ units, and tetramethylsilane was used as an internal reference ($\delta = 0$). Infrared spectra were acquired on a BOMEM MB-100 variable-resolution FTIR spectrophotometer. Mass spectra were obtained on a ZAB-E high-resolution mass spectrometer (VG, Manchester, England), on a Hewlett-Packard GC/MS/DS Model HP-5988A instrument, or on a JEOL SX-102 high-resolution mass spectrometer. Elemental analyses were provided by Oneida Research Services (Whitesboro, NY).

4-Fluoro-3-(trimethylsilyl)toluene (1). To a solution of 3-bromo-4-fluorotoluene (5 g, 26.5 mmol) in dry THF (65 mL), cooled in a dry ice/ether bath, was gradually added 16.5 mL of 1.6 M *n*-butyllithium in hexanes. After the mixture was stirred for 15 min, 3.3 mL (1 equiv) of trimethylsilyl chloride (distilled over calcium hydride) was added dropwise. The solution was warmed gradually to room temperature and left stirring for 20 h. Most of the THF from the reaction mixture was removed by rotary evaporation, and the residue was partitioned between water and ether. The aqueous layer was further extracted with ether, and the pooled ethereal layer was washed with brine, dried with sodium sulfate, and evaporated to obtain 4 g of clear yellow oil. This was purified by silica gel chromatography to yield 1.5 g (31%) of a clear oil: $^1\text{H-NMR}$ (CDCl_3) δ 0.25 (s, 9H, SiMe_3), 2.70 (s, 3H, CH_3), 6.35 (t, 1H, aromatic), 7.15 (m, 2H, aromatic); MS (CI) m/z 181, 165, 151, 136, 109.

4-Fluoro-3-(trimethylsilyl)benzyl Bromide (2). A mixture of **1** (4.4 g, 24.2 mmol), *N*-bromosuccinimide (2.2–4.3 g, 14.1–24.2 mmol), and benzoyl peroxide (24 mg, 0.1 mmol) in 15 mL of carbon tetrachloride was heated under reflux for 2–4 h. The solids from the cooled reaction mixture were separated by filtration. The filtrate was washed with 5% sodium hydroxide and brine, dried with sodium sulfate, and evaporated. The residue was purified by silica gel chromatography to yield 1.7 g (27%) of a clear to pale yellow oil: $^1\text{H-NMR}$ (CDCl_3) δ 0.32 (s, 9H, SiMe_3), 4.49 (s, 2H, CH_2Br), 6.96 (t, 1H, aromatic), 7.38 (m, 2H, aromatic); MS (CI) m/z 262, 245, 182, 165, 119.

4-Fluoro-3-(trimethylsilyl)- α -phthalimido-toluene (3). Potassium phthalimide (1.31 g, 7 mmol) and **2** (1.67 g, 6.4 mmol), were taken in 10 mL of acetone, and the mixture was heated at reflux for 1 h. The mixture was filtered, and the residue was washed with acetone. Evaporation of acetone from the filtrate yielded 1.89 g (90%) of a fluffy solid: mp 94–95 $^\circ\text{C}$; $^1\text{H-NMR}$ (CDCl_3) δ 0.29 (s, 9H, SiMe_3), 4.80 (s, 2H, CH_2NH), 6.91 (t, 1H, aromatic), 7.46 (m, 2H, aromatic), 7.70 (m, 2H, aromatic), 7.84 (m, 2H, aromatic); MS (CI) m/z 345 ($\text{M} + \text{NH}_4^+$), 328 (MH^+).

[4-Fluoro-3-(trimethylsilyl)benzyl]amine (4). A solution of hydrazine (460 mg, 9.2 mmol) in ethanol (0.4 mL) was added to a solution of **3** (1.9 g, 5.8 mmol) in 10 mL of hot ethanol. The mixture was heated in an oil bath maintained at 80–100 $^\circ\text{C}$. Within 5–6 min, the reaction mixture could no longer be stirred and was heated as

such for an additional 2–3 h. The reaction mixture was acidified with 1 N hydrochloric acid, filtered, and washed with cold water. The filtrate was made basic with 1 N sodium hydroxide and extracted with ether. The pooled ethereal extract was dried with anhydrous magnesium sulfate. To the above solution was added, with vigorous stirring, ethereal hydrogen chloride. The precipitate formed was isolated by filtration, washed with ether, and dried to yield 150 mg (12%) of a white solid: mp 206–209 $^\circ\text{C}$; $^1\text{H-NMR}$ (CD_3OD) δ 0.35 (s, 9H, SiMe_3), 4.10 (s, 2H, CH_2NH_2), 7.10 (t, 1H, aromatic), 7.55 (m, 2H, aromatic); MS (CI) m/z 198, 182, 108.

[4-Fluoro-3-(trimethylsilyl)benzyl]guanidine (5). A dry mixture of **4** (155 mg, 0.66 mmol) and cyanamide (88 mg, 2 mmol) was heated at 100 $^\circ\text{C}$ in an oil bath for 4 h. The residue was dissolved in 150 μL of water, and the product was precipitated as a bicarbonate salt by the addition of 150 μL of potassium bicarbonate solution in water (35 mg, 0.35 mmol). The bicarbonate salt was filtered and recrystallized from 2 N sulfuric acid to obtain 96 mg (50%) of white crystals: mp 255–257 $^\circ\text{C}$; $^1\text{H-NMR}$ (CD_3OD) δ 0.30 (s, 9H, SiMe_3), 4.35 (s, 2H, CH_2NH), 7.01 (t, 1H, aromatic), 7.37 (m, 2H, aromatic); MS (FAB^+) m/z 240, 181; HRMS calcd for $\text{C}_{11}\text{H}_{19}\text{N}_3\text{FSi}$ (MH^+) m/z 240.1332, found 240.1335. Anal. Calcd for $\text{C}_{11}\text{H}_{19}\text{FN}_3\text{O}_2\text{Si}_{1/2}$: C, 45.81; H, 6.64; N, 14.57. Found: C, 45.71; H, 6.54; N, 14.45. The purity of **5** was further checked by HPLC. A Waters μ Bondapak C18 (10 μm , 3.9 $\times$ 300 mm) column was used and eluted with 78/22 (v/v) 0.2 M $\text{NH}_4\text{H}_2\text{PO}_4$ /tetrahydrofuran at a flow rate of 1 mL/min ($t_R = 22$ –23 min). The purity was more than 99%, and HPLC failed to detect any [3-(trimethylsilyl)benzyl]guanidine (TMSBG, $t_R = 14$ –15 min).

Conversion of 5 to Unlabeled FIBG, an Attempt To Isolate the Byproduct. In order to characterize the byproduct (possibly by mass spectroscopy) formed during the radioiodination of **5** (*vide infra*), the iodination reaction was conducted using sodium iodide. To a solution of sodium iodide in 0.1 N NaOH (1 μL of 0.36 M solution, 0.36 μmol) was added 10 μL of *N*-chlorosuccinimide in trifluoroacetic acid (0.3 M) followed by 5 μL of **5** in trifluoroacetic acid (0.1 M, 0.5 μmol). After 5 min, the reaction mixture was injected onto the HPLC column. A major peak corresponding to FIBG was formed, with no product eluting with a retention time corresponding to that of MIBG detectable. The results were the same when the reaction was allowed to proceed for 30 min.

Determination of Relative Reactivity of 5 and TMSBG toward Iodination. To 1 μL (0.356 μmol) of sodium iodide in 0.1 N NaOH were added 5 μL of a 0.6 M solution of *N*-chlorosuccinimide in trifluoroacetic acid and 15 μL of trifluoroacetic acid solution of **5** and TMSBG (each 33 mM, 0.5 μmol). After 5 min at room temperature, the reaction mixture was injected onto the HPLC column. The major peak corresponded to MIBG, and there was no detectable peak corresponding to FIBG.

Radioiodination of 5 Using *N*-Chlorosuccinimide. To a Reacti vial containing 0.1–2 mCi of [^{131}I]iodide in 1–3 μL of 0.1 N NaOH was added 10 μL of a solution of *N*-chlorosuccinimide (0.3 M in trifluoroacetic acid) followed by 5 μL of a 0.14 M solution of **5** in trifluoroacetic acid. The mixture was vortexed and left at room temperature for 5 min. The radioiodinated (4-fluoro-3-iodobenzyl)guanidine was purified by HPLC using solvent system A. The retention time of the product was about 35 min. Radiochemical yields were in the range of 50–60% of the activity injected into the HPLC column.

About 30% of the injected activity was eluted with a retention time corresponding to that of an MIBG standard (25 min). This byproduct also had a retention time

similar to that of MIBG (14 min) when solvent system B was used. To investigate whether MIBG is formed due to the defluorination of FIBG, the stability of [^{131}I]FIBG under the reaction conditions was determined. About 100–200 μCi of [^{131}I]FIBG (purified by HPLC and processed using a C18 Sep Pak, *vide infra*) in 50–100 μL of methanol was added to a Reacti vial, and the methanol was evaporated to dryness using an argon stream. To the residual dry activity were added 3 μL of 0.1 N NaOH, 10 μL of 0.3 M *N*-chlorosuccinimide in trifluoroacetic acid, and 5 μL of a 0.14 M solution of **5** in trifluoroacetic acid. Aliquots of this mixture were analyzed periodically by HPLC.

Radioiodination of **5** Using Hydrogen Peroxide.

To 50 μL of a saturated solution of **5** in water was added 100–500 μCi of [^{131}I]iodide in 1–2 μL of 0.1 N NaOH followed by 8.3 μL of 6 N HCl and 5 μL of 6% hydrogen peroxide in water. The mixture was heated at 50 $^{\circ}\text{C}$ for 30 min, and the [^{131}I]FIBG was isolated by HPLC in 75–80% radiochemical yield. The byproduct corresponding to MIBG was formed in less than 7% radiochemical yield.

Preparation of [^{211}At]AFBG. Astatine-211 was produced on the Duke University Medical Center cyclotron by 27–28 MeV α -particle-beam bombardment of a natural bismuth target via the $^{209}\text{Bi}(\alpha, 2n)^{211}\text{At}$ reaction as described (Zalutsky *et al.*, 1989). The activity was distilled and trapped into chloroform. Activity from aliquots of chloroform solution was extracted into 2–3 μL of 0.1 N sodium hydroxide (Vaidyanathan and Zalutsky, 1992). To about 100 μCi of the ^{211}At activity was added 10 μL of a 0.3 M solution of *N*-chlorosuccinimide in trifluoroacetic acid followed by 10 μL of a 0.07 M solution of the precursor **5** in trifluoroacetic acid. Initially, the reaction was conducted at room temperature; however, poor radiochemical yields were obtained. So the reaction mixture was heated at 70–75 $^{\circ}\text{C}$ for various periods of time. The yield was determined by reverse-phase HPLC using solvent system B.

Paired-Label Binding of Authentic No-Carrier-Added [^{131}I]MIBG and [^{125}I]Labeled Byproduct Obtained from the Radioiodination of **5.** SK-N-SH human neuroblastoma cells were cultured and plated as described previously (Vaidyanathan and Zalutsky, 1993). To 4×10^5 SK-N-SH cells per well were added 150 nCi of both nca [^{131}I]MIBG and the [^{125}I]labeled byproduct in a total volume of 500 μL of medium. The cells were incubated at 37 $^{\circ}\text{C}$ for 2 h in an incubator. At the end of the incubation period, the medium was aspirated and the cells were washed twice with phosphate-buffered saline. The cells were solubilized by incubation with 0.5 N NaOH for 0.5 h. Vials containing the solubilized cells were counted for ^{131}I and ^{125}I in a dual-channel γ -counter to determine the cell-bound activity. Nonspecific binding was determined by preincubation of cells for 0.5 h with 1.5 μM desipramine, an uptake-1 inhibitor, before addition of the radiolabeled agents as described previously (Vaidyanathan *et al.*, 1995). Each experiment was performed in quadruplicate.

RESULTS AND DISCUSSION

Although radioiodinated MIBG has been a valuable radiopharmaceutical in clinical nuclear medicine, there is considerable interest in development of improved (halobenzyl)guanidine derivatives for diagnostic and therapeutic applications. For example, the physical characteristic of ^{131}I was found to be suboptimal in therapy, especially for the micrometastatic tumors (Sisson *et al.*, 1990), leading a number of investigators to propose ^{211}At as an alternative radiolabel (Kemshead *et al.*, 1990; Mairs *et al.*, 1991; Shapiro *et al.*, 1987). For

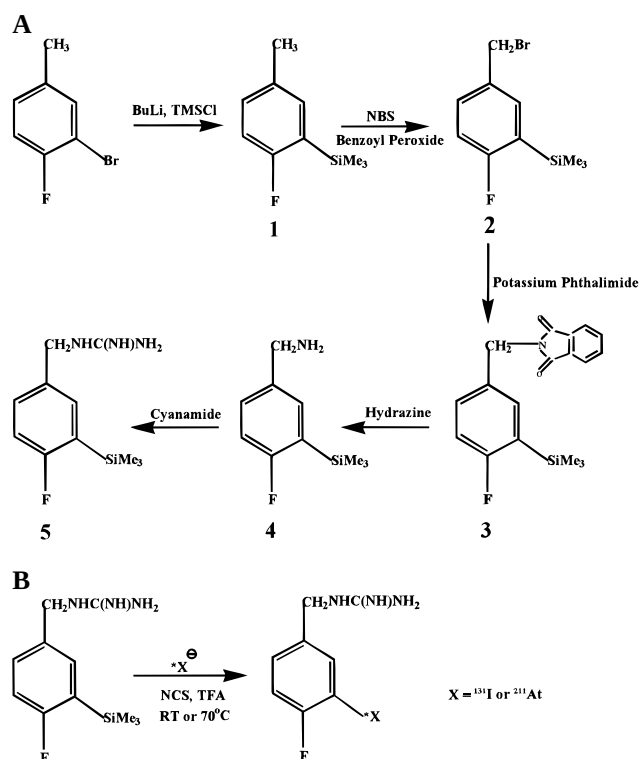


Figure 2. (A) Synthesis scheme for the preparation of silicon precursor **5**. (B) Radiohalogenation of silicon precursor **5**.

imaging, [^{123}I]MIBG in combination with single photon emission computed tomography has been found to have advantages over the [^{131}I]MIBG (Shulkin *et al.*, 1986), suggesting that better images may be obtained by combination of the distribution properties of MIBG with the superior imaging capabilities of PET. In order to be useful for PET, a ^{18}F -labeled analog of this agent ([^{18}F]FIBG) was developed (Vaidyanathan *et al.*, 1994a). The encouraging results obtained with this new derivative motivated the development of radioiodinated and astatinated analogs of FIBG. A recent review indicated that [^{125}I]FIBG has excellent adrenal uptake in dog (Wieland, 1989); however, no details with regard to the synthesis of this tracer have been published.

Since no-carrier-added MIBG and [^{211}At]MABG were prepared in excellent yields from a silicon precursor (Vaidyanathan and Zalutsky, 1992, 1993), an analogous precursor was sought for the preparation of nca [^{131}I]FIBG and [^{211}At]AFBG. The scheme for the synthesis of the required precursor is shown in Figure 2A. The conversion of 3-bromo-4-fluorotoluene to 4-fluoro-3-(trimethylsilyl)toluene (**1**) via the sodium for bromine exchange was not successful. When this conversion was attempted via a lithio derivative, a 30% isolated yield of the required compound was obtained. Subsequent steps proceeded in similar fashion as those reported previously for the synthesis of TMSBG (Vaidyanathan and Zalutsky, 1993). The yields for the preparation of **2**–**5** were 27, 90, 12, and 50%, respectively. The final compound was characterized by various spectroscopic techniques as well as by the combustion analysis. These data were in full agreement with its structure.

When radioiodination conditions similar to those used successfully in the preparation of nca [^{131}I]MIBG (*N*-chlorosuccinimide as the oxidant in a trifluoroacetic acid medium) were used for the conversion of **5** to [^{131}I]FIBG (Figure 2B), 50–60% radiochemical yields were obtained in less than 5 min at room temperature. These yields were much lower than the >90% yields obtained routinely for nca [^{131}I]MIBG. However, another radioactive

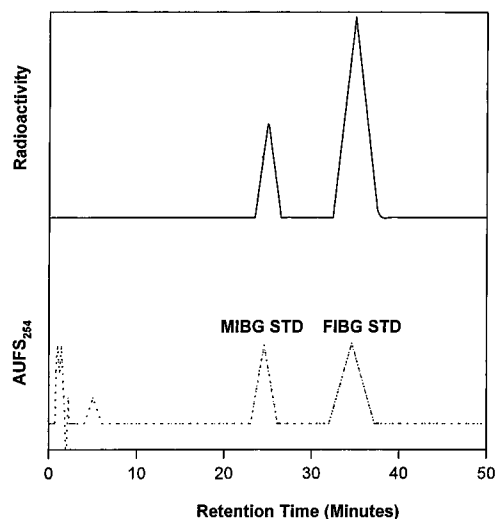


Figure 3. Typical HPLC profile (using solvent system A) of a reaction mixture of radioiodination of **5** coinjected with MIBG and FIBG standards. There is a delay of about 0.4 min between the UV and radioactivity traces.

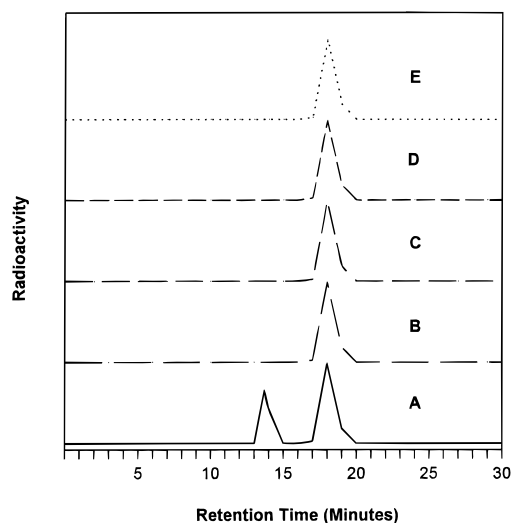


Figure 4. (A) HPLC profile of the radioiodination reaction mixture in the conversion of **5** to $[^{131}\text{I}]$ FIBG. (B–E) HPLC pattern of $[^{131}\text{I}]$ FIBG subjected to reaction conditions for (B) 30 min, (C) 60 min, (D) 120 min, and (E) 180 min.

peak, in an amount roughly equal to half that of $[^{131}\text{I}]$ -FIBG activity, was also formed in this reaction. This secondary radioactive peak eluted with a retention time corresponding to that of MIBG using both solvent systems (Figures 3 and 4A). The retention time of MIBG is about 25 min with solvent system A and about 14 min with solvent system B. The corresponding values for FIBG are about 35 and 18 min, respectively. Another indication that the byproduct could be $[^{131}\text{I}]$ MIBG was obtained from a paired-label binding assay of nca $[^{131}\text{I}]$ -MIBG and the byproduct obtained from the ^{125}I labeling of **5** with SK-N-SH cells. Very high specific uptake of MIBG by the SK-N-SH cell line has been demonstrated (Buck *et al.*, 1985; Smets *et al.*, 1989; Vaidyanathan and Zalutsky, 1993). As shown in Figure 5, the percent of input activity bound was 62 ± 2 and $69 \pm 2\%$, respectively, for authentic nca $[^{131}\text{I}]$ MIBG and the byproduct (purified by HPLC). Furthermore, in both cases, the binding was reduced to about 6% when the cells were pretreated with the uptake-1 inhibitor, desipramine.

If this byproduct is indeed MIBG, it is not clear how it could have been formed. One possibility is presence of trace amounts of TMSBG in **5**. Since halodesilylation is

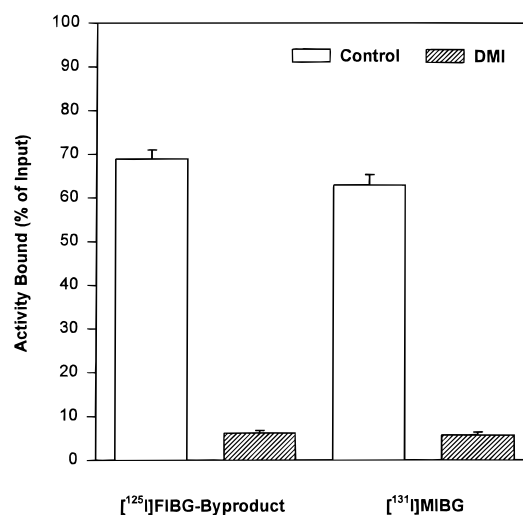


Figure 5. Paired-label binding of nca $[^{131}\text{I}]$ MIBG and ^{125}I -labeled byproduct obtained from the radioiodination of **5** ($[^{125}\text{I}]$ -FIBG-byproduct) to SK-N-SH neuroblastoma cells.

an electrophilic reaction, one would expect TMSBG to be much more reactive than **5** due to the strong electron-withdrawing nature of fluorine. To verify that this indeed was the case, cold iodination of an equimolar mixture of TMSBG and **5** was conducted using less than 1 equiv of iodide. Under these conditions, only MIBG was produced, suggesting that TMSBG is many times more reactive than **5** toward iodination. However, accurate elemental analysis has been obtained for **5**, and HPLC of it failed to show any evidence of TMSBG. Other investigators have prepared nca $[^{131}\text{I}]$ MIBG from TMSBG using hydrogen peroxide as the oxidant in aqueous conditions in excellent yields (Joshua, A. V., Edmonton Radiopharmaceutical Center, Alberta, Canada, personal communication). Since we have been able to obtain similar yields for nca $[^{131}\text{I}]$ MIBG using this procedure, we decided to investigate these conditions for the production of $[^{131}\text{I}]$ FIBG. Interestingly, 75–80% of $[^{131}\text{I}]$ FIBG was formed under these conditions, and the byproduct was formed in less than 7% yield. Although the greater reactivity of TMSBG suggests that only trace levels of this impurity could be responsible for the observed byproduct, this is insufficient evidence in itself to assert unequivocally that the byproduct was generated from the radioiodination of trace amounts of TMSBG.

To investigate whether defluorination of $[^{131}\text{I}]$ FIBG under the reaction conditions accounted for the byproduct, $[^{131}\text{I}]$ FIBG isolated by HPLC and subsequently concentrated by solid-phase cartridge (*vide infra*) was subjected to the reaction conditions and followed periodically by HPLC. As shown in Figure 4, $[^{131}\text{I}]$ FIBG was very stable under reaction conditions during the 3 h observation period. This suggests that MIBG must have been produced by an alternate reaction pathway. In order to characterize this byproduct, isolation of it was attempted by conducting the reaction using carrier iodide. Surprisingly, byproduct formation was not seen at a carrier-added level.

A remote possibility is the formation of a benzyne intermediate resulting from a β -elimination and the conversion of the benzyne intermediate to MIBG. This type of elimination of β -halosilanes generally occurs under strongly basic or nucleophilic conditions. Generation of benzyne by fluoride-induced elimination of aromatic β -halosilanes has been reported (Hudrlik and Agwarambo, 1988), and this could be relevant if residual fluoride were present in trifluoroacetic acid. However,

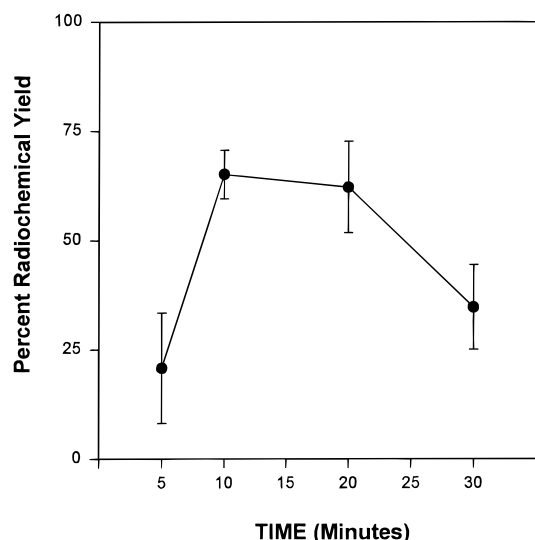


Figure 6. $[^{211}\text{At}]$ AFBG production. Dependence of radiochemical yield on time of reaction.

the formation of benzyne under the highly acidic conditions employed for the current reaction is highly unlikely. In order for MIBG to form from the benzyne generated from the elimination reaction of **5**, an electrophilic substitution of I^+ would have to be invoked. Although benzyne generally undergo nucleophilic substitution, electrophilic substitution is not uncommon (Hoffmann, 1967). However, to complete the reaction, a hydride ion is necessary, the presence of which is not possible in an acidic medium.

To date, a maximum of 800 μCi of $[^{131}\text{I}]$ FIBG has been produced. No coeluting carrier has been detected in the HPLC UV trace. The lower limit of detection under these HPLC conditions is about 2 nmol. Thus, the specific activity of $[^{131}\text{I}]$ FIBG is estimated to be more than 400 Ci/mmol.

As observed previously with TMSBG, the astatodesilylation of **5** using *N*-chlorosuccinimide did not take place when the reaction was conducted at room temperature. However, excellent yields have been obtained for $[^{211}\text{At}]$ MABG when the reaction was performed at 70 °C over a period of 5 min. In contrast, a 5 min reaction time was not sufficient to obtain an appreciable yield of $[^{211}\text{At}]$ AFBG. In order to find the optimum time, a yield versus reaction duration study was performed, and the results are shown in Figure 6. Although 65–70% yield was obtained by conducting the reaction for 10 min, no further improvement was seen by increasing the reaction time to more than 10 min. In fact, the yield actually decreased when the reaction was conducted for 30 min. This may be due to deastatination of $[^{211}\text{At}]$ AFBG as the relative amount of activity corresponding to $[^{211}\text{At}]$ -astatide was higher as determined by HPLC. For this reason, a 10–15 min reaction time is recommended for optimum yield.

It is interesting to note that the ratio of $[^{211}\text{At}]$ AFBG to its byproduct is slightly higher than that of $[^{131}\text{I}]$ FIBG to its byproduct obtained for the iodination of **5**. For example, after a 10–15 min reaction, the XFBG/byproduct ratio was 3.5/1 for ^{211}At compared with 2/1 for ^{131}I . The reason for this is not known at this time. The slower rate of reaction in the formation of $[^{211}\text{At}]$ AFBG from **5** may be due to several reasons. Two of these, which also pertain to astatodesilylation of TMSBG, are the steric bulk and the lower (about 30-fold) concentration of ^{211}At compared with that of ^{131}I . An additional factor comes into play in the case of $[^{211}\text{At}]$ AFBG. In the transition

state of the latter reaction, the carbon-bearing fluorine will have net positive charge which is energetically unfavorable. Astatination of **5** under aqueous conditions using hydrogen peroxide as the oxidant did not yield any product, nor can these conditions be adaptable for the formation of $[^{211}\text{At}]$ MABG from TMSBG.

Although the yields obtained for the target compounds are less than those obtained for $[^{131}\text{I}]$ MIBG and $[^{211}\text{At}]$ -MABG from their silicon precursors, most of the remainder of activity is converted to a byproduct with a specificity for neuroblastoma cells similar to that of $[^{131}\text{I}]$ -MIBG. The target cell uptake/retention and tissue distribution properties of FIBG were not identical to those of MIBG (Vaidyanathan *et al.*, 1994b), so it is important to separate them from the mixture in order to better understand the differences between FIBG and MIBG. The HPLC systems described, especially system A, could separate these two compounds very well. As performed with other (halobenzyl)guanidines (Vaidyanathan and Zalutsky, 1992, 1993; Vaidyanathan *et al.*, 1994a), HPLC fractions containing $[^{131}\text{I}]$ FIBG and $[^{211}\text{At}]$ AFBG can be desalted and concentrated using a C18 solid-phase cartridge. The concentrated activity can be reconstituted in a buffer suitable for biological assays.

In conclusion, methods for the preparation of no-carrier-added $[^{131}\text{I}]$ FIBG have been developed. Using the same silicon precursor, the astatinated analog, $[^{211}\text{At}]$ -AFBG, could also be prepared in good yields. Preliminary results have indicated that $[^{131}\text{I}]$ FIBG, like $[^{18}\text{F}]$ -FIBG, binds specifically in vitro to SK-N-SH human neuroblastoma cells to a higher degree than MIBG (Vaidyanathan *et al.*, 1994b). Moreover, about 70–80% of the initially bound activity was observed to be retained by SK-N-SH cells for up to 3 days, whereas by this time period, less than 15% of the bound MIBG activity was retained. Although ^{211}At has a half-life of only 7.2 h, it will be interesting to see whether $[^{211}\text{At}]$ AFBG will also be retained for a prolonged time by the SK-N-SH cells and whether such retention has any therapeutic advantage. Further evaluations of the in vitro binding properties of $[^{131}\text{I}]$ FIBG and $[^{211}\text{At}]$ AFBG are in progress and will be reported in a subsequent publication.

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LITERATURE CITED

- Buck, J., Bruchelt, G., Girgert, R., Treuner, J., and Niethammer, D. (1985) Specific Uptake of *m*- $[^{125}\text{I}]$ iodobenzylguanidine in the human neuroblastoma cell line SK-N-SH. *Cancer Res.* 45, 6366–6370.
- Garg, P. K., Garg, S., and Zalutsky, M. R. (1994) Synthesis and preliminary evaluation of *para*- and *meta*- $[^{18}\text{F}]$ fluorobenzylguanidine. *Nucl. Med. Biol.* 21, 97–103.
- Glowniak, J. V., Turner, F. E., Gray, L. L., Palac, R. T., Lagunas-Solar, M. C., and Woodward, W. R. (1989) Iodine-123 metaiodobenzylguanidine imaging of the heart in idiopathic congestive cardiomyopathy and cardiac transplants. *J. Nucl. Med.* 30, 1182–1191.
- Hoffmann, R. W. (1967) *Dehydrobenzenes and Cycloalkynes*, *Organic Chemistry*, Vol. 11, p 182, Academic Press, New York.
- Hudrlík, P. F., and Agwaramgbo, E. L. O. (1988) Elimination reactions of β -functional organosilicon compounds. In *Silicon Chemistry* (J. Y. Corey, E. R. Corey, and P. P. Gaspar, Eds.) pp 95–104, Ellis Horwood Limited, Chichester, England.
- Kemshead, J. T., Pizer, P. L., and Patel, K. (1990) Neuroblastoma: Perspectives for future research. In *Neuroblastoma:*

- Tumor Biology and Therapy* (C. Pochedly, Ed.) pp 381–395, CRC Press, Boca Raton, FL.
- Loc'h, C., Mardon, K., Valette, H., Brutesco, C., Merlet, P., Syrota, A., and Maziere, B. (1994) Preparation and pharmacological characterization of [^{76}Br]-meta-bromobenzylguanidine ([^{76}Br]MBBG). *Nucl. Med. Biol.* 21, 49–55.
- Mairs, R., Angerson, W. J., Babich, J. W., and Murray, T. (1991) Differential penetration of targeting agents into multicellular spheroids derived from human neuroblastoma. In *Advances in Neuroblastoma Research 3 (Progress in Clinical and Biological Research, Vol. 366)* (A. E. Evans, G. J. D'Angio, A. G. Knudson, Jr., and R. C. Seeger, Eds.) pp 495–501, Wiley-Liss, Inc., New York.
- McEwan, A. J., Wyeth, P., and Ackery, D. (1986) Radioiodinated iodobenzylguanidines for diagnosis and therapy. *Appl. Radiat. Isot.* 37, 765–775.
- O'Donoghue, A., Wheldon, T. E., Babich, J. W., Moyes, J. S. E., Barret, A., and Meller, T. (1991) Implications of the uptake of [^{131}I]-radiolabelled meta-iodobenzylguanidine (MIBG) for the targeted radiotherapy of neuroblastoma. *Br. J. Radiol.* 64, 428–434.
- Ott, R. J., Tait, D., Flower, M. A., Babich, J. W., and Lambrecht, R. M. (1992) Treatment planning for [^{131}I]-MIBG radiotherapy of neural crest tumors using [^{124}I]-MIBG positron emission. *Br. J. Radiol.* 65, 787–791.
- Shapiro, B., and Gross, M. D. (1987) Radiochemistry, biochemistry, and kinetics of [^{131}I]-metaiodobenzylguanidine (MIBG) and [^{123}I]-MIBG: Clinical implications of the use of [^{123}I]-MIBG. *Med. Pediatr. Oncol.* 15, 170–177.
- Shulkin, B. L., Shapiro, B., Francis, I., Door, R., Shew, S.-W., and Sisson, J. C. (1986) Primary extra-adrenal pheochromocytoma positive I-123 MIBG imaging with negative I-131 MIBG imaging. *Clin. Nucl. Med.* 11, 851–854.
- Sisson, J. C., Hutchinson, R. J., Shapiro, B., Zasadny, K. R., Normolle, D., Wieland, D. M., Wahl, R. L., Singer, D. A., Mallette, S. A., and Mudgett, E. E. (1990) Iodine-125–MIBG to treat neuroblastoma: Preliminary report. *J. Nucl. Med.* 31, 1479–1485.
- Smets, L. A., Loesberg, C., Janssen, M., Metwally, E. A., and Huiskamp, R. (1989) Active uptake and extravesicular storage of m-iodobenzylguanidine in human neuroblastoma SK-N-SH cells. *Cancer Res.* 49, 2941–2944.
- Strickland, D. K., Vaidyanathan, G., and Zalutsky, M. R. (1994) Cytotoxicity of alpha-particle-emitting m-[^{211}At]astatobenzylguanidine on human neuroblastoma cells. *Cancer Res.* 54, 5414–5419.
- Vaidyanathan, G., and Zalutsky, M. R. (1992) 1-(m-[^{211}At]-Astatobenzyl)guanidine: Synthesis via Astatine Demetalation and Preliminary in Vitro and in Vivo Evaluation. *Bioconjugate Chem.* 3, 499–503.
- Vaidyanathan, G., and Zalutsky, M. R. (1993) No-carrier-added synthesis of meta-[^{131}I]iodobenzylguanidine. *Appl. Radiat. Isot.* 44, 621–628.
- Vaidyanathan, G., Affleck, D. J., and Zalutsky, M. R. (1994a) (4-[^{18}F]Fluoro-3-iodobenzyl)guanidine, a Potential MIBG Analogue for Positron Emission Tomography. *J. Med. Chem.* 37, 3655–3662.
- Vaidyanathan, G., Affleck, D. J., Slade, S. A., Welsh, P., and Zalutsky, M. R. (1994b) No-carrier-added 4-fluoro-3-iodobenzylguanidine labeled with [^{131}I]FIBG and [^{18}F]FIBG: Potential MIBG analogs for PET imaging and therapy of neuroendocrine tumors. *XVIth International Cancer Congress*, October 30–November 5, New Delhi, India.
- Vaidyanathan, G., Affleck, D. J., and Zalutsky, M. R. (1995) Validation of 4-[^{18}F]fluoro-3-iodobenzylguanidine as a positron-emitting analog of MIBG. *J. Nucl. Med.* 36, 644–650.
- Valette, H., Loc'h, C., Mardon, K., Bendriem, B., Merlet, P., Fuseau, C., Sabry, S., Raffel, D., Maziere, B., and Syrota, A. (1993) Bromine-76-metabromobenzylguanidine: A PET radiotracer for mapping sympathetic nerves of the heart. *J. Nucl. Med.* 34, 1739–1744.
- Wieland, D. M. (1986) Radiopharmaceutical Design: The Adrenal Medulla and Its Diseases. In *Radiopharmaceuticals: Progress and Clinical Perspectives* (A. R. Fritzberg, Ed.) Vol. 1, pp 117–153, CRC Press, Inc., Boca Raton, FL.
- Zalutsky, M. R., Garg, P. K., Friedman, H. S., and Bigner, D. D. (1989) Labeling monoclonal antibodies and F(ab')₂ fragments with the alpha particle emitting nuclide astatine-211: preservation of immunoreactivity and in vivo localizing capacity. *Proc. Natl. Acad. Sci. U.S.A.* 86, 7149–7153.

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Probing Nucleic Acid Geometries: Oligonucleotides Containing 2'-O-Phenethyladenosine at Specific Sites

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The synthesis of 2'-O-phenethyladenosine and its incorporation into an oligodeoxyribonucleotide and a chimeric oligodeoxy/oligoribonucleotide are described. The study was designed to determine the consequences of site-specific induction of such a small, flexible hydrophobic ligand into a nucleic acid. Through the use of optical (T_m , circular dichroism) spectroscopy and nuclear magnetic resonance, it was discovered that this substitution destabilized the duplex and that the benzene ring of the phenethyl group lies in the major groove of both the DNA analog and the DNA–RNA duplex structure. The plane of the benzene ring lies perpendicular to the stacked bases of the oligonucleotide which, in other respects, exhibits relatively normal B-type geometry for the oligodeoxynucleotide and a modified A-type geometry in the DNA–RNA duplex. This finding has implications for the synthesis of oligodeoxynucleotides containing major groove binders as ligands.

INTRODUCTION

The specificity inherent in Watson–Crick and Hoogsteen hydrogen bonding among the purines and pyrimidines which comprise interacting single-stranded nucleic acids has stimulated great interest in both academic and industrial laboratories. A major focus of this interest has been the design and synthesis of antisense oligodeoxynucleotides and their utilization in the inhibition of gene expression by duplex formation with complementary single-stranded regions of messenger RNA (1–7). Additionally, substantial work has been carried out using Hoogsteen hydrogen bonding in the design and synthesis of triplex-forming oligonucleotides (1, 8–11).

A number of investigators have recognized that attachment of intercalating (12, 13), alkylating (14), cleaving (15–17), or photo cross-linking (18, 19) functional groups to the 3'- or 5'-end of antisense oligonucleotides should afford enhanced affinity and/or cleavage specificity for the oligonucleotide-directed interruption of gene expression. In very few cases, however, has any exploration of the fate of ligands attached to the 2'-hydroxyl of a ribonucleotide incorporated into an oligonucleotide been undertaken.

While a substantial number of studies have been published on 2'-O-alkyl oligos (20–22), the primary motivation for these efforts was enhanced stability toward ribonuclease hydrolysis of synthetic RNA molecules. It has been reported that anthraquinone moieties may be linked to the 2'-hydroxyl of uridine via a methylene linker (23) or to U, C, and A via a more complex and considerably longer linker (24).

In both cases, the anthraquinone was said to intercalate on the basis of observed duplex stabilization. A similar observation was made for substitution of an anthraquinone moiety at the 1'-position of 2'-deoxyuridine on a 10-atom tether (25). On the other hand, attachment of the larger pyrene group to the 2'-OH of uridine led to destabilization of the duplex when the modified nucleoside was placed at an internal position (26). Recently, we have synthesized 2'-O-(anthraquinone-2-ylmethyl)adenosine, incorporated it into oligodeoxynucleotides, and demonstrated unequivocally using NMR

and other techniques that intercalation does occur, even with only a one-carbon tether (27).

However, no study has been reported that describes the consequences of site-specific introduction of a small, flexible hydrophobic ligand, such as phenethyl, at the 2'-position of a ribonucleotide incorporated into a nucleic acid. In principle, the benzene ring in such a construct could assume one of four orientations: (1) it could intercalate between the base of the nucleoside to which it is attached and its 3'-neighboring base, (2) it could lie along the minor groove of the duplex, (3) it could reside in the major groove of the duplex, or (4) it could extend into the aqueous solution.

In the present paper, we will demonstrate a result that was to us counterintuitive. That is, in both DNA duplexes and DNA–RNA hybrids, the benzene ring resides in the major groove of the duplex in an orientation with the plane of the phenyl ring essentially perpendicular to that of the planes of the stacked bases.

EXPERIMENTAL PROCEDURES

General Methods. ^1H -NMR spectra were recorded on either an IBM AF 200 MHz FT-NMR spectrometer or a Varian Unity 500 MHz spectrometer. NMR samples of nucleosides were prepared using $(\text{CD}_3)_2\text{SO}$ or CDCl_3 , with tetramethylsilane (TMS) or solvent peak as internal standard. Electrospray mass spectra were recorded on a Vestec 201 ionization instrument with a quadrupole mass analyzer. Thin-layer chromatography (TLC) was performed on Kieselgel 60 F₂₅₄ aluminum-backed silica gel sheets. CD spectra were recorded on a Jasco J-720 spectropolarimeter. Elemental analyses were performed by M-H-W Laboratories in Phoenix, AZ. Mass spectra were recorded on either a Finnegan MAT 95 or a VG 7050E mass spectrometer. The oligonucleotides were synthesized on an Applied Biosystems 380B instrument.

Materials. All chemicals were purchased from Aldrich Chemical Co. unless otherwise specified. The enzymes, snake venom phosphodiesterase (SVP) and bacterial alkaline phosphatase (BAP), were purchased from Sigma.

Preparation of Oligonucleotides. Four oligonucleotides were prepared using standard machine chemistry and commercially available reagents except for the 2'-O-phenethyladenosine derivative described below. For initial studies, including T_m measurements, syntheses

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Table 1. Sequences of Various Oligonucleotides and Their T_m s ($^{\circ}\text{C}$)

	sequence	name	T_m
5	5'd(CGC ACA TGT GCG)3'	standard 12-mer	41.6
6	5'd(CGC A*CA TGT GCG)3' A* = 2'-O-phenethyladenosine	phenethyl 12-mer	29.4
7	5'd(CGC ACA) r(UGU GCG)3'	standard hybrid	36
8	5'd(CGC A*CA) r(UGU GCG)3' A* = 2'-O-phenethyladenosine	phenethyl hybrid	31

were carried out on a 1 μmol scale. Subsequently, 10 μmol syntheses were performed in order to obtain sufficient material for NMR studies. As expected, yields at steps corresponding to ribonucleotide introduction were somewhat lower than those for standard DNA oligonucleotide synthesis. The four self-complementary oligonucleotides used to study the role of 2'-O-phenethyl substitution are given in Table 1, along with their T_m values.

T_m Determination. All T_m values were determined on a Hewlett-Packard diode array spectrophotometer equipped with an electronic temperature-controlling device. Absorbance at 260 nm was measured at 1 $^{\circ}\text{C}$ intervals, and the cuvette was equilibrated at each temperature for 2 min. For duplex formation, the solution was heated to $\sim 70^{\circ}\text{C}$ and then cooled slowly. Duplexes were kept at 5 $^{\circ}\text{C}$ overnight before T_m measurements were carried out. Duplexes were formed at 1.58×10^{-4} M concentration (stock solution) in 100 mM NaCl containing 20 mM phosphate buffer (pH 7). The stock solution was diluted 50-fold at 4 $^{\circ}\text{C}$ with the same buffer for T_m measurement.

High-Performance Liquid Chromatography (HPLC) Purification and Analysis of Oligomers. All HPLC purifications and analyses were performed after detritylation on a Hitachi D-6200 HPLC system equipped with an L-3000 diode array spectrophotometer. Oligonucleotides synthesized on a 1 μmol scale were purified on a preparative Whatman Partisil ODS reverse-phase column. Oligomers prepared on a 10 μmol scale were purified on a Sephadex G25 column (100 $\times$ 2.5 cm). Elution was performed with distilled water at a flow rate of 20 mL/h. Purified oligomers were analyzed on a Rainin Microsorb C₁₈ analytical column (25 cm $\times$ 4.4 mm). Solvents used for elution were as follows: solvent A, 95% 50 mM ammonium bicarbonate buffer (pH 7)/5% acetonitrile; and solvent B: 80% 50 mM ammonium bicarbonate buffer (pH 7)/20% acetonitrile.

The following gradient was used for purification of oligomers: 100% A (0–4 min, linear 100% A to 100% B (4–15 min), 100% B (15–25 min), linear 100% B to 100% A (25–32 min), re-equilibration with 100% A (32–40 min); flow rate, 2 mL/min.

The following gradient was used for analysis of oligomers: 100% A (0–4 min), linear 100% A to 100% B (4–10 min, 100% B (10–15 min), linear 100% B to 100% A (15–20 min), re-equilibration with 100% A (20–30 min); flow rate, 1 mL/min.

Nuclease Digestion and Analysis of Modified and Unmodified Oligomers. A mixture of oligonucleotide (0.5 OD units) with 50 mM Tris-HCl (pH 8), 10 mM MgCl₂, SVP (3 units), and BAP (3 units in 100 μL of distilled water) was incubated at 37 $^{\circ}\text{C}$ for 5 h; 15 μL of 5 M sodium acetate was added to the hydrolysis mixture, followed by the addition of 230 μL of absolute ethanol. This mixture was chilled at -70°C for 30 min and centrifuged at 30000g for 5 min. The supernatant was diluted with 1 mL of 95% ethanol, chilled, centrifuged, decanted, and evaporated to dryness. The residue was redissolved in distilled water for HPLC analysis.

HPLC analysis for unmodified oligonucleotide hydrolysates was performed on a C₁₈ column; a C₈ column was used for the analysis of modified oligonucleotide hydrolysates. Elution was performed isocratically with a 50 mM phosphate buffer (pH 4)/methanol linear gradient.

NMR Experiments on Oligonucleotides. One- and two-dimensional (1D and 2D) proton data sets were collected on a Varian Unity 500 MHz spectrometer. NMR samples were prepared to a concentration of oligonucleotide of ~ 1.6 mM. One-dimensional temperature-dependent 500 MHz ^1H data sets were collected in 90% H₂O buffer/10% D₂O. One-dimensional proton-decoupled 500 MHz ^{31}P data sets were collected in 100% buffered D₂O solution. The oligomers were lyophilized twice from 99.996% D₂O and then dissolved in 99.996% D₂O containing 100 mM sodium chloride and 20 mM phosphate buffer (pH 7).

Two-dimensional phase-sensitive NOESY spectra of all the oligomers were collected using a 250 or 400 ms mixing time. All data sets were acquired in the hypercomplex mode, with 256 increments in the t_1 dimension, 32 or 64 scans per free induction decay (fid), and 2048 complex points in t_2 . The t_2 increments were zero-filled to 2048 points and transformed with a Gaussian apodization function.

Two-dimensional proton phase-sensitive double-quantum filtered COSY (DQF-COSY) experiments were collected using the standard pulse sequence with a 2 μs pulse repetition time and homospoil of 1 ms. Data sets were collected in hypercomplex mode with 256 t_1 increments, 32 or 64 scans per fid, and 2048 complex points in T_2 . The t_2 dimension was processed with a Gaussian filter; the t_1 increments were zero filled to 2048 points and transformed with a Gaussian apodization function.

Molecular Modeling. Molecular modeling studies were performed using Silicon Graphics IRIS or Indigo workstations. Molecules were visualized using BIOSYM software; minimizations and dynamics were performed using the DISCOVER program. For simulation of nucleosides, cvff potentials were used. All nucleosides were first minimized using the steepest gradient for 500 iterations and then a biconjugate gradient for 2000 iterations. To obtain a global minimum, the molecular dynamic simulations were used. All dynamics were performed at 300 K for 50 ps, including a 20 ps equilibration period. The low-energy conformers obtained in the molecular dynamics simulation were further minimized to obtain a family of low-energy conformers. The protocol used for initial minimization was used for these minimizations also.

Oligonucleotides were visualized using the bipolymer module of the InsightII software. AMBER potentials were used during minimization of oligonucleotide conformations. When the phenethyl groups were attached to the 2'-OH, the software was unable to assign a potential for C5 of adenosine. Potential "CB" was assigned to this carbon manually. Only minimizations were performed on the oligonucleotides. The phenethyl group was placed in the major groove by torsioning the bonds between the benzene ring and the 2'-hydroxyl. The torsions were removed, and the oligonucleotides were minimized first by 500 iterations of the steepest gradient minimizer and then by 2000 iterations of the conjugate gradient minimizer. Partial charges on the backbone phosphorus nuclei were reduced to 0.8, while those on oxygen were reduced to -0.5 . In some instances, all the hydrogen-bonded atoms of the first three base pairs were fixed in space before minimization.

2'-O-Phenethyladenosine (1). Adenosine (10 g, 37.4 mmol) was dissolved in anhydrous DMF (250 mL), and

the solution was cooled to 0 °C. Sodium hydride (1.1 g, 45 mmol, prewashed with hexanes) was added to the adenosine solution. The reaction mixture was vigorously stirred for 90 min. Phenethyl tosylate (10 g, 36 mmol) in anhydrous DMF (100 mL) was added in portions over a period of 2 h, during which time the reaction mixture was allowed to warm to room temperature. After 2 h at room temperature, the mixture was heated to 60 °C and another 8.0 g of phenethyl tosylate was added in portions over a 2 h period. The reaction mixture was then allowed to cool to room temperature, and 1.0 g of NaH (60% dispersion in mineral oil) was added. The reaction mixture was again heated to 60 °C, a final aliquot of phenethyl tosylate (8 g) was added, and the mixture was stirred overnight at 60 °C. Solvent was evaporated *in vacuo* to yield a residue to which water (75 mL) was added. The aqueous layer was washed with hexanes (3 × 50 mL) to remove styrene produced from phenethyl tosylate and then with ethyl acetate. The ethyl acetate was dried over sodium sulfate and evaporated *in vacuo* to afford an oil. The product was crystallized from ethanol to provide 2.15 g (15.2%): UV (H₂O) λ_{max} 254 nm (ϵ = 21 880); ¹H-NMR [(CD₃)₂SO] δ 8.37 (s, 1H, H8), 8.14 (s, 1H, H2), 7.39 (s, 2H, NH₂), 7.08 (m, 5H, Ar), 6.00 (d, 1H, 1'-H, $J_{1',2'} = 6.2$ Hz), 5.45 (m, 1H, 5'-OH), 5.22 (d, 1H, 3'-OH), 4.52 (m, 1H, 2'-H), 4.33 (m, 1H, 3'-H), 4.00 (m, 1H, 4'-H), 3.85–3.38 (complex m, 4H, 5'-H, 5''-H, OCH₂), 2.72 (t, 2H, CH₂Ph); MS m/z 372 (M + 1)⁺. Anal. Calcd for C₁₈H₂₁N₁₅O₄: C, 58.20; H, 5.70; N, 18.86. Found: C, 58.06; H, 5.74; N, 18.79.

N⁶-Benzoyl-2'-O-Phenethyladenosine (2). The title compound was synthesized using the transient protection procedure of Jones (28). 2'-O-Phenethyladenosine (2.70 g, 5.6 mmol) was dissolved in pyridine (50 mL). Trimethylsilyl chloride (5.05 mL, 39.6 mmol) was added, and the solution was stirred for 45 min. Benzoyl chloride (3.24 mL, 38 mmol) was added, and the reaction mixture was allowed to stir for 3.5 h. The mixture was cooled in an ice bath, and 10 mL of cold water was added. After 5 min, 20 mL of 30% aqueous ammonia was added, and the mixture was stirred at room temperature for 30 min.

The solvent was removed under vacuum, and the residue was suspended in water and extracted with ethyl acetate (3 × 50 mL). The combined organic layers were washed with water (2 × 50 mL), followed by brine, dried over sodium sulfate, and evaporated to dryness. The compound was not isolated but used directly in the next reaction. Judging from the NMR spectrum, the yield of the reaction was about 75%: ¹H-NMR [(CD₃)₂SO] δ 11.22 (s, 1H, NH), 8.74 and 8.70 (2s, 2H, H8, H2), 6.12 (d, 1H, 1'-H, $J_{1',2'} = 6.0$ Hz); MS m/z 476 (M + 1)⁺.

N⁶-Benzoyl-5'-(dimethoxytrityl)-2'-O-phenethyladenosine (3). Dimethoxytritylation of **2** was carried out according to the procedure of Wu et al. (29). Compound **2** (1.95 g, 5.4 mmol) was dried by coevaporation with pyridine and dissolved in pyridine (20 mL). Dimethoxytrityl chloride (2.92 g, 8.6 mmol) was added to the solution, and the reaction mixture was allowed to stir for 12 h at 4 °C. Thin-layer chromatography indicated the presence of starting material; therefore, an additional 500 mg of dimethoxytrityl chloride was added, and the reaction continued for an additional 12 h. Methanol (5 mL) was added to quench the reaction; the solvent was evaporated *in vacuo*, and the residue was dissolved in ethyl acetate (50 mL). The ethyl acetate layer was washed with 5% sodium bicarbonate (3 × 75 mL), water (75 mL), and brine and dried over sodium sulfate. The product was purified by silica gel chromatography with a gradient of hexane in chloroform (20 to 0%). Triethylamine (0.1%) was added to the eluting solvent to prevent

degradation of the tritylated product: yield 2.4 g (75%); UV (H₂O) λ_{max} 234 nm (ϵ 27 175), 282 (ϵ 16 736); ¹H-NMR [(CD₃)₂SO] δ 11.30 (s, 1H, NH), 8.62 (s, 1H, H8), 8.55 (s, 1H, H2), 6.20 (d, 1H, 1'-H), 5.34 (d, 1H, 3'-OH), 4.74 (t, 1H, 2'-H), 4.48 (m, 1H, 3'-H), 4.18 (m, 1H, 4'-H), 3.95–3.57 (2m, 2H, 5',5''-H), 3.70 (s, 6H, OCH₃); MS m/z 777.3141 (M + 1)⁺, calcd 777.3162 (difference = 2.1 mmu). The purity of the compound was confirmed by normal-phase HPLC.

N⁶-Benzoyl-5'-(dimethoxytrityl)-2'-O-phenethyladenosine 3'-O-(Cyanoethyl)-N,N-diisopropylphosphoramidite (4). The title compound was synthesized according to the general procedure of Kierzek et al. (30). Acetonitrile (15 mL) and β -(cyanoethyl)-N,N,N',N'-tetraisopropyl phosphordiamidite (0.47 mL, 1.4 mmol) were added to **2** (1 g, 1.28 mmol) and diisopropylammonium tetrazolide (109 mg, 0.64 mmol, dried *in vacuo* for 3 h). After 12 h, thin-layer chromatography indicated the presence of some starting material; hence, an additional 0.1 mL of phosphordiamidite was added, and the reaction was continued for an additional 12 h. The reaction mixture was diluted with saturated sodium bicarbonate (50 mL) and extracted with dichloromethane (2 × 50 mL). Combined organic extracts were washed three times with brine, dried over sodium sulfate, and concentrated *in vacuo* to a dry foam. The foam was purified by silica gel chromatography using 20% hexane in chloroform and 1% triethylamine: yield 700 mg (56%); ³¹P-NMR δ 150.86, 150.22.

RESULTS

Chemistry. Synthesis of the nucleoside required for this study, 2'-O-phenethyladenosine, was rather difficult. The 3',5'-O-tetraisopropylidisiloxane protection/strong organic base-mediated alkylation methodology of Sproat (31) was unsuccessful in this case, as was the 2',3'-O-stannylene chemistry devised by Moffatt (32) and successfully applied to the synthesis of 2'-O-(anthraquinon-2-yl methyl)adenosine by Yamana et al. (23). The simple approach of Ts'o (33) succeeded, albeit giving rise to 2'-O-phenethyladenosine in a modest 15% yield.

The obvious difficulty leading to poor yields in this reaction is the favored elimination giving rise to styrene under the strongly basic (sodium hydride) conditions of this reaction. A number of leaving groups were examined, most of which were either too unreactive (Br) or eliminated too readily (OMs, I). The tosyloxy leaving group was found to be the best compromise and permitted us to obtain adequate amounts of 2'-O-phenethyladenosine.

Since the 3'-O-phenethyladenosine is also a product of the reaction, it was necessary to establish unequivocally the site of alkylation. This was accomplished by means of 2D COSY and NOESY experiments. Briefly, both NOESY and COSY spectra revealed that the signal at 6.00 δ corresponding to H1' was coupled to H2' at 4.52 which, in turn, was coupled to H3' at 4.33. H3' showed strong NOESY and COSY cross-peaks to the OH signal at 5.22 δ , whereas the H2' signal showed no connectivity to exchangeable OH protons. This unequivocally established the position of substitution of the phenethyl group at 2'.

Protection of 2'-O-phenethyladenosine using standard chemistry provided N⁶-benzoyl-5'-O-(dimethoxytrityl)-2'-O-phenethyladenosine (cyanoethyl)-N,N-diisopropylphosphoramidite. This synthon was used in a standard machine synthesis to give the DNA 12-mer **6** and the DNA-RNA hybrid **8**. Predictably, yields at the step incorporating 2'-O-phenethyladenosine and yields of the RNA components of the hybrids were modest, but reac-

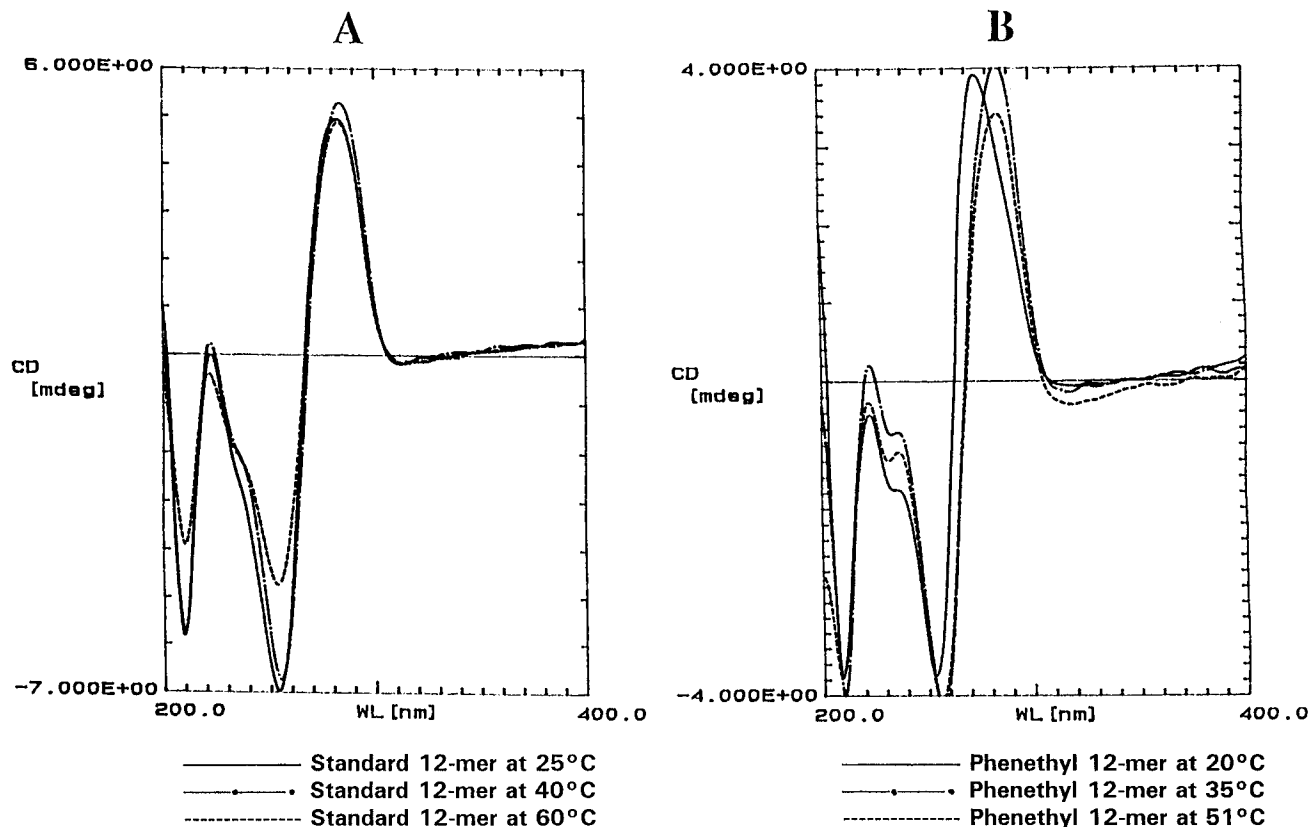


Figure 1. Circular dichroism spectra for the standard 12-mer (A) and the phenethyl 12-mer (B) at various temperatures.

tions carried out on a 10 μ M scale afforded sufficient material for all the physicochemical measurements undertaken.

Melting temperatures were obtained for both standard oligomers **5** and **7** and the modified oligomers **6** and **8**, and the data are presented in Table 1. Substitution of phenethyloxy for proton (**6** vs **5**) led to a significant destabilization of the duplex. In order to determine whether this was a result arising largely from inclusion of a ribonucleoside unit as opposed to a deoxyribonucleoside unit, melting curves were also obtained on oligomers in which the modified nucleoside was adenosine ($T_m = 37.4$ °C), 2'-O-methyladenosine ($T_m = 34.6$ °C), and 2'-O-benzyladenosine ($T_m = 29.0$ °C). Clearly, the data reveal that the observed destabilization seems to result largely from the steric bulk of the substituent rather than from conformational alterations imposed by the 2'-OH. It is worth noting in this regard that the anthraquinon-2-ylmethyl substitution previously reported (23, 27) led to significant stabilization of the duplex resulting from intercalation of the anthraquinone moiety on the 3'-side of the adenosine residue.

Circular Dichroism Spectropolarimetry. The CD spectra for the standard 12-mer and the phenethyl 12-mer at various temperatures are shown in Figure 1. It is readily apparent that, although subtle differences exist in the spectra of compounds **5** and **6**, both spectra at all temperatures are highly characteristic of B-DNA and show little evidence of significant deviations from normal DNA duplex formation (34).

NMR Spectroscopy of Oligonucleotides. Solutions for 1D NMR in which imino proton signals were to be observed were prepared in H₂O solutions containing 10% D₂O. The water peak was suppressed using a water suppression pulse sequence. The imino proton spectra for the standard 12-mer at various temperatures are shown in Figure 2. The self-complementary structure of the

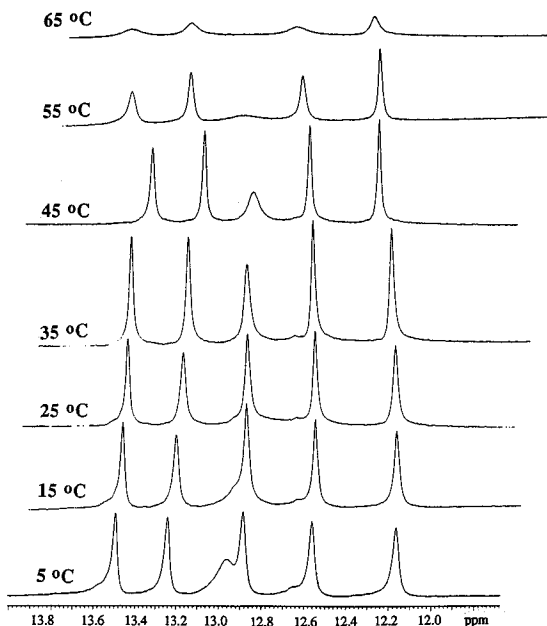


Figure 2. Imino proton spectra for the standard 12-mer at various temperatures.

oligonucleotide divides the duplex into identical halves; consequently, only six imino proton resonances should be observed when all bases of the duplex are paired. At 5 °C, all six imino protons were observed. The two downfield resonances correspond to AT base pairs, and the four upfield resonances correspond to GC base pairs (35).

The downfield signal of the central pair of resonances is the first to melt out between 5 and 15 °C, indicative of fraying of the terminal GC base pair. Peaks sharpen with increasing temperature and the consequent increased tumbling motion of the molecules to about 45 °C, at which time melting begins and peaks again broaden

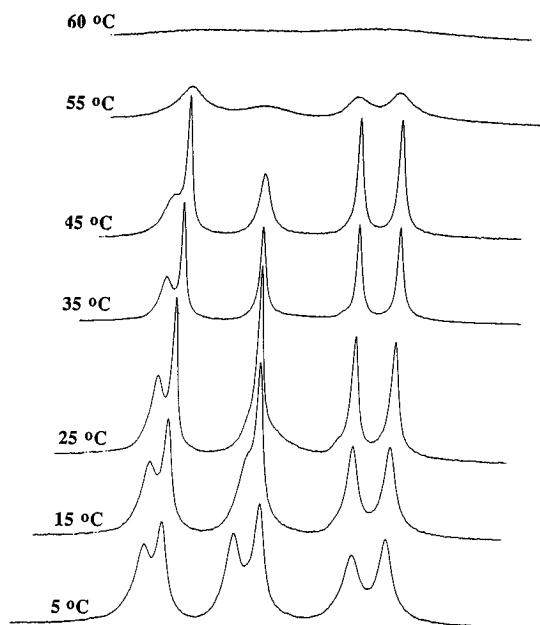


Figure 3. Imino proton spectra for the phenethyl 12-mer at various temperatures.

and disappear. The remaining central resonance is the next to disappear as predicted for the penultimate GC base pair.

The T_m plot derived from this information reveals that the melting temperature is between 50 and 55 °C. This value is somewhat higher than that from the UV melting experiments, a typical result considering the much higher concentration of oligomer driving the equilibrium toward the duplex form (27).

The imino proton spectra for the phenethyl 12-mer at various temperatures are shown in Figure 3. Although the spectra are generally similar in appearance to those obtained for oligo 5, the differences are significant. At 5 °C, three pairs of broad signals are observed. Again, the most downfield signals correspond to AT base pairs. One-

dimensional NOE difference spectroscopy was used to assign these two resonances (data not shown). The hydrogen-bonded N_3H of T is close to the adenine H2 proton. Consequently, irradiation of N_3H leads to a change of intensity in the H2 proton signal. Irradiation of the upfield resonance of the pair gave rise to change in the intensity of a peak at 7.26 δ . This peak was identified (*vide infra*) by two-dimensional NMR spectroscopy as H2 of A6, identifying the upfield resonance as T7- N_3H . Selective irradiation of the more downfield imino resonance could not be accomplished because of peak overlap. Irradiation at 13.4 δ led to a change in the intensities of two peaks, one of which was H2 of A6. The second peak was later identified as H2 of A4 by 2D NOESY spectroscopy. Thus, the most downfield proton corresponds to T9- N_3H which hydrogen bonds to A4.

As the temperature is increased, the resonances become sharper until 25 °C, at which time peak broadening indicates the onset of melting. Careful inspection of the spectra reveals that the peaks corresponding to the G2-C11 imino proton and the T9-A4 imino proton are broadening simultaneously. The former was anticipated since it forms the penultimate base pair. Broadening of the A4-T9 imino proton signal indicates local denaturation of the helix, which must be resulting from the influence of the phenethyl group. The NMR-derived melting temperature is between 35 and 40 °C, also higher than that observed by UV spectroscopy.

For two-dimensional spectra, oligomers 5 and 6 were dissolved in D_2O containing 0.1 M NaCl and 20 mM phosphate buffer (pH 7) and lyophilized several times from 99.996% D_2O . The oligomers were annealed by heating each sample to 65 °C and slowly cooling to room temperature. Spectra for oligomer 5 were collected at 5 °C, and because of significant overlap at lower temperatures, spectra for oligomer 6 were collected at 15 °C. A mixing time of 250 ms was used in DNA oligomer NOESY experiments.

Figure 4 shows the expanded contour plot of the NOESY spectrum for the standard oligo 5, showing

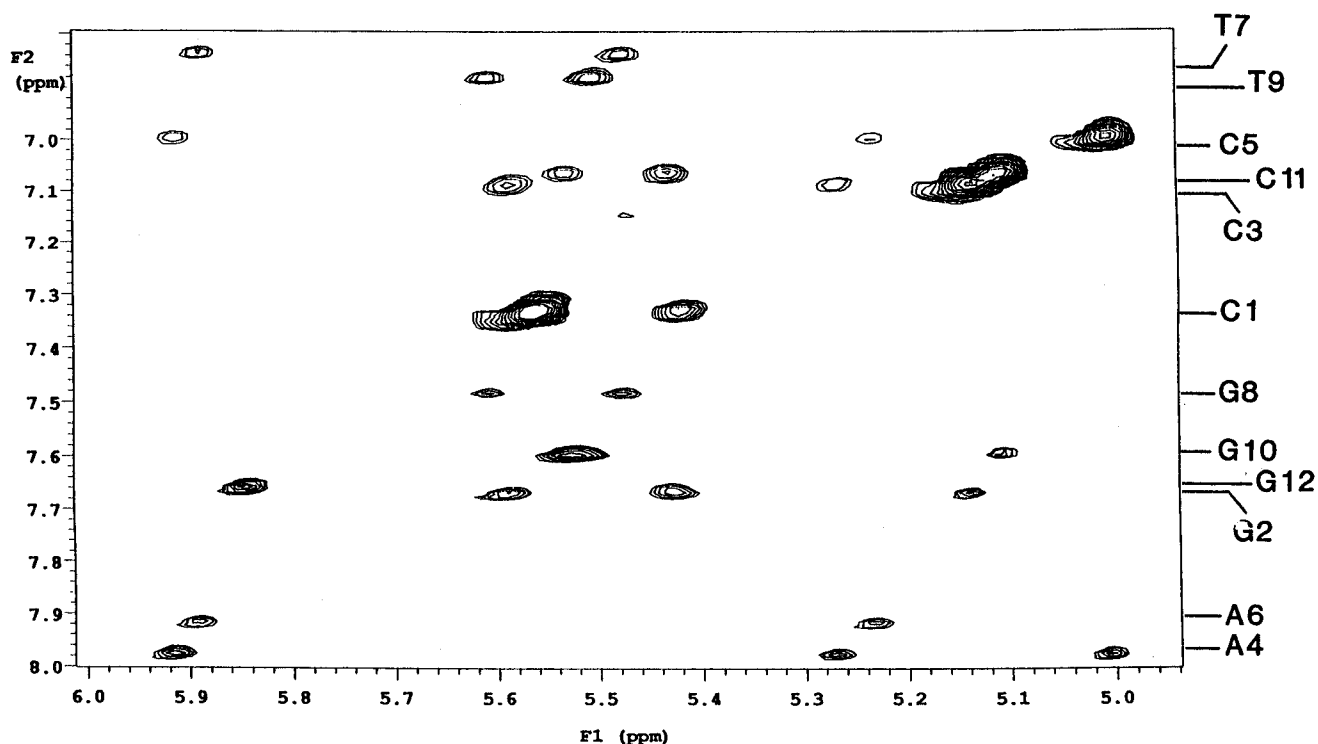


Figure 4. Expanded contour plot (aromatic H1' region) of the NOESY spectrum for the standard 12-mer.

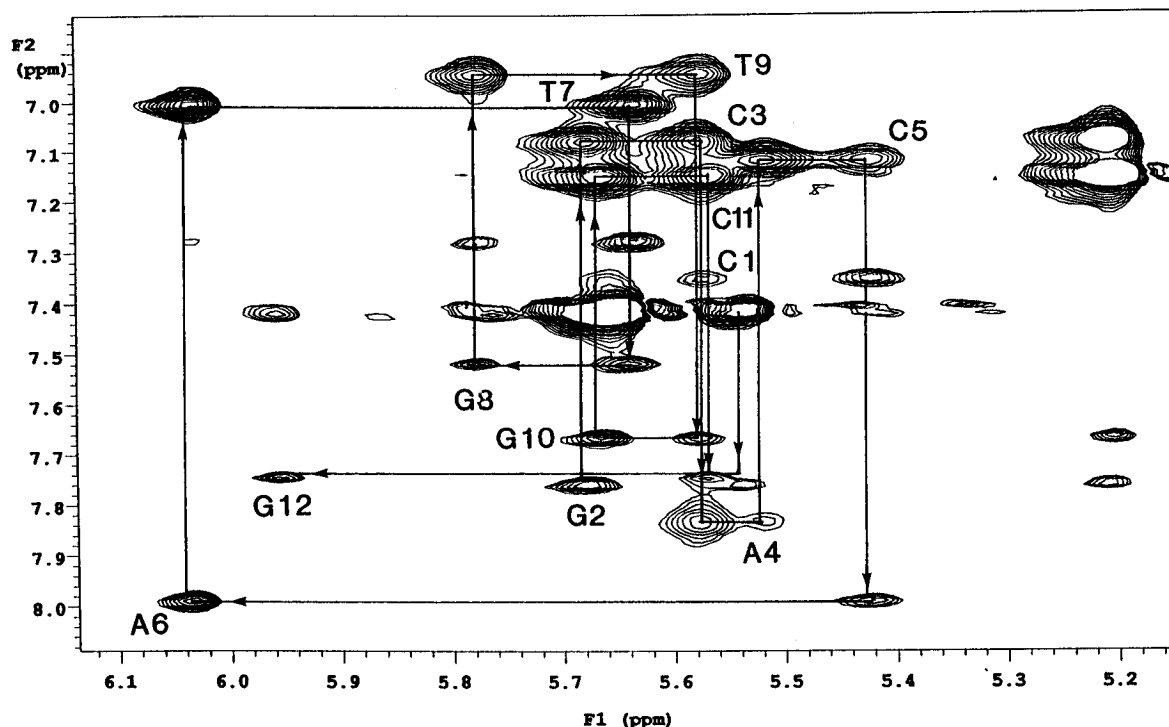


Figure 5. Expanded contour plot (aromatic H1' region) of the NOESY spectrum for the phenethyl 12-mer.

connectivities between aromatic protons and H1' protons. One may easily observe that the peaks are of rather uniform intensity, characteristic of normal B-DNA geometry with each aromatic (H6/H8) proton almost equidistant from its own H1' and the previous H1' (36). This region of the spectrum was used to assign all the base and H1' protons. The aromatic methyls of T7 and T9 provide convenient starting points for the assignments. Each methyl shows cross-peaks with the H6 proton of its own base and the aromatic proton of the preceding base. This resulted in quick identification of A6, T7, G8, and T9. The rest of the nucleosides were identified by performing a "NOESY-walk" in this region, as previously described (36), giving rise to the assignments illustrated in Figure 4. The assignments were confirmed using the aromatic H2' region of the NOESY spectrum and by using DQF-COSY spectroscopy (data not shown).

In Figure 5, similar data for the aromatic H1' region of the NOESY spectrum of oligo **6**, the phenethyl 12-mer, are presented. Assignments were made in the same way as for oligo **5** and, in general, are consistent with overall B-DNA geometry. Examination of the NOESY data reveals the cross-peak between H8 of A4 and its own H1' is considerably weaker than the rest of the peaks. Since the intensity of the cross-peak depends upon the distance between the protons, the distance between H8 and H1' of A4 must be longer than normal. This could result from a change in sugar pucker or from a change in the glycosyl bond angle. The second factor is regarded as less likely since a change in bond angle would alter the distance between A4-H8 and C3-H1'. No such change was observed.

The sugar pucker of A4 was qualitatively analyzed in a DQF-COSY experiment. The intensity of a cross-peak between two protons in this experiment depends on the scalar coupling constant between them which, in turn, depends upon the dihedral angle.

Figure 6 shows the expanded H1'-H2' region of the DQF-COSY spectrum for the phenethyl 12-mer. The arrow in the figure points at the cross-peak for H1'-H2' of A4, whereas the other peaks correspond to the remain-

ing H1'-H2' cross-peaks. Most studies involving DNA-RNA hybrids have found the ribo sugar pucker to be quite typical of A-type or 3'-endo conformation (37-39) (*vide infra*).

If the sugar pucker for A4 were close to C3'-endo, the signal corresponding to the H1'-H2' cross-peak (Figure 6) would have been absent. The appearance of this peak, albeit weaker than the others, suggests that A4 has a conformation significantly different from C3'-endo. Molecular modeling (*vide infra*) is in accord with this conclusion, showing an envelope conformation which has more C2'-endo than C3'-endo character.

The information available from the NOESY spectra also addresses the issue of possible intercalation of the benzene ring. If intercalation were to occur, as was recently shown to be the case for the anthraquinon-2-ylmethyl derivative (27), cross-peaks would be observed between the benzene aromatic protons and the H1' protons of A4 and C5. Furthermore, cross-peaks should appear between the benzene aromatic protons and the aromatic protons of A4 and C5. Neither was observed in the NOESY spectrum. Also, because intercalation would increase the distance between A4 and C5, the loss of cross-peaks between H1' of A4 and H6 of C5 would be observed. However, that cross-peak was clearly visible in the aromatic H1' region of the NOESY spectrum (Figure 5).

Further evidence regarding the conformational preference of the phenethyl group may be derived from the aromatic H2' region of the NOESY spectrum (Figure 7). The arrow in Figure 7 points toward two peaks. The upper cross-peak shows the through-space connectivity between benzylic protons of the phenethyl group and H6 of C5. The lower peak reveals connectivity between benzylic protons and the aromatic protons of the benzene ring. Molecular modeling suggests these NOE conditions can be satisfied only when the benzene ring lies outside the helix. The DQF-COSY spectrum confirms the location of the benzene ring as being inside the major groove.

Figure 8A shows the cross-peaks between the H5 and H6 protons of the cytidines of the standard 12-mer. The

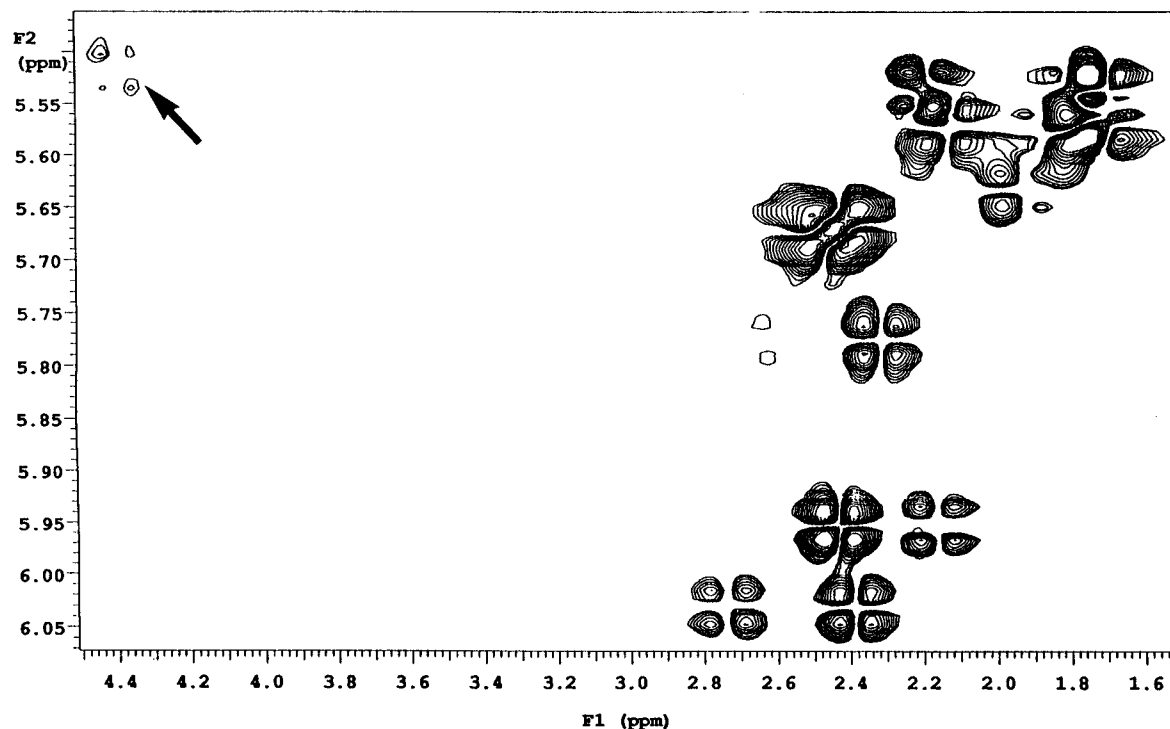


Figure 6. Expanded contour plot ($H1'-H2'$ region) of the DQF-COSY spectrum for the phenethyl 12-mer. The arrow indicates the $H1'-H2'$ cross-peak for A4.

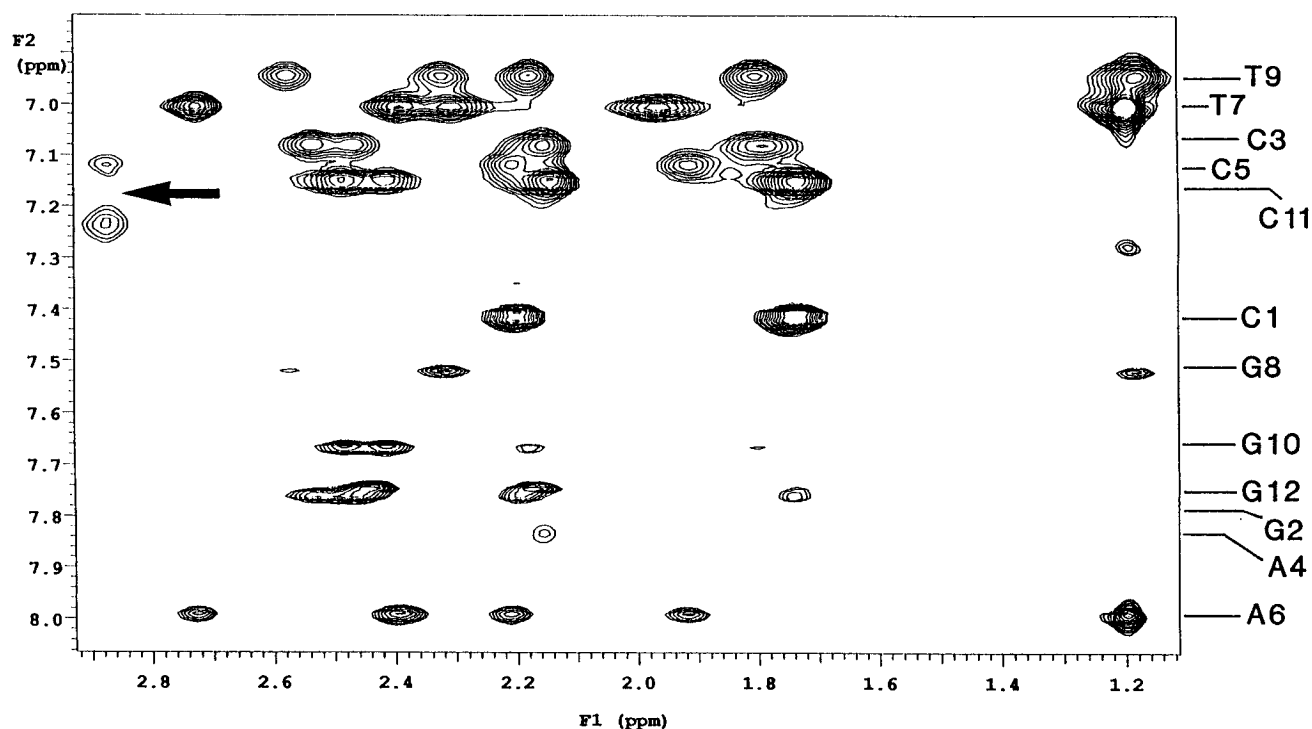


Figure 7. Expanded contour plot (aromatic $H2'$ region) of the NOESY spectrum for the phenethyl 12-mer. The arrow indicates connectivities of the benzylic proton.

cross-peaks are uniform in intensity, which is expected since the distance between H5 and H6 is invariant. Differences in the chemical shifts arise from differential shielding and can be predicted from the location of cytidines within the duplex.

Figure 8B reveals that the signal for C5 is substantially shielded relative to that in the standard oligomer and, most importantly, is of greatly reduced intensity. This may be accounted for by locating the benzene ring in the

major groove in such a way that it is perpendicular to the planes of the stacked bases and places H5 of C5 within its shielding cone. This results in an upfield chemical shift, and the motion of the benzene ring causes C5 to experience a variable magnetic field, such that the H5 resonance is considerably broadened. This broadening results in the apparent weak intensity of the H5-H6 cross-peak. The stacked plot clearly reveals this broadening relative to the other cytidine peaks in the set

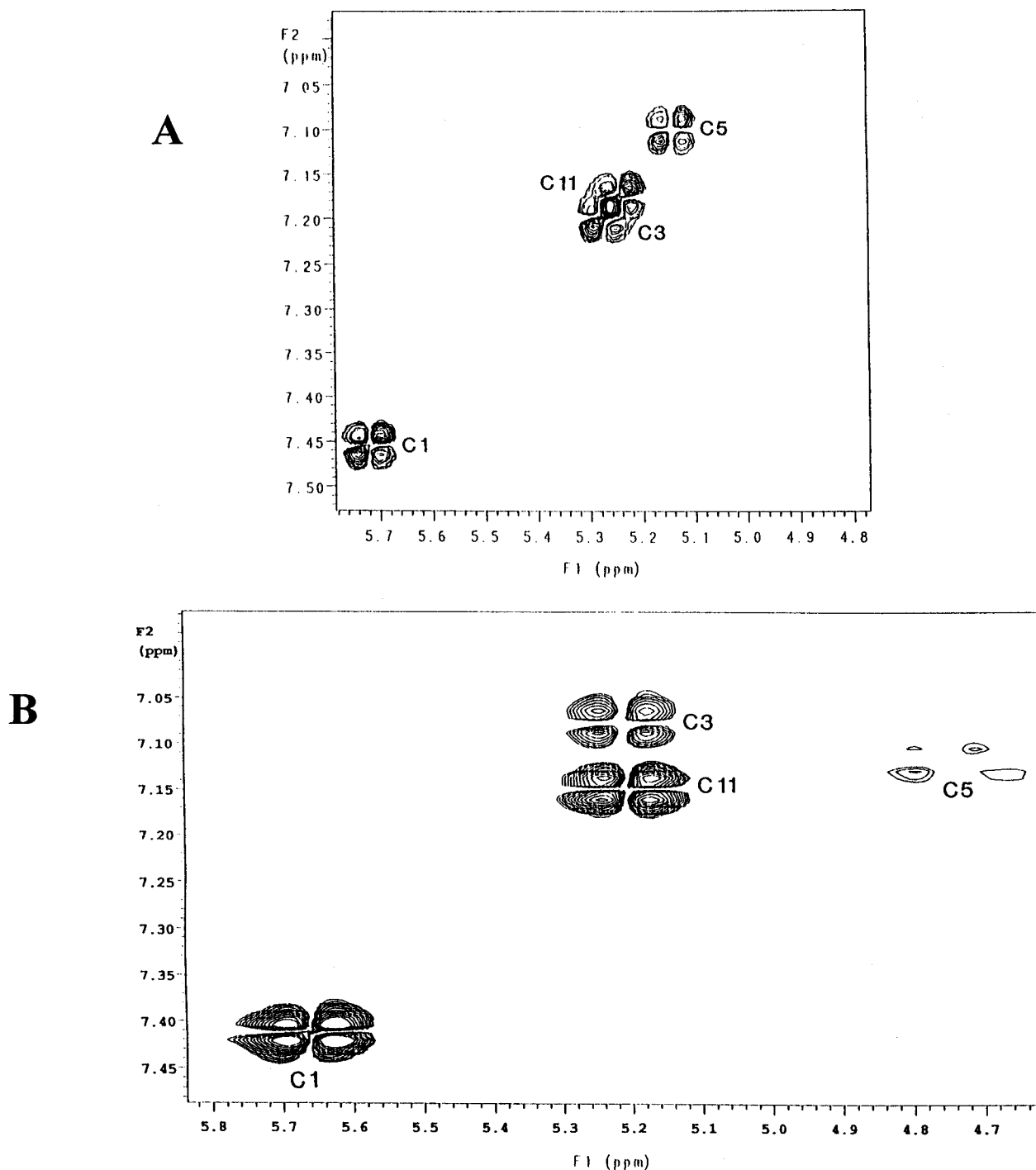


Figure 8. Expanded contour plots (H5–H6 region) of the DQF-COSY spectrum (A) for the standard 12-mer and (B) for the phenethyl 12-mer.

(Figure 9). The molecular model shown as a stereoplot in Figure 10 in which the arrow points at the benzene ring is fully consistent with these data.

The NMR studies on the chimeric DNA–RNA hybrid structures **7** and **8** were performed in much the same way as described above for the DNA oligomers. However, it has been demonstrated that the increased relaxation time of RNA-type protons may give spurious results if mixing times in NOESY experiments are too short. All NOESY experiments on the hybrids, therefore, were performed using three mixing times: 250, 400, and 600 ms. The NOESY experiments with 400 ms mixing times showed all the required connectivities, and no additional connectivities were observed in the 600 ms experiments. All discussions presented here will be taken from NOESY experiments performed with a 400 ms mixing time.

The expanded contour plot on the NOESY spectrum for the standard hybrid **7** is shown in Figure 11 in the aromatic H1' region. The presence of ribonucleotides has spread the aromatic H1' region over a large chemical shift range. Peak intensities are not uniform, and the assignment of all peaks reveals that peaks corresponding to RNA connectivities are considerably weaker than those for DNA connectivities, as expected. The chemical shifts for DNA and RNA H1' differ significantly, and one can divide the aromatic H1' region at 5.69 ppm. All ribonucleotide resonances are upfield from 5.69 ppm, whereas all DNA H1', except C5–H1', are downfield.

Evaluation of the standard 12-mer hybrid **7** reveals that the structure generally resembles the modified A-form geometry described by Reid and his colleagues (37). The A-form character of the RNA component is

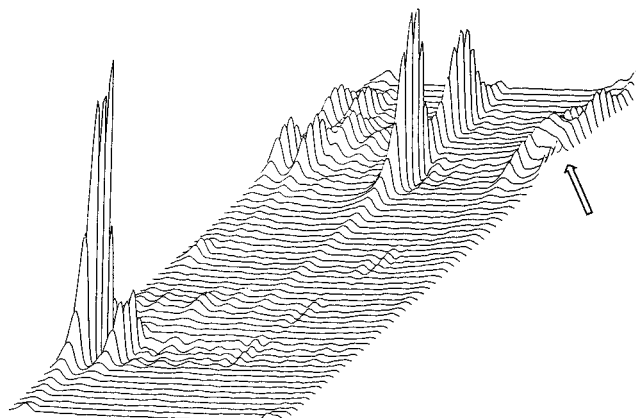


Figure 9. Stacked plot (H5-H6 region) of the DQF-COSY spectrum for the phenethyl 12-mer. The arrow indicates H5 of C5.

confirmed by the presence of strong cross-peaks from aromatic H8/H6 to H2' of the (N1) sugar and the lack of similar intranucleoside cross-peaks (data not shown). As

noted by asterisks in Figure 11, the H2 protons of A4 and A6 demonstrate connectivity with H1' of G10 and G8, respectively.

Such intrastrand NOEs are not associated with B-form DNA but arise with narrowing of the minor groove (40). H2 of A6 and A4 also show weak connectivities with their own H1', another observation which is not found in B-DNA geometry. On the other hand, H2 of A6 shows clear connectivity with H1' of U7, a finding more typical of DNA duplex geometry. This alteration of the A-type pattern at the junction may be responsible for the modest destabilization of the hybrid noted by melting studies as compared to standard DNA.

The expanded contour plot for the H1'-H2' region of the DQF-COSY spectrum for the standard hybrid is shown in Figure 12. In general, the C3'-endo sugar pucker of RNA strands results in very small $J_{1',2'}$ values, and in fact, those cross-peaks are missing in this spectrum, suggesting that most of the ribo sugars assume the expected C3'-endo sugar pucker. The exceptions are G12, the terminal nucleoside in which rapid equilibration of conformers gives rise to strong 1',2'-couplings and, in-

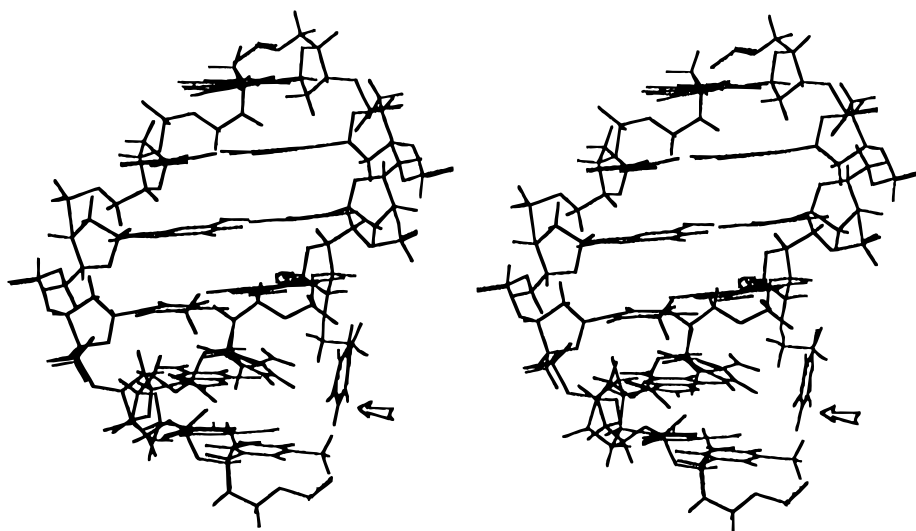


Figure 10. Stereoview of the model for the phenethyl 12-mer showing proximity of the benzene ring to C5.

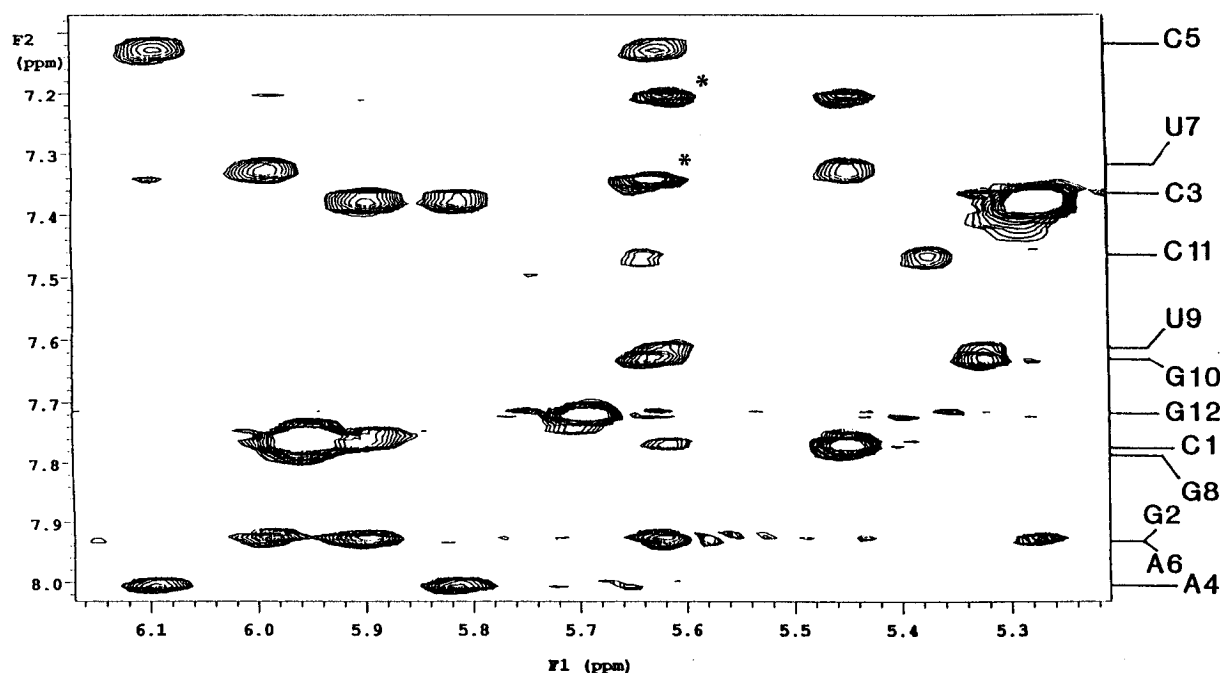


Figure 11. Expanded contour plot (aromatic H1' region) of the NOESY spectrum for the standard hybrid.

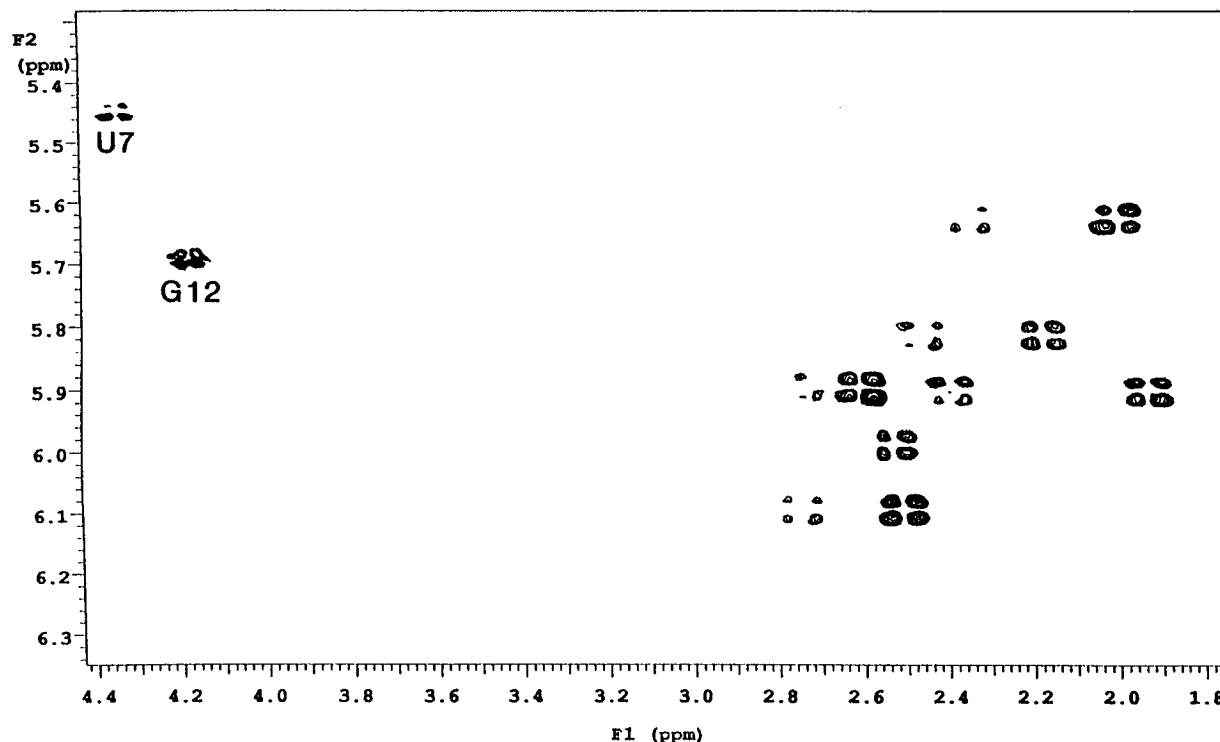


Figure 12. Expanded contour plot (H1'–H2' region) of the DQF-COSY spectrum for the standard hybrid.

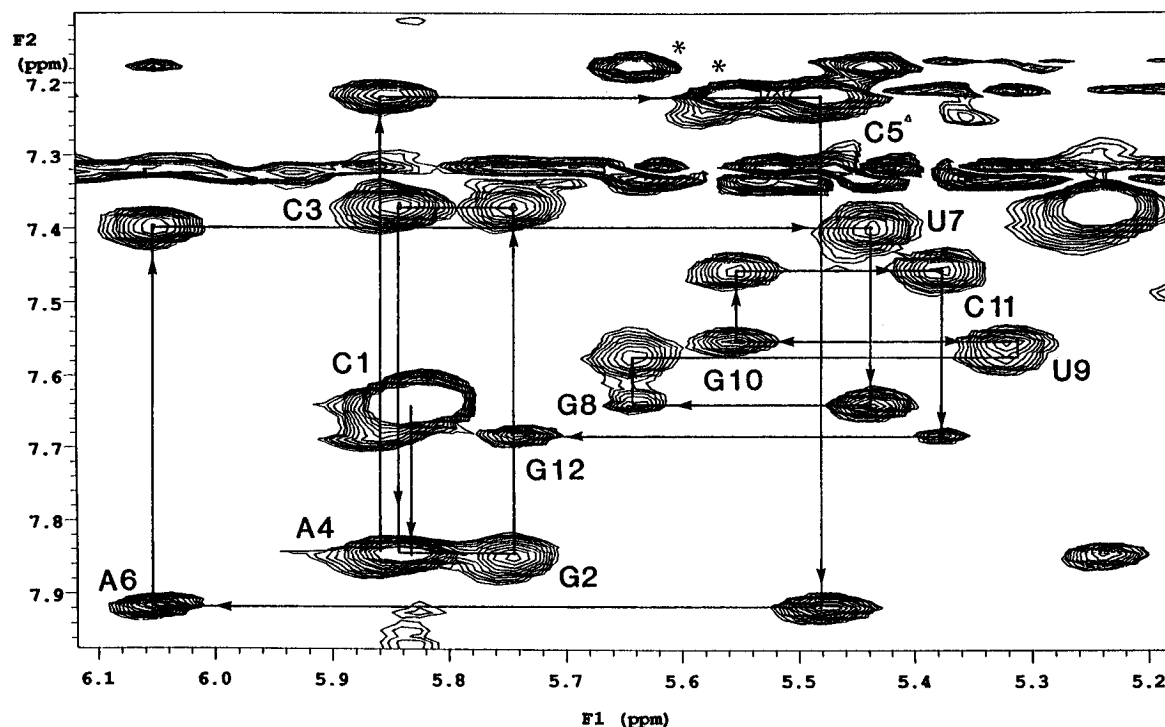


Figure 13. Expanded contour plot (aromatic H1' region) of the NOESY spectrum for the phenethyl hybrid.

terestingly, U7 which apparently assumes much more of a C2'-endo conformation, presumably because of its position at the junction.

The expanded contour plot for the aromatic H1' region of the NOESY spectrum for phenethyl hybrid **8** is shown in Figure 13. The assignments are illustrated on the figure. Data are entirely consistent with the conformation observed for the standard hybrid. Thus, the A–H2 protons show the intrastrand connectivities as marked by asterisks, and H2 of A6 also shows the previously identified intrastrand connectivity with H1' of U7. The close similarity between **7** and **8** conformations is also

apparent from the DQF-COSY spectrum of the 1',2'-region shown in Figure 14, leaving the final issue to be resolved as the location of the benzene ring from the phenethyl nucleoside.

This was determined using NOESY and DQF-COSY experiments, as described above, for the phenethyl DNA **6**. In Figure 15, the aromatic H2' cross-peaks may be seen. The arrow points to the peaks corresponding to connectivity between the benzylic proton of the phenethyl group and H6 of C5 (upper signal) and between the benzylic protons and the aromatic protons of the phenethyl group (lower signal).

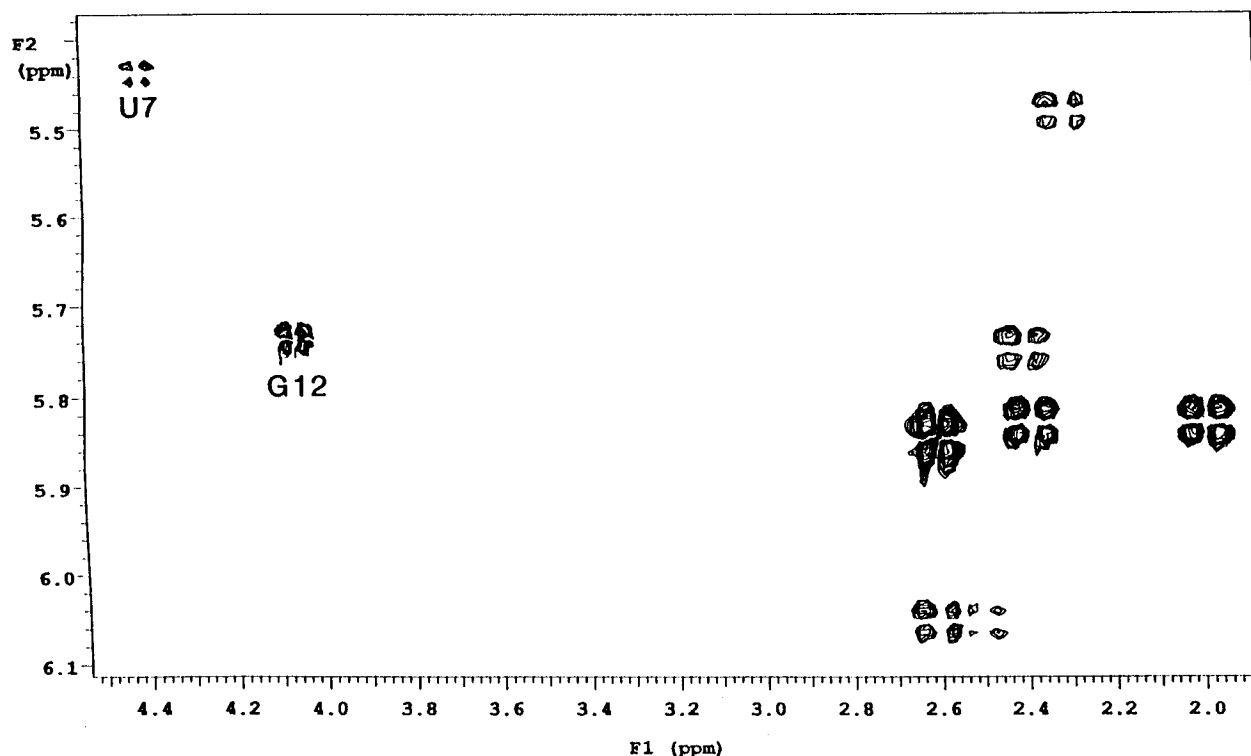


Figure 14. Expanded contour plot (H1'–H2' region) of the DQF-COSY spectrum for the phenethyl hybrid.

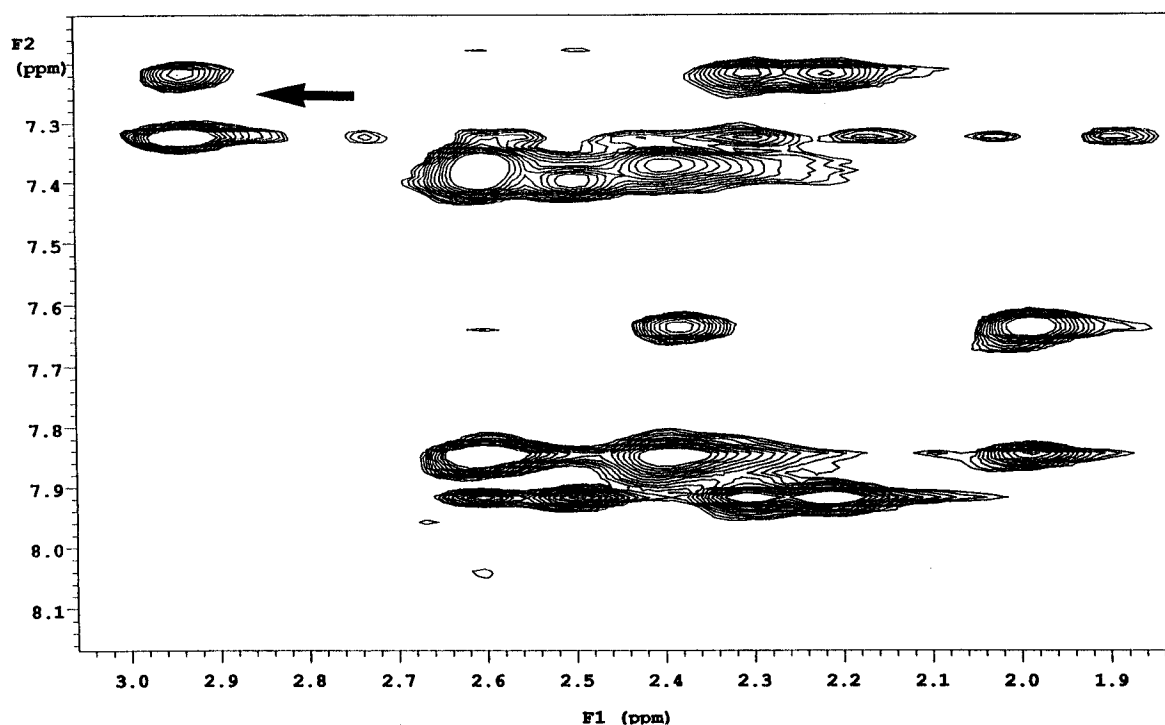


Figure 15. Expanded contour plot (aromatic H2' region) of the NOESY spectrum for the phenethyl hybrid. The arrow indicates connectivities of the benzylic proton.

Finally, in Figure 16, comparison of the data for the standard hybrid with that for the phenethyl hybrid reveals, just as seen previously, a marked reduction in signal intensity for the H5–H6 coupling of C5, again consistent with the benzene ring residing in the major groove and exerting an anisotropic effect on the H6 proton of C5.

The only significant difference noted between substitution of 2'-O-phenethyladenosine into DNA as opposed to

the DNA–RNA hybrid lies in the observation of an H1'–H2' cross-peak for A4 in the former but not in the latter. While one might normally consider the lack of coupling in the latter case to be associated with C3'-endo sugar geometry, a pure C3'-endo is not consistent with the demonstration that the benzene ring is introduced into the major groove; rather, such a conformation would place the benzene ring firmly in the minor groove. Therefore, one must assume that the conformations

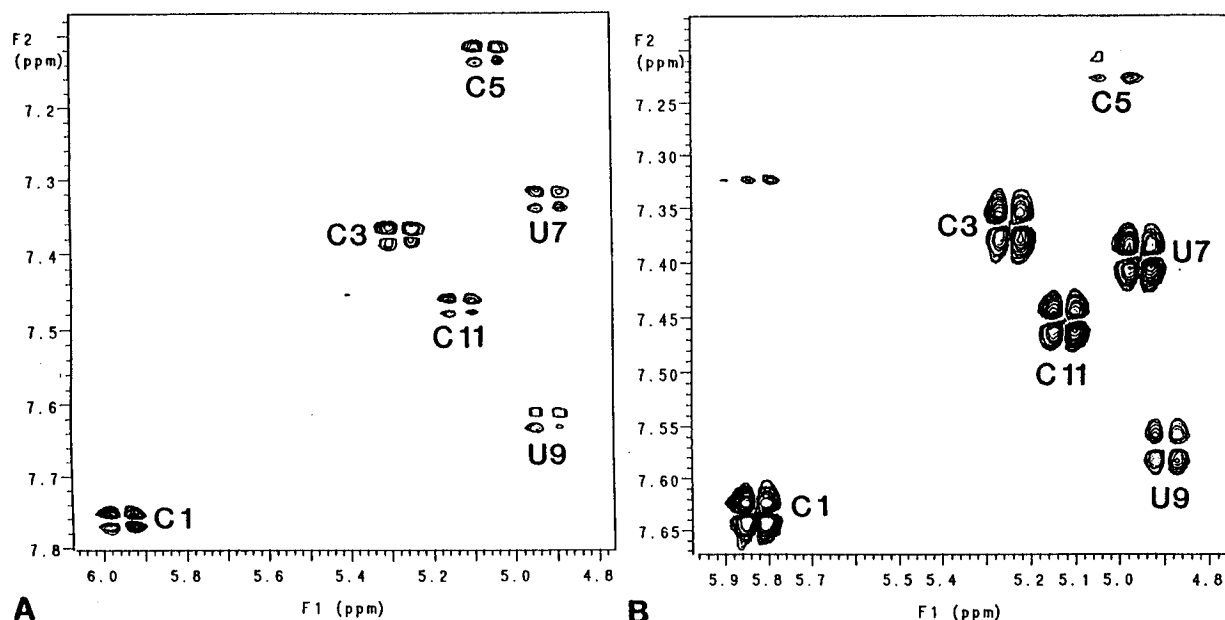


Figure 16. Expanded contour plot (H5–H6 region) of the DQF-COSY spectrum for the standard hybrid (A) and the phenethyl hybrid (B).

available for 2'-*O*-phenethyladenosine in the hybrid **4** lie between O4'-exo and C2'-exo, or in the "northwest" quadrant of the pseudorotational cycle (41).

DISCUSSION

As noted in the Introduction, the introduction of a small, flexible hydrophobic group into the 2'-position of a ribonucleoside and inclusion of that nucleoside into an oligodeoxynucleotide or a chimeric DNA–RNA hybrid could, in principle, give rise to four locations of the benzene ring in the complex: (1) it could extend into the aqueous solution, (2) it could lie in the minor groove, (3) it could intercalate, or (4) it could reside in the major groove. Although initially we regarded the last option as the least likely, it turns out that in both oligonucleotide duplexes this is precisely what takes place. Although there are slight differences in the conformation of the 2'-*O*-phenethylribose in the two circumstances as reflected by the existence of weak H1'–H2' coupling in the DNA and lack of such coupling in the hybrid, it remains a fact that the benzene ring must reside in the major groove because of its profound impact on the H6 proton of C5.

It is interesting that the substitution of the phenethyloxy group for the proton in DNA, although it leads to some destabilization of the duplex, does not create gross distortions in the oligonucleotide backbones. That is, the NMR data are fully consistent with B-type geometry in the DNA oligomers and a modified A-type geometry, as previously described (37–40), in the DNA–RNA duplex. This markedly unusual introduction of an aromatic substituent into the major groove has obvious implications in the design of oligonucleotides containing DNA major groove binding molecules. This area is under intense evaluation in this laboratory at the present time.

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LITERATURE CITED

- (1) Uhlmann, E., and Peyman, A. (1990) Antisense Oligonucleotides: A New Therapeutic Principle. *Chem. Rev.* 90, 543–584.
- (2) Beaucage, S. L., and Iyer, R. P. (1993) The Synthesis of Modified Oligonucleotides by the Phosphoramidite Approach and Their Applications. *Tetrahedron* 49, 6123–6194.
- (3) Beaucage, S. L., and Iyer, R. P. (1993) The Synthesis of Specific Ribonucleotides and Unrelated Phosphorylated Biomolecules by the Phosphoramidite Method. *Tetrahedron* 49, 10441–10488.
- (4) Tonkinson, J. L., and Stein, C. A. (1993) Antisense Nucleic Acids—Prospects for Antiviral Intervention. *Antiviral Chem. Chemother.* 4, 193–200.
- (5) Stein, C. A. and Cheng, Y.-C. (1993) Antisense Oligonucleotides as Therapeutic Agents—Is the Bullet Really Magical? *Science* 261, 1004–1012.
- (6) Beaucage, S. L., and Iyer, R. P. (1992) Advances in the Synthesis of Oligonucleotides by the Phosphoramidite Approach. *Tetrahedron* 48, 2223–2311.
- (7) Milligan, J. F., Matteucci, M. D., and Martin, J. C. (1993) Current Concepts in Antisense Drug Design. *J. Med. Chem.* 36, 1923–1937.
- (8) Cheng, Y.-K., and Pettitt, B. M. (1992) Hoogsteen versus Reversed-Hoogsteen Base Pairing: DNA Triple Helices. *J. Am. Chem. Soc.* 114, 4465–4474.
- (9) Thuong, N. T., and Hélène, C. (1993) Sequence-Specific Recognition and Modification of Double-Helical DNA by Oligonucleotides. *Angew. Chem., Int. Ed. Engl.* 32, 666–690.
- (10) Hobbs, C. A., and Yoon, K. (1994) Differential Regulation of Gene Expression *in vivo* by Triple Helix-Forming Oligonucleotides as Detected by a Reporter Enzyme. *Antisense Res. Dev.* 4, 1–8.
- (11) Clarenc, J.-P.; Lebleu, B., and Léonetti, J.-P. (1994) Base Changes and Triple-Helix Hybridization Properties of GT containing Third Strands: A Systematic Study. *Nucleosides Nucleotides* 13, 799–809.
- (12) Sun, J.-S., François, J.-C., Montenay-Garestier, T., Saison-Behmoaras, T., Roig, V., Thuong, N. T., and Hélène, C. (1989) Sequence-Specific Intercalating Agents: Intercalation at Specific Sequences on Duplex DNA via Major Groove Recognition by Oligonucleotide-Intercalator Conjugates. *Proc. Natl. Acad. Sci. U.S.A.* 86, 9198–9202.

- (13) Mann, J. S., Shibata, Y., and Meehan, T. (1992) Synthesis and Properties of an Oligodeoxynucleotide Modified with a Pyrene Derivative at the 5'-Phosphate. *Bioconjugate Chem.* 3, 554-558.
- (14) Vlassov, V. V., Gaidamakov, S. A., Zarytova, V. F., Knorre, D. G., Levina, A. S., Nikonova, A. A., Podust, L. M., and Fedorova, O. S. (1988) Sequence-Specific Chemical Modification of Double-Stranded DNA with Alkylating Oligodeoxyribonucleotide Derivatives. *Gene* 72, 313-322.
- (15) Boidot-Forget, M., Chassignol, M., Takasugi, M., Thuong, N. T., and Hélène, C. (1988). Site-Specific Cleavage of Single-Stranded and Double-Stranded DNA Sequences by Oligodeoxyribonucleotides Covalently Linked to an Intercalating Agent and an EDTA-Fe Chelate. *Gene* 72, 361-371.
- (16) Lin, S.-B., Blake, K. R., Miller, P. S., and Ts'o, P. O. P. (1989) Use of EDTA Derivatization To Characterize Interactions between Oligodeoxyribonucleoside Methylphosphonates and Nucleic Acids. *Biochemistry* 28, 1054-1061.
- (17) Iverson, B. L., and Dervan, P. B. (1987) Nonenzymatic Sequence-Specific Cleavage of Single-Stranded DNA to Nucleotide Resolution. DNA Methyl Thioether Probes. *J. Am. Chem. Soc.* 109, 1241-1243.
- (18) Levis, J. T., and Miller, P. S. (1994) Properties of Exonuclease-Resistant, Psoralen-Conjugated Oligodeoxyribonucleotides *in vitro* and in Cell Culture. *Antisense Res. Dev.* 4, 231-241.
- (19) Godard, G., François, J.-C., Duroux, I., Asseline, U., Chassignol, M., Thuong, N., Hélène, C., and Saison-Behmoaras, T. (1994) Photochemically and Chemically Activatable Antisense Oligonucleotides: Comparison of Their Reactivities towards DNA and RNA Targets. *Nucleic Acids Res.* 22, 4789-4795.
- (20) Morvan, F., Porumb, H., Degols, G., Lefebvre, I., Pompon, A., Sproat, B. S., Rayner, B., Malvy, C., Lebleu, B., and Imbach, J.-L. (1993) Comparative Evaluation of Seven Oligonucleotide Analogues as Potential Antisense Agents. *J. Med. Chem.* 36, 280-287.
- (21) Lesnik, E. A., Guinasso, C. J., Kawasaki, A. M., Sasmor, H., Zounes, M., Cummins, L. L., Ecker, D. J., Cook, P. D., and Freier, S. M. (1993) Oligodeoxynucleotides Containing 2'-O-Modified Adenosine: Synthesis and Effects on Stability of DNA:RNA Duplexes. *Biochemistry* 32, 7832-7838.
- (22) Lamond, A. I., and Sproat, B. S. (1993) Antisense Oligonucleotides Made of 2'-O-AlkylRNA: Their Properties and Applications in RNA Biochemistry. *FEBS Lett.* 325, 123-127.
- (23) Yamana, K., Nishijima, Y., Ikeda, T., Gokota, T., Ozaki, H., Nakano, H., Sangen, O., and Shimidzu, T. (1990) Synthesis and Interactive Properties of an Oligonucleotide with Anthraquinone at the Sugar Fragment. *Bioconjugate Chem.* 1, 319-324.
- (24) Keller, T. H., and Häner, R. (1993) Synthesis and Hybridization Properties of Oligonucleotides Containing 2'-O-Modified Ribonucleotides. *Nucleic Acids Res.* 21, 4499-4505.
- (25) Dan, A., Yoshimura, Y., Ono, A., and Matsuda, A. (1993) Nucleosides & Nucleotides. 118. Synthesis of Oligonucleotides Containing a Novel 2'-Deoxyuridine Analogue that Carries an Aminoalkyl Tether at 1'-Position; Stabilization of Duplex Formation by an Intercalating Group Accommodated in the Minor Groove. *Bioorg. Med. Chem. Lett.* 3, 615-618.
- (26) Yamana, K., Gokota, T., Ozaki, H., Nakano, H., Sangen, O., and Shimidzu, T. (1992) Enhanced Fluorescence in the Binding of Oligonucleotides with a Pyrene Group in the Sugar Fragment to Complementary Polynucleotides. *Nucleosides Nucleotides* 11, 383-390.
- (27) Deshmukh, H. M., Joglekar, S. P., and Broom, A. D. (1995) Self-Complementary Oligodeoxyribonucleotides Containing 2'-O-(Anthraquinone-2-methyl)adenosine. *Bioconjugate Chem.* 6, 578-586.
- (28) Ti, G. S., Gaffney, B. L., and Jones, R. A. (1982) Transient Protection: Efficient One-Flask Syntheses of Protected Deoxynucleosides. *J. Am. Chem. Soc.* 104, 1316-1319.
- (29) Wu, T., Ogilvie, K. K., and Pon, R. T. (1989) Prevention of Chain Cleavage in the Chemical Synthesis of 2'-Silylated Oligoribonucleotides. *Nucleic Acids Res.* 17, 3501-3517.
- (30) Kierzek, R., Caruthers, M. H., Longfellow, C. E., Swinton, D., Turner, D. H., and Freier, S. M. (1986) Polymer-Supported RNA Synthesis and Its Application To Test the Nearest-Neighbor Model for Duplex Stability. *Biochemistry* 25, 7840-7846.
- (31) Sproat, B. S., Beijer, B., and Iribarren, A. (1990) New Synthetic Routes to Protected Purine 2'-O-Methylriboside-3'-O-phosphoramidites Using a Novel Alkylation Procedure. *Nucleic Acids Res.* 18, 41-49.
- (32) Wagner, D., Verheyden, J. P. H., and Moffatt, J. G. (1974) Preparation and Synthetic Utility of Some Organotin Derivatives of Nucleosides. *J. Org. Chem.* 39, 24-30.
- (33) Yano, J., Kan, L. S., and Ts'o, P. O. P. (1980) A Simple Method of the Preparation of 2'-O-Methyladenosine. Methylation of Adenosine with Methyl Iodide in Anhydrous Alkaline Medium. *Biochim. Biophys. Acta* 629, 178-183.
- (34) Gray, D. M., Ratliff, R. L., and Vaughan, M. R. (1992) Circular Dichroism Spectroscopy of DNA. *Methods Enzymol.* 211, 389-405.
- (35) Tonelli, A. E., and Patel, D. J. (1974) Proton Nuclear Magnetic Resonance Investigations and Ring Current Calculations of Guanine N-1 and Thymine N-3 Hydrogen-Bonded Protons in Double-Helical Deoxyribonucleotides in Aqueous Solution. *Proc. Natl. Acad. Sci. U.S.A.* 71, 1945-1948.
- (36) Reid, B. R. (1987) Sequence-Specific Assignments and Their Use in NMR Studies of DNA Structure. *Q. Rev. Biophys.* 20, 1-34.
- (37) Salazar M., Fedoroff, O. Y., Miller, J. M., Ribeiro, N. S., and Reid, B. R. (1993). The DNA Strand in DNA-RNA Hybrid Duplexes is Neither B-Form nor A-Form in Solution. *Biochemistry* 32, 4207-4215.
- (38) Wang, A. H. J., Fujii, S., van Boom, J. H., van der Marel, G. A., van Boeckel, S. A. A., and Rich, A. (1982) Molecular Structure of r(GCG)d(TATACGC):DNA-RNA Hybrid Helix Joined to Double Helical DNA. *Nature* 299, 601-604.
- (39) Happ, C. S., Happ, E., Clore, G. M., and Gronenborn, A. M. (1988) Refinement of the Structure of RNA-DNA Hybrid 5'-[r(GCA)d(TGC)]₂. *FEBS Lett.* 236, 62-70.
- (40) Fedoroff, O. Y., Salazar, M., and Reid, B. R. (1993) Structure of a DNA:RNA Hybrid Duplex. Why RNase H Does Not Cleave Pure RNA. *J. Mol. Biol.* 233, 509-523.
- (41) Hosur, R. V., Ravikumar, M., Chary, K. V., Sheth, A., Govil, G., Zu-Kun, T., and Miles, H. T. (1986) Solution Structure of d-GAATTCGAATTC by 2D-NMR. A New Approach to Determination of Sugar Geometries in DNA Segments. *FEBS Lett.* 205, 71-76.

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Synthesis and Purification of Thermally Sensitive Oligomer–Enzyme Conjugates of Poly(*N*-isopropylacrylamide)–Trypsin

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Using chain-transfer polymerization, we have synthesized oligomers of poly(*N*-isopropylacrylamide) [poly(NIPAAm)] with one carboxyl group at the end of each oligomer chain. The lower critical solution temperature (LCST) of the oligomers is very close to that of homo-poly(NIPAAm) lacking the end carboxyl group. The carboxyl groups were activated in methylene chloride using *N,N'*-dicyclohexylcarbodiimide (DCC) and *N*-hydroxysuccinimide (NHS). A conjugate of trypsin with the preactivated oligomer has been prepared. We studied the effect of oligomer to enzyme (O/E) ratio in the feed on the O/E ratio of the conjugate (the average number of oligomer chains conjugated to one trypsin molecule), assuming that only the primary amino groups of lysine residues and the amino terminal of trypsin would react. The O/E ratio of the conjugate was estimated by determination of the remaining primary amine groups on the trypsin molecule. More than 95% of the conjugate can be recovered by thermally induced precipitation.

INTRODUCTION

There is growing interest in covalent conjugates of biomolecules and soluble synthetic polymers for applications in drug delivery, affinity separations, diagnostics, and bioprocesses. One of the first applications of this type of conjugate has been the conjugation of poly(ethylene glycol) (PEG) to therapeutic enzymes to protect the enzyme from recognition as a foreign molecule by the body's immune system (1, 2). Others have conjugated drugs as pendant groups (3, 4), sometimes via biodegradable or hydrolyzable linkages (5), to the backbone of various water soluble polymers. A targeting moiety may also be conjugated to the same polymer backbone (6).

Ligands such as antibodies, carbohydrates, peptide and protein cell receptor ligands, and biotin have been conjugated to a variety of polymers. We have developed a novel immunoassay based on phase separation of a thermally sensitive polymer conjugated to an antibody which is then complexed to an antigen and a second (labeled) antibody (7) and applied the same principle to affinity separation of IgG by protein A (8). Other affinity separations have been based on thermally induced (9, 10) or pH-induced precipitations (11–13) and polyligand–affinity receptor coacervation or gelation (14–16). Conjugates of ligands and polymers have also been used for affinity separations by (a) two-phase, liquid–liquid partitioning (17–19) or (b) liquid–solid (20, 21) partitioning.

In an enzyme bioprocess, it is usually desirable to separate the enzyme and product, recover the product, and recycle the enzyme. Compared with dialysis, membrane ultrafiltration, or gel chromatographic separation, phase separation of a soluble polymer–enzyme conjugate by causing the polymer to precipitate is a more convenient way to separate the enzyme from the product. The product can then be recovered from the supernatant and the enzyme conjugate redissolved and recycled for further

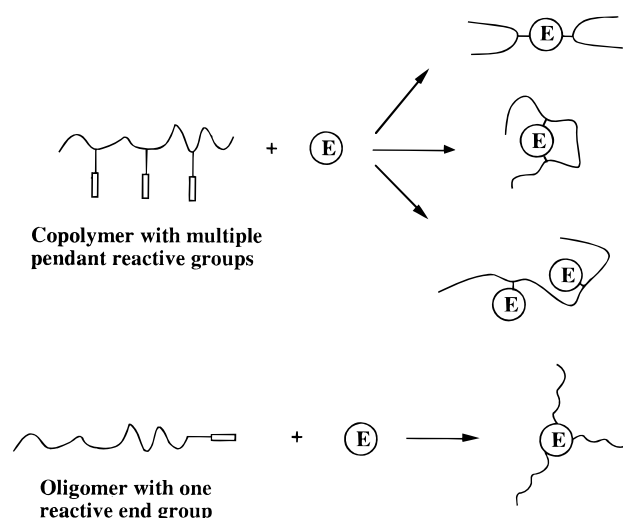


Figure 1. Difference between two conjugation protocols.

reaction with fresh substrate. We have proposed the use of thermally sensitive, soluble–insoluble polymers for this purpose (22, 23). Others have used pH changes to induce precipitation of a polymer–enzyme conjugate (24–26). More recently, we (27, 28) and others, especially Okano and co-workers (29–32), have developed oligomers of thermally sensitive polymers having one reactive end group, which can be used for conjugation to a protein. This method is more controlled and well-defined for conjugation of polymers to proteins (e.g. see Figure 1) and is similar in principle to the previously reported method of conjugation of PEG to therapeutic enzymes (1, 2). As an example of the controllability of this conjugation reaction, we have more recently reported on the site-specific conjugation of a temperature-sensitive oligomer by its reactive end group to a genetically engineered cysteine SH site which was deliberately located either away from the active site of cytochrome *b*₅ (33) or within the binding pocket of streptavidin (SAV) (34). In the latter case, biotin–SAV binding was reversibly controlled by the temperature and its effect on the polymer coil size (34).

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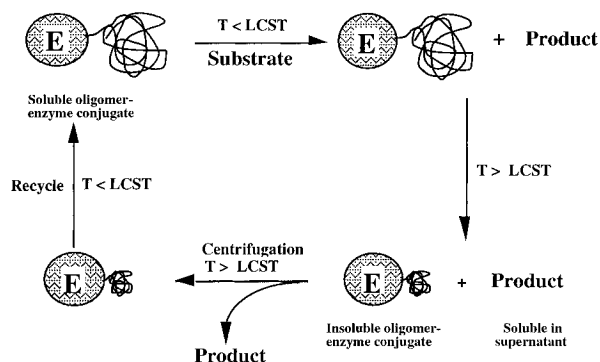


Figure 2. Thermal recycling process for recovery of product and recycle of the oligomer-enzyme conjugate.

Table 1. Preparation and Analysis of Poly(NIPAAm)

[M]:[S]:[I] ^a	<i>M_n</i> ^b	LCST (°C) ^c
100:4:0.5	4900	33.8 ± 0.4
100:2:0.25	7600	33.2 ± 0.5
100:1:0.13	11000	33.4 ± 0.4
100:0.5:0.07	19000	33.5 ± 0.3

^a Mole ratio of monomer [M] (NIPAAm), chain transfer agent [S] (MPA), and initiator [I] (AIBN). ^b The molecular weight of the oligomer was determined by vapor pressure osmometry (VPO).

^c Lower critical solution temperature (LCST) was determined spectroscopically at 500 nm in 0.25 wt % oligomer-H₂O solutions.

In the present study, we report on the synthesis and conjugation of end-reactive oligomers of *N*-isopropylacrylamide (NIPAAm) to trypsin, as a model enzyme. Our process for separation, recovery, and recycle of the enzyme conjugate is shown schematically in Figure 2. The enzymatic properties of this conjugate will be reported in a subsequent publication.

EXPERIMENTAL PROCEDURES

Materials. *N*-Isopropylacrylamide (NIPAAm, Eastman Kodak, Rochester, NY) was purified by recrystallization from *n*-hexane. 2,2'-Azobisisobutyronitrile (AIBN, J. T. Baker, Phillipsburg, NJ) was recrystallized from methanol. 3-Mercaptopyruvic acid (MPA, Aldrich, Milwaukee, WI) was purified by distillation under reduced pressure. *N*-Hydroxysuccinimide (NHS), *N,N'*-dicyclohexylcarbodiimide (DCC), and trypsin (type III, from bovine pancreas) were purchased from Sigma (St. Louis, MO). Fluorescamine (Eastman Kodak, Rochester, NY) was used as received. All other reagents were of analytical grade.

Preparation and Activation of Poly(NIPAAm). Poly(NIPAAm) terminated with a carboxyl group at one end was prepared by chain transfer polymerization using MPA as a chain transfer agent, as described by Chen and Hoffman (27). Briefly, 11.3 g of monomer, NIPAAm, plus various amounts of chain transfer agent, MPA, and initiator, AIBN, were dissolved in 50 mL of *tert*-butyl alcohol. The ratios of NIPAAm/MPA/AIBN used are shown in Table 1. The solutions were degassed by freezing and evacuating three times and then sealed. Polymerization was carried out at 60 °C for 3 h. The oligomers were collected by precipitation in diethyl ether. In order to obtain an oligomer with a narrow molecular weight distribution, fractionation was conducted using acetone as solvent and *n*-hexane as nonsolvent at 30 °C (35). The molecular weight of the oligomer was determined by vapor pressure osmometry (VPO, model OSV111 from Knauer, Germany).

The active-ester oligomer was prepared by reaction of the carboxyl terminal group with NHS using DCC as an

activating reagent in methylene chloride at room temperature, as described by Chen and Hoffman (28).

Conjugation of Trypsin with NHS-Esterified Poly(NIPAAm). NHS-esterified oligomer (40 mg) was dissolved in *N,N*-dimethylformamide (200 μL) (DMF), which was previously dried with molecular sieves, at room temperature. Up to 40 mg of trypsin was dissolved in 1 mL of 0.1 M sodium tetraborate buffer (pH 9.0) at 4 °C. The two solutions were mixed and gently shaken at 4 °C for 4 h and then at room temperature for 2 h to allow the conjugation to proceed.

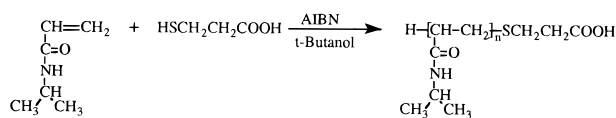
The conjugation solution was then diluted to 8 mL with a 0.1 M sodium tetraborate buffer (pH 9.0). To this solution was added 0.5 mL of saturated ammonia sulfate aqueous solution to reduce the LCST below 30 °C (21, 28). The conjugate was separated from unconjugated trypsin and other water-soluble chemicals by thermally induced precipitation from the solution at 30 °C and recovered by centrifugation at 11000*g* at 30 °C for 30 min. The recovered conjugate was dissolved in 8 mL of Tris-HCl buffer (pH 8.2, 0.05 M containing 0.02 M CaCl₂), in which trypsin was of maximum catalytic activity. This procedure was repeated three times. The conjugate was finally dissolved in fresh Tris-HCl buffer to reach a protein concentration about 150 mg/mL and stored at 4 °C. The protein content was determined by both means of UV absorption at 280 nm and trypsin active site titration (36).

Estimation of the O/E Ratio of the Conjugate.

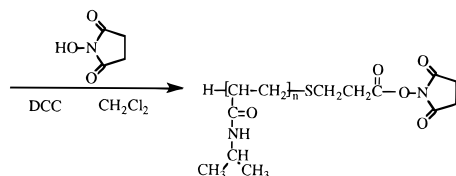
Each oligomer chain attached on a trypsin molecule consumes only one primary amine because there is only one reactive group for conjugation per oligomer. By measuring the remaining primary amine groups in the conjugate, we were able to estimate the O/E ratio of the conjugate, or the average number of oligomer chains conjugated to one trypsin molecule. Trypsin has fifteen primary amine groups, fourteen from lysine residues and one from the N-terminus, which may be utilizable for conjugation. The maximum value of the O/E ratio of the conjugate is 15 if all amines are reacted with the oligomer.

Primary amine groups were determined by a fluorometric assay (37). Conjugates were dissolved in 0.1 M sodium phosphate buffer (pH 8.0). Fluorescamine-dry DMF solution (0.5 mL), with a concentration of 0.3 mg/mL, was added to 1.5 mL of the conjugate solution while vortexing. After 7 min, the fluorescence of the solutions was measured on a fluorescence spectrometer (model F-4500 from Hitachi) with an excitation wavelength of 390 nm and an emission wavelength of 483 nm. The excess fluorescamine is rapidly destroyed by hydrolysis and does not contribute any signal before or after hydrolysis. The fluorescence is stable for several hours. A calibration curve was derived, on the basis of the total titratable amine content in native trypsin using exactly the same procedures.

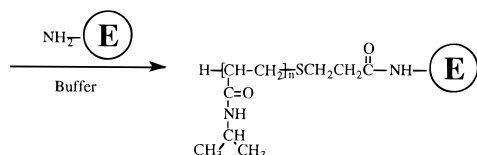
Thermal Recycle of the Conjugate. Iodine-125-labeled trypsin was conjugated with oligomer using the same protocol as used for native trypsin conjugation. Radiolabeled conjugate (1.0 mL), at a concentration of 50 μg/mL in 0.05 M Tris-HCl buffer (pH 8.2) containing 0.02 M CaCl₂, was precipitated at 30 °C after addition of 0.1 mL of saturated ammonium sulfate. The precipitate was collected by centrifugation at 11000*g* at 30 °C for 15 min. Trypsin content in the supernatant was determined by measuring the radioactivity from the trypsin in a γ spectrometer (GammaTrac 1185R, TmAnalytic Inc., U.S.A.). The precipitate was dissolved in 1.0 mL of Tris-HCl buffer at 4 °C for the next cycle. Protein recovery



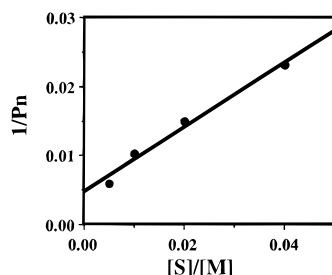
Step I . Synthesis of oligomer



StepII . Activation of oligomer



Step III . Conjugation

Figure 3. Chain transfer initiation synthesis of poly(NIPAAm) with one carboxyl end group and its conjugation with enzyme.**Figure 4.** Plot of the reciprocal of polymerization degree ($1/P_n$) against the molar ratio of chain transfer agent (S) (where S = 3-mercaptopropionic acid) to NIPAAm monomer $[S]/[M]$.

was calculated with following formula:

$$\text{protein recovery (\%)} = \frac{C_i}{C_{i-1}} \times 100$$

where C is the trypsin concentration ($\mu\text{g/mL}$) and i is the number of cycles.

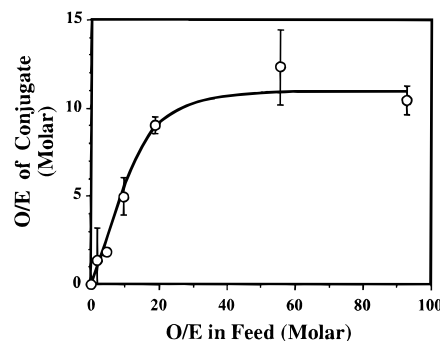
RESULTS AND DISCUSSION

Oligomer Synthesis. Two advantages of chain transfer polymerization are that it is easy both to control and vary the molecular weight and it is also a convenient way to introduce a functional group at one polymer chain end. We employed this method to prepare COOH-terminated poly(*N*-isopropylacrylamide) with different molecular weights. The synthesis of poly(NIPAAm) and activation of its carboxyl end group are schematically shown in Figure 3.

tert-Butyl alcohol was used as solvent for the polymerization because of its low chain transfer constant and good solubility for both monomer and oligomer. Figure 4 shows that the reciprocal of the polymerization degree ($1/P_n$) is proportional to the molar ratio of chain transfer agent to monomer $[S]/[M]$. This linear relationship between $1/P_n$ and $[S]/[M]$ agrees with the theoretical description (38):

$$1/P_n = 1/P_{n_0} + C_s([S]/[M])$$

where P_n and P_{n_0} = number average degree of polymerization obtained with or without chain transfer agent, respectively, $[S]$ and $[M]$ = concentration of monomer and

**Figure 5.** Effect of O/E ratio in the feed on the O/E ratio of the conjugate, or the average number of oligomer chains conjugated to each trypsin molecule, which was estimated by a fluorometric assay. The M_n of the oligomer was 5100. To vary the O/E ratio in the feed, the concentration of trypsin was changed while that of the oligomer was kept constant, at 33 mg/mL.

chain transfer agent, respectively, and C_s = chain transfer constant. The chain transfer constant, which is the ratio of the rate constant for chain transfer reaction to that for propagation, is 0.47, as calculated from Figure 4. Table 1 summarizes the M_n s of the different oligomers synthesized.

The oligomers are soluble in aqueous solutions at temperatures below their LCSTs and precipitate from the solutions above their LCSTs (the temperature at the onset of turbidity in a diluted solution of the oligomer is defined as the LCST). The LCSTs of the oligomers (shown in Table 1) are very close to that of homo-poly-(NIPAAm) (32 °C), which lacks a carboxyl group at the end of the polymer chain.

The oligomers were first activated by reacting with DCC and simultaneously coupling with an excess of NHS in methylene chloride, rather than conjugating the polymer directly to the enzyme in the presence of carbodiimide. There were two reasons for this. First of all, preactivation of the oligomers can avoid the direct activation of enzyme by carbodiimide and, therefore, eliminate the conjugation between enzyme molecules. Second, high conversion of the PNIPAAm carboxyl end group to the NHS active ester can be achieved since the activation reaction is carried out in an organic solvent, methylene chloride, where hydrolysis of the NHS active ester is avoided. The NHS-activated oligomers were very stable when stored in a desiccator at -18 °C. The reaction activity of the NHS-activated oligomers was unchanged after storage for several months.

Effect of the O/E Ratio in the Feed on the O/E Ratio of the Conjugate. The O/E ratio of the conjugate should play an important role in both the bioactivity of the enzyme and the recovery efficiency of the conjugate. Figure 5 shows that the maximum O/E ratio of the conjugate is about 11 (out of fifteen total primary amines in trypsin). We were not able to get a higher O/E ratio of the conjugate, probably because of steric hindrance. That is, as the O/E ratio of the conjugate increases, the surface of the trypsin gets more and more crowded with oligomers, which sterically block access of other activated oligomers to any remaining and accessible primary amine groups. It is also possible that the remaining primary amine groups are either buried or not readily accessible to the oligomers.

Effect of pH on Conjugation. When *N*-hydroxysuccinimide ester is employed for protein conjugation, it is generally recommended to use a buffer with pH of 8.0–9.0 (39). If the pH is too low, most of the primary amine groups are positively charged, since the pK_a of the lysine

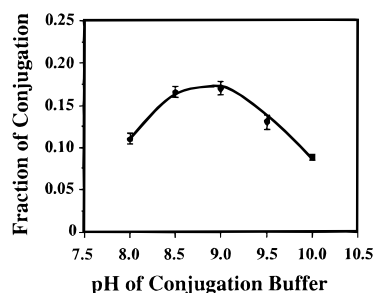


Figure 6. Fraction of conjugation as a function of buffer pH. The buffer used was 0.1 M sodium borate. The M_n of the oligomer was 5100. The O/E ratio in the feed was 9.3.

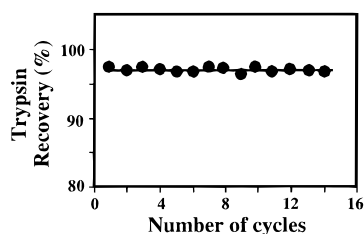


Figure 7. Recovery of trypsin–PNIPAAm conjugate after each cycle using cyclic precipitation–dissolution steps. Molecular weight of the oligomer was 5100. O/E in the feed was 9.3. The buffer was 0.1 M Tris–HCl (pH 8.2) containing 0.02 M CaCl_2 . Recycling temperatures were 0 and 30 °C.

amine group is about 9.3. The positively charged amine has a very low reactivity with the NHS ester, and therefore, a low conjugation yield may be obtained. On the other hand, the NHS ester is susceptible to hydrolysis at high pH. Selecting an optimum pH for conjugation is essential. Figure 6 shows the pH profile of the conjugation of poly(NIPAAm) with trypsin. It can be seen that pH ca. 8.5–9.0 results in a maximum fraction of conjugation (defined as the fraction of trypsin in the conjugation reaction vessel which is conjugated), even with trypsin, which has a pI of 10.5.

Recovery of Conjugate. We selected a conjugate with an O/E ratio of ca. five oligomers per trypsin molecule and thermally cycled in Tris–HCl buffer (pH 8.2, 0.1 M containing 20 mM CaCl_2) between 0 and 30 °C. Figure 7 shows that more than 95% of the trypsin conjugate can be recovered after each precipitation–dissolution cycle. A control experiment in which a mixture of native trypsin and free poly(NIPAAm) were thermally cycled instead of the conjugate was carried out. No protein could be found in the precipitate after two cycles. These demonstrated that the oligomer chains conjugated to the trypsin were very effective in phase separating the protein from the solution above the LCST and redissolving it below the LCST.

CONCLUSIONS

Using chain transfer polymerization, we have successfully synthesized 5000–19000 MW poly(NIPAAm) oligomers, each with one carboxyl end group. The LCSTs of these oligomers are the same as that of poly(NIPAAm) without the functional group at the chain end. We have then conjugated these oligomers to the enzyme trypsin, by preactivation of the oligomer carboxyl end group followed by reaction with primary amine groups of trypsin. About eleven out of fifteen primary amine groups in trypsin react with the active poly(NIPAAm). The optimum pH for conjugation is in the range of 8.5–9.0. When the conjugate was thermally cycled above and below the LCST, the conjugate recovery was over 95% after each precipitation, in each of 15 cycles.

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LITERATURE CITED

- (1) Abuchowski, A., McCoy, J. R., Palczuk, N. C., Es., T. V., and Davis, F. F. (1977) Effect of covalent attachment of polyethylene glycol on immunogenicity and circulating life of bovine liver catalase. *J. Biol. Chem.* 252, 3582–2586.
- (2) Fuertges, F., and Abuchowski, A. (1990) The clinical efficacy of poly(ethylene glycol)-modified proteins. *J. Controlled Release* 11, 139–148.
- (3) Maeda, H., Takeshita, J., and Kanamaru, R. (1979) A lipophilic derivative of neocarzinostatin: A polymer conjugation of an antitumor protein antibiotic. *Int. J. Pept. Protein Res.* 14, 81–87.
- (4) Maeda, H., Matsumoto, T., Konno, T., Iwai, K., and Ueda, M. (1984) Tailor-making of protein drugs by polymer conjugation for tumor targeting: a brief review on smancs. *J. Protein Chem.* 3, 181–193.
- (5) Krinick, N. L., Sun, Y., Joyner, D., Spikes, J. D., Straight, R. C., and Kopecek, J. (1994) A polymeric drug delivery system for the simultaneous delivery of drugs activatable by enzymes and/or light. *J. Biomater. Sci. Polym. Ed.* 5, 303–324.
- (6) O'Hare, K. B., Hume, I. C., Scarlett, L., Chytr'y, V., Kopeckov'a, P., Kopecek, J., and Duncan, R. (1989) Effect of galactose on interaction of *N*-(2-hydroxypropyl)methacrylamide copolymers with hepatoma cells in culture: preliminary application to an anticancer agent, daunomycin. *Hepatology* 10, 207–214.
- (7) Monji, N., and Hoffman, A. S. (1987) A novel immunoassay system and bioseparation process based on thermal phase separating polymers. *Appl. Biochem. Biotechnol.* 14, 107–120.
- (8) Chen, J. P., and Hoffman, A. S. (1990) Polymer-protein conjugates. II. Affinity precipitation separation of human IgG by a poly(*N*-isopropylacrylamide)-protein A conjugate. *Biomaterials* 11, 631–634.
- (9) Nguyen, A. L., and Luong, J. H. T. (1989) Syntheses and application of water soluble reactive polymers for purification and immobilization of biomolecules. *Biotechnol. Bioeng.* 34, 1186–1190.
- (10) Galaev, I. Y., and Mattiasson, B. (1993) Affinity thermoprecipitation: Contribution of the efficiency and access of the ligand. *Biotechnol. Bioeng.* 41, 1101–1106.
- (11) Senstad, C., and Mattiasson, B. (1989) Affinity-precipitation using chitosan as ligand carrier. *Biotechnol. Bioeng.* 33, 216–220.
- (12) Senstad, C., and Mattiasson, B. (1989) Purification of wheat germ agglutinin using affinity flocculation with chitosan and a subsequent centrifugation or flotation step. *Biotechnol. Bioeng.* 34, 387–393.
- (13) Schneider, M., Guillot, C., and Lamy, B. (1981) The affinity precipitation technique. Application to the isolation and purification of trypsin from bovine pancreas. *Ann. N. Y. Acad. Sci.* 369, 257–263.
- (14) Pecs, M., Eggert, M., and Schuegerl, K. (1991) Affinity precipitation of extracellular microbial enzymes. *J. Biotechnol.* 21, 137–142.
- (15) Morris, J. E., Hoffman, A. S., and Fisher, R. R. (1993) Affinity precipitation of proteins by polyligands. *Biotechnol. Bioeng.* 41, 991–997.
- (16) Larsson, P. O., and Mosbach, K. (1979) Affinity precipitation of enzymes. *FEBS Lett.* 98, 333–338.
- (17) Karr, L. J., Donnelly, D. L., Kozlowski, A., and Harris, J. M. (1994) Use of poly(ethylene glycol)-modified antibody in cell extraction. *Methods Enzymol.* 228, 377–390.
- (18) Karr, L. J., Shafer, S. G., Harris, J. M., van Alstine, J. M., and Snyder, R. S. (1986) Immuno-affinity partition of cells in aqueous polymer two-phase systems. *J. Chromatogr.* 3354, 269–282.
- (19) Sharp, K. A., Yalpani, M., Howard, S. J., and Brooks, D. E. (1986) Synthesis and application of a poly(ethylene glycol)-

- antibody affinity ligand for cell separations in aqueous polymer two-phase systems. *Anal. Biochem.* 154, 110–117.
- (20) Monji, N., Cole, C. A., Tam, M., Goldstein, L., Nowinski, R. C., and Hoffman, A. S. (1990) Application of a thermally-reversible polymer-antibody conjugate in a novel membrane-based immunoassay. *Biochem. Biophys. Res. Commun.* 172, 652–660.
- (21) Monji, N., Cole, C. A., and Hoffman, A. S. (1994) Activated, *N*-substituted acrylamide polymers for antibody coupling: Application to a novel membrane-based immunoassay. *J. Biomater. Sci., Polym. Ed.* 5, 407–420.
- (22) Hoffman, A. S. (1987) Thermally reversible polymers and hydrogels in therapeutics and diagnostics. *J. Controlled Release* 6, 297.
- (23) Cole, C. A., Schreiner, S. M., Priest, J. H., Monji, N., and Hoffman, A. S. (1987) *N*-isopropylamide and *N*-acryloxysuccinimide copolymer: a thermally reversible water-soluble, activated polymer for protein conjugation. In *Reversible Polymeric Gels and Related Systems* (P. Russo, Ed.) p 245, Symposium Series 350, American Chemical Society, ACS Washington, DC.
- (24) Okamura, K., Ikura, K., Yoshikawa, M., Sakaki, R., and Chiba, H. (1984) Soluble-insoluble interconvertible enzymes. *Agric. Biol. Chem.* 48, 2435–2440.
- (25) Taniguchi, M., Kobayashi, M., and Fujii, M. (1989) Properties of a reversible soluble-insoluble cellulase and its application to repeated hydrolysis of crystalline cellulose. *Biotechnol. Bioeng.* 34, 1092–1097.
- (26) Taniguchi, M., Hoshino, K., Watanabe, K., Sugai, K., and Fujii, M. (1992) Production of soluble sugar from cellulosic materials by repeated use of a reversibly soluble-autoprecipitating cellulase. *Biotechnol. Bioeng.* 39, 287–292.
- (27) Chen, G. H., and Hoffman, A. S. (1992) Synthesis of carboxylated poly(NIPAAm) oligomers and its application to form thermoreversible polymer-enzyme conjugates. *Polym. Prepr.* 33, 468–469.
- (28) Chen, G. H., and Hoffman, A. S. (1993) Preparation and properties of thermoreversible, phase-separating enzyme-oligo(*N*-isopropylacrylamide) conjugates. *Bioconjugate Chem.* 4, 509–514.
- (29) Takei, Y., Aoki, T., Sanui, K., Ogata, N., Okano, T., and Sakurai, Y. (1993) Temperature-responsive bioconjugates. 1. Synthesis of temperature-responsive oligomers with reactive end groups and their coupling to biomolecules. *Bioconjugate Chem.* 4, 42–46.
- (30) Takei, Y., Aoki, T., Sanui, K., Ogata, N., Okano, T., and Sakurai, Y. (1993) Temperature-responsive bioconjugates. 2. Molecular design for temperature-modulated bioseparations. *Bioconjugate Chem.* 4, 341–346.
- (31) Takei, Y., Matsukata, M., Aoki, T., Sanui, K., Ogata, N., Kikuchi, A., Sakurai, Y., and Okano, T. (1994) Temperature-responsive bioconjugates. 3. Antibody–poly(*N*-isopropylacrylamide) conjugates for temperature-modulated precipitations and affinity bioseparations. *Bioconjugate Chem.* 5, 577–582.
- (32) Matsukata, M., Takei, Y., Aoki, T., Sanui, K., Ogata, N., Sakurai, Y., and Okano, T. (1994) Temperature modulated solubility-activity alterations for poly(*N*-isopropylacrylamide)-lipase conjugates. *J. Biochem.* 116, 682–688.
- (33) Chilkoti, A., Chen, G. H., Stayton, P. S., and Hoffman, A. S. (1994) Site-specific conjugation of a temperature-sensitive polymer to a genetically-engineered protein. *Bioconjugate Chem.* 5, 504–507.
- (34) Stayton, P. S., Shimoboji, T., Long, C., Chilkoti, A., Chen, G., Harris, J. M., and Hoffman, A. S. (1995) Control of protein-ligand recognition using a stimuli-responsive polymer. *Nature* 378, 472–474.
- (35) Fujishige, S. (1987) Intrinsic viscosity-molecular weight relationships for poly(*N*-isopropylacrylamide) solutions. *Polym. J.* 19, 297–300.
- (36) Chase, T., and Shaw, E. (1970) Titration of trypsin, plasmin, and thrombin with *p*-nitrophenyl *p*'-guanidinobenzoate HCl. *Methods Enzymol.* 19, 20–27.
- (37) Stocks, S. J., Jones, A. J. M., Ramey, C. W., and Brooks, D. E. (1986) A fluorometric assay of the degree of modification of protein primary amines with polyethylene glycol. *Anal. Biochem.* 154, 232–234.
- (38) Berger, K. C., and Brandrup, G. (1989) Transfer constants to monomer, polymer, catalyst, solvent, and additive in free radical polymerization. In *Polymer Handbook* (J. Brandrup and E. H. Immergut, Eds.) 3rd ed. 11/81, Wiley-Interscience, New York.
- (39) Brinmley, M. (1992) A brief survey of methods for preparing protein conjugates with dyes, haptens, and cross-linking reagents. *Bioconjugate Chem.* 3, 2–13.

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On–Off Switching of Enzymatic Reaction by Recombinant Calmodulin on a Solid-Phase Matrix

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A fusion protein consisting of human calmodulin (CaM) and glutathione S-transferase (GST) was produced by gene fusion. The fusion protein was overexpressed in *Escherichia coli* as a soluble form and purified with one-step affinity chromatography using glutathione–Sephadex. The protein had the modulating activity of CaM and the binding capability to glutathione of GST. Phosphodiesterase, which is a CaM dependent enzyme, was activated by the fusion protein, with the Ca^{2+} level equal to the level equivalent to a native CaM. Furthermore, CaM could be immobilized on a solid-phase matrix through the use of GST moiety while its modulating activity was retained. Phosphodiesterase activity was switched on and off by the immobilized CaM with or without Ca^{2+} , and repeated use of CaM was demonstrated.

INTRODUCTION

Advances in protein engineering have led us to design a variety of fusion proteins by assembling several functions of proteins. For example, two enzymes, which catalyze sequential reactions, have been fused to perform at an enhanced reaction rate due to the proximity of the enzymes (1, 2). There is an advantage in facilitating the sequential catalysis by two or more enzymes for the biotechnological production process. Another approach for utilizing the fusion protein is using a binding protein for biological analysis and protein purification. Some reports have demonstrated the fusion protein as a reagent for enzyme immunoassay (3, 4). In previous reports, we have shown an applicability of the fusion protein between marker enzymes and protein A, which binds to IgG molecules, in enzyme immunoassay (5, 6). The gene fusion systems for the expression and purification of recombinant proteins have been studied extensively (7–9). In these systems, the structural gene of interest is connected in frame to a gene encoding a protein which binds to a ligand by affinity, and the resulting protein can be readily purified only through the use of affinity chromatography.

Another expected advantage of the fusion protein containing a binding protein is the capability of immobilizing the functional protein on a matrix. Through fusion of a binding protein to a functional protein such as an enzyme, we expected that the functional protein, while controlling the orientation of an active site in the molecule, can be assembled. Such hybridization of proteins by gene fusion has been attracting attention as a useful technique for immobilization and assembly of functional proteins.

Immobilization of biological materials offers several advantages, for instance, stabilization of the intrinsically unstable materials and increasing the efficiency of the reaction by assembling the functional molecules. More-

over, it is possible to use rather expensive biological materials continuously and repeatedly. Immobilizing techniques have already been utilized for immobilization of enzymes or antibodies for the manufacturing of biosensors and bioreactors (10). Besides, the ligands immobilized on a matrix are very useful tools for purification of materials which have affinities for ligands as described above.

Calmodulin (CaM)¹ undergoes remarkable conformational change when it binds Ca^{2+} (11, 12), which results in the modulation of many important biological reactions (13, 14). This regulation is one of the key steps in a variety of cellular processes controlled by Ca^{2+} . By immobilizing CaM while retaining its activity to a solid-phase matrix, we found that it is a feasible to control the conformational change of CaM and the activity of CaM dependent enzymes by Ca^{2+} , continuously and reversibly. One successful approach for such immobilization of CaM has been demonstrated by chemical coupling. CaM immobilized on Sephadex has been used for the affinity purification of CaM dependent proteins (15–17). However, in these cases, CaM molecules are immobilized on a matrix with random orientation. For the exhibition of the efficient enzymatic reaction, it is important not only to bind the target proteins for the purification but also to immobilize CaM molecule while controlling their orientation. For construction of such a system, we prepared a monolayer membrane of CaM modified with lipid at the air–water interface by the Langmuir–Blodgett method and showed that the conjugated protein film activates the CaM dependent enzyme, depending on Ca^{2+} concentration, in our previous work (18).

In this study, we prepared a recombinant CaM–glutathione S-transferase fusion protein for the purpose of immobilizing a modulating protein while controlling its orientation on a solid-phase matrix. It is possible to immobilize CaM on a glutathione-immobilized matrix through the use of glutathione S-transferase (GST),

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¹ Abbreviations: CaM, calmodulin; GST, glutathione S-transferase; IPTG, isopropyl β -D-thiogalactoside; PDE, 3',5'-cyclic-nucleotide phosphodiesterase; cAMP, cyclic 3',5'-monophosphate; ADA, adenosine deaminase; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

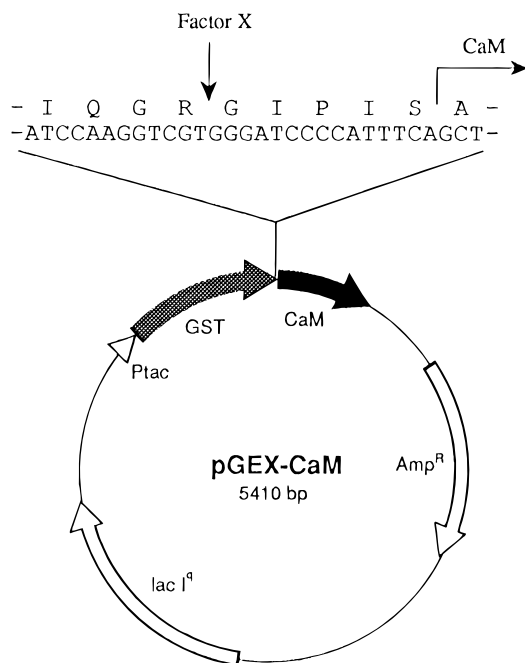


Figure 1. Map of the plasmid pGEX-CaM: GST, structural gene for glutathione S-transferase; CaM, structural gene for CaM; Amp^R, ampicillin resistance gene; lacI^q, lac repressor gene; and Ptac, tac promoter. Arrows indicate the direction of transcription. The sequences of nucleotides and amino acids in the junction region between GST and CaM are also shown. Factor X indicates the factor Xa site in the resulting fusion protein.

because GST can bind to matrix-bound glutathione. Furthermore, on-off switching of phosphodiesterase activity by CaM with or without Ca²⁺ was investigated. This approach is expected to apply not only to a bioreactor system for CaM dependent enzymes but also to the delivery of drugs such as calcitonin and parathyroid hormone, which are responsible for the regulation of Ca²⁺ concentration in serum.

EXPERIMENTAL PROCEDURES

Materials. Plasmid pGEX-3X was purchased from Pharmacia (Uppsala, Sweden). Restriction enzyme, *Xmn*I, was obtained from New England Biolabs (Beverly, MA), and other restriction enzymes, ligase, T4 DNA polymerase, and factor Xa, were purchased from Takara Shuzo (Shiga, Japan). 3',5'-Cyclic-nucleotide phosphodiesterase was from Sigma (St. Louis, MO). Glutathione-Sepharose beads were from Pharmacia, and all other chemicals were of analytical grade.

Construction of Plasmid. The GST-CaM gene fusion vector, pGEX-CaM, was constructed as follows. The coding region of human CaM cDNA (19) was amplified by polymerase chain reaction using synthetic oligonucleotides, 5'-AGGGAAGGATTTTCAGCTGACCAACT-GACTGAAG and 5'-CAGGTCGACTCACTTTGCTGCTGTC-ATCATTTG. The amplified fragment was digested with *Xmn*I and *Sall*I, and subsequently, both terminal ends were blunted with T4 DNA polymerase. The isolating gene fragment of CaM was ligated to the *Sma*I site of the pGEX-3X with T4 DNA ligase. In the resulting plasmid, the gene of CaM was fused to the gene of GST in frame. The constructing plasmid is schematically drawn in Figure 1.

Expression and Purification of the Fusion Protein. The *Escherichia coli* strain JM105 cells transformed with plasmid, pGEX-CaM, were cultured in 400 mL of LB medium containing ampicillin (50 mg mL⁻¹)

at 37 °C with shaking. In the midexponential phase, isopropyl β-D-thiogalactoside (IPTG) was added to a final concentration of 0.1 mM and the mixture cultured for another 3 h. The bacterial cells were harvested by centrifugation at 4000g for 10 min at 4 °C, and the cell pellet was washed twice with cold STE buffer [0.1 M NaCl, 10 mM Tris-HCl, and 1 mM ethylenediaminetetraacetic acid (EDTA) (pH 7.8)] and resuspended in 10 mL of the same buffer containing 1% Triton-X100. The cell suspension was sonicated for 30 min and centrifuged at 5000g for 10 min to remove the cell debris. The fusion protein was purified through the use of glutathione-Sepharose beads as described by the manufacturer (Pharmacia). The resulting supernatant was mixed with an equal volume of glutathione-Sepharose beads and incubated for 30 min at room temperature. The beads were washed three times with PBS [150 mM NaCl, 16 mM Na₂HPO₄, and 4 mM NaH₂PO₄ (pH 7.3)] and then mixed with elution buffer [50 mM Tris-HCl (pH 8.0) containing 50 mM glutathione]. The protein molecules bound to the beads were released competitively from the beads with the elution buffer. The solution containing the fusion protein was obtained from the supernatant by centrifugation.

Isolation of the CaM Moiety. There is a recognition site of factor Xa, which is a site specific protease (20), between GST and CaM (Figure 1). For cutting off the protein by factor Xa, the concentration of Ca²⁺ in the protein solution was adjusted to 1 mM with CaCl₂. Then factor Xa was added at a 1:100 (factor Xa:fusion protein) molar ratio and the mixture incubated for 2 h at 25 °C. When the reaction mixture was applied to glutathione-Sepharose beads again, the GST moiety bound to the beads and only the CaM moiety remained in the solution.

Activity Measurement of PDE. The activity of CaM was estimated as an activator for 3',5'-cyclic-nucleotide phosphodiesterase (PDE), which is one of the CaM dependent enzymes (21). The measurement was followed by the method of Schiefer (22). PDE is activated in the presence of CaM which binds Ca²⁺ and catalyzes the hydrolysis reaction of cyclic adenosine 3',5'-monophosphate (cAMP) to adenosine 5'-monophosphate (AMP). Then the AMP formed from cAMP in the reaction is dephosphorylated to adenosine in the presence of alkaline phosphatase. Subsequently, adenosine is hydrolyzed to inosine in the presence of adenosine deaminase (ADA). The PDE activity was determined by monitoring the decrease in absorbance at 265 nm which accompanies consumption of cAMP.

CaM solution (10 μL) was mixed thoroughly with a glycylglycine buffer (100 mM, pH 7.5) containing MgSO₄ (1.2 mM), CaCl₂ (2.1 mM), ADA (4 ku/L), alkaline phosphatase (30 ku/L), and PDE (3u/L) and the mixture incubated for 10 min at 30 °C. Finally, a solution of cAMP was added to a final concentration of 87 mM, and the final volume of the assay mixture was 1.0 mL.

Immobilization of CaM on a Mixture. The fusion protein was immobilized on glutathione-Sepharose beads which were used in the purification process. Cell lysate containing the fusion protein was mixed with glutathione-Sepharose and incubated for 30 min. The beads were washed well with PBS to remove proteins which adsorbed nonspecifically on a matrix. The resulting beads were directly used as the fusion protein-immobilized solid-phase matrix.

RESULTS AND DISCUSSION

Expression of the Fusion Protein. The constructing plasmid, pGEX-CaM, possessed the fusion gene of GST and CaM under the control of the tac promoter

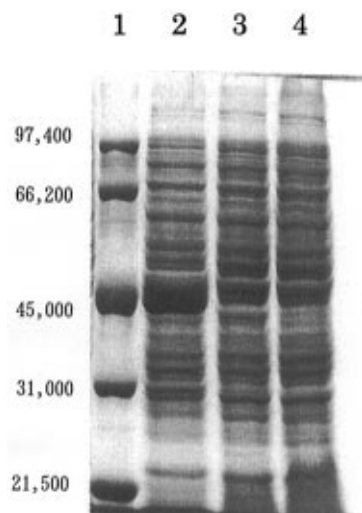


Figure 2. SDS-PAGE analysis of the fusion protein. *E. coli* JM105 cells, with or without plasmid pGEX-CaM, were lysed with sonication, and soluble proteins were analyzed: lane 1, standard samples (molecular mass is indicated on the left); lane 2, JM105/pGEX-CaM cell lysate induced by IPTG; lane 3, JM105/pGEX-CaM cell lysate (noninduced); and lane 4, JM105 cell lysate.

(Figure 1). The resulting fusion protein was designed by forming a site-to-site binding, that is, the C-terminal site of GST fused to the N-terminal site of CaM. The fused gene encodes a single polypeptide of GST and CaM. The sequences of nucleotides and resulting amino acids in the junction region between GST and CaM are shown in Figure 1. The fusion protein deduced from the DNA sequence has a molecular mass of ca. 43 kDa.

The cell extracts obtained from *E. coli* JM105 harboring pGEX-CaM were analyzed by a 10% SDS-PAGE. Figure 2 shows SDS-PAGE analysis of cell extracts of host JM105 (lane 4), transformed cells with pGEX-CaM (lane 3), and IPTG-induced transformed cells (lane 2). From the bands of marker proteins (lane 1), the main band in the lane 2 was ascribed to the fusion protein with a molecular mass of ca. 43 kDa. The band was not observed in lanes 3 and 4. The protein was produced in large amounts in soluble form from only the transformed cells induced with IPTG.

Purification of the Fusion Protein. The fusion protein could be purified in one step through the use of glutathione-Sepharose beads. About 6.4 mg of the purified fusion protein was obtained from 400 mL of culture. The purified fusion protein was analyzed by a 10% native PAGE, in the presence and in the absence of Ca^{2+} (Figure 3). The fusion protein could be purified with the affinity chromatography as shown in one band by staining with Coomassie brilliant blue. The migration in the presence of Ca^{2+} (lane 1) was a little faster than that in the absence of Ca^{2+} (lane 2). This result indicates that the CaM moiety of the fusion protein binds Ca^{2+} and changes its conformation to a compact folding.

In the fusion protein, there is a recognition site of factor Xa, which is a site specific protease, between GST and CaM. So the GST moiety and CaM moiety could be separated easily from the fusion protein by cutting off with factor Xa.

Ca^{2+} Dependent PDE Activation by the Fusion Protein. PDE is one of the typical CaM dependent enzymes. This enzyme is activated in the presence of Ca^{2+} -binding CaM and catalyzes the hydrolysis of cAMP to AMP. CaM-deficient PDE will catalyze this reaction at a low rate, and CaM activates PDE only in the presence of Ca^{2+} . Thus, the rate of reaction of PDE can



Figure 3. PAGE analysis of the purified fusion protein. The fusion protein was purified with glutathione-Sepharose beads: lane 1, the purified fusion protein in the presence of Ca^{2+} (treated with 2 mM CaCl_2); and lane 2, the fusion protein in the absence of Ca^{2+} [treated with 3 mM 1,2-bis(2-aminoethoxy)ethane- N,N,N',N' -tetraacetic acid (EGTA)].

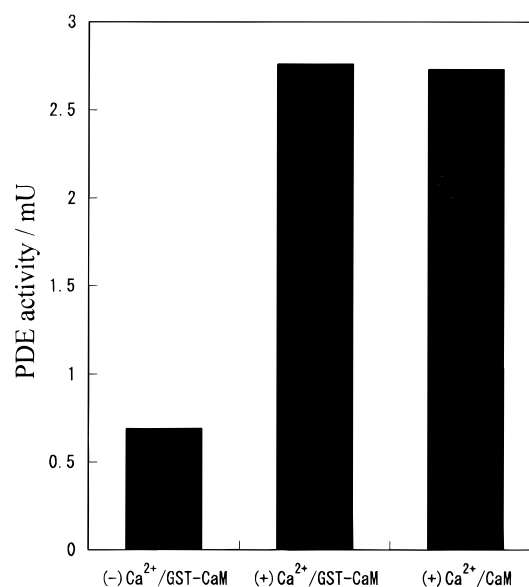


Figure 4. PDE activation by GST-CaM fusion protein. The fusion protein and PDE were incubated at 30 °C for 10 min in the assay mixture, in the absence [(-) Ca^{2+} /GST-CaM] and presence [(+) Ca^{2+} /GST-CaM] of Ca^{2+} . PDE activity with CaM which was digested from the fusion protein by factor Xa was also measured [(+) Ca^{2+} /CaM].

be used to estimate the amount of CaM present in the reaction mixture.

Figure 4 shows the PDE activity with the fusion protein in the absence and presence of Ca^{2+} . The activity of PDE in the presence of Ca^{2+} is about 4-fold higher than the activity in the absence of Ca^{2+} . Moreover, the PDE activity with CaM (not fusing with GST) which was separated from the fusion protein by factor Xa was almost the same as the activity by the fusion protein. These results indicate that the CaM moiety in the fusion protein functioned as an activator of PDE without losing its activity by fusing with GST.

Reversible Reactivity of Calmodulin on a Matrix.

In order to confirm the possibility for on-off switching of enzymatic activity by CaM with or without Ca^{2+} , the fusion protein was immobilized on a solid-phase matrix. The glutathione-Sepharose beads were used as a matrix for immobilization of the fusion protein through the use of the GST moiety. Then the PDE activity which is modulated by immobilized CaM was investigated. A

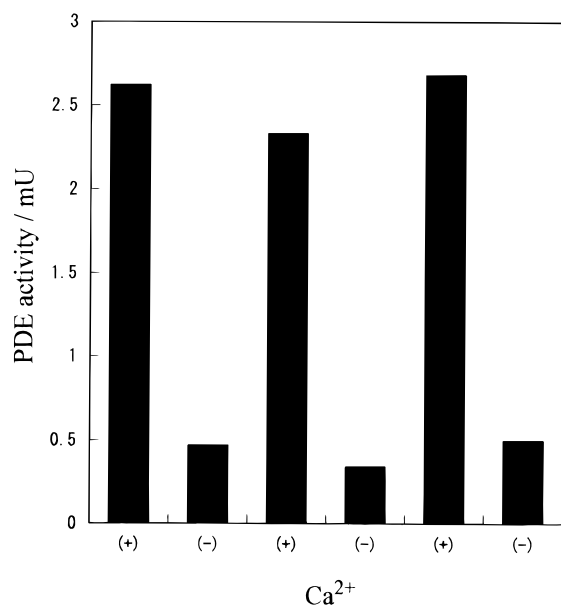


Figure 5. PDE activation by GST-CaM fusion protein in immobilized form. The fusion protein on the solid-phase matrix was incubated with PDE assay mixture. The reaction mixture in the presence (+) and absence (-) of Ca^{2+} was incubated alternately, and PDE activity in each stage was determined.

solution of the glutathione beads (50 μL) which immobilized the fusion protein was used instead of a CaM solution in the assay mixture described in Experimental Procedures. After incubation for 10 min at 30 $^{\circ}\text{C}$, cAMP was added to a final concentration of 87 mM. The change in absorbance for 10 min at 265 nm was measured, and PDE activity was determined. Figure 5 shows the PDE activity with the fusion protein in the presence and in the absence of Ca^{2+} . In the presence of Ca^{2+} , the PDE activity was almost the same as that activated by free CaM (comparing with Figure 3). Then the beads were washed three times with 100 mM glycylglycine buffer (pH 7.5) containing 3 mM EGTA for chelating Ca^{2+} . After centrifugation, the beads were resuspended in 970 mL of the reaction buffer containing EGTA instead of CaCl_2 , and then a solution of cAMP was added. In this case, the PDE activity was restricted at a lower level. Then the beads were washed again with 100 mM glycylglycine buffer (pH 7.5) and suspended in a reaction buffer containing 2 mM CaCl_2 . The PDE activity was recovered to the initial level. These experiments were repeated three times. Almost the same PDE activity could be obtained in each stage of the experiment. These results suggest that the fusion protein could be used repeatedly by immobilization on a solid-phase matrix.

SUMMARY

The present paper describes an approach toward construction of a system of controlling an enzymatic activity continuously and reversibly by assembling a modulating protein. For this purpose, GST-CaM fusion protein was selected and prepared by a genetic method. The resulting fusion protein had a binding affinity to matrix-bound glutathione with GST and a modulating activity with CaM in its immobilized form. One of the CaM dependent enzymes, PDE, was activated in a reversible manner by matrix-bound CaM with or without Ca^{2+} . On-off switching of enzymatic reaction by Ca^{2+} was demonstrated. This also indicates the possibility of the repeated use of CaM for the CaM dependent enzymes. Although the work present here is a simple approach

toward a molecular assembly, the data obtained in this study will open a new field for assembly of functional protein. Such Ca^{2+} responsible molecular assembly may be useful as a bioreactor system or new intelligent materials for the delivery of drugs such as calcitonine and parathyroid hormone, which are responsible for the regulation of Ca^{2+} concentration in serum.

LITERATURE CITED

- (1) Bülow, L. (1987) Characterization of an artificial bifunctional enzyme, β -galactosidase/galactokinase, prepared by gene fusion. *Eur. J. Biochem.* 163, 443–448.
- (2) Ljungcrantz, P., Carlsson, H., Mansson, M.-O., Buckel, P., Mosbach, K., and Bülow, L. (1989) Construction of an Artificial Bifunctional Enzyme, β -Galactosidase/Galactose Dehydrogenase, Exhibiting Efficient Galactose Channeling. *Biochemistry* 28, 8786–8792.
- (3) Peterhans, A., Mecklenburg, M., Meussdoerffer, F., and Mosbach, K. (1987) A simple competitive enzyme-linked immunosorbent assay using antigen- β -galactosidase fusions. *Anal. Biochem.* 163, 470–475.
- (4) Lindblad, C., Persson, M., Bülow, L., Stahl, S., and Mosbach, K. (1987) The design of a simple competitive ELISA using human proinsulin-alkaline phosphatase conjugate prepared by gene fusion. *Biochem. Biophys. Res. Commun.* 149, 607–614.
- (5) Kobatake, E., Nishimori, Y., Ikariyama, Y., Aizawa, M., and Kato, S. (1990) Application of a fusion protein, metaprotease/protein A, to an enzyme immunoassay. *Anal. Biochem.* 186, 14–18.
- (6) Kobatake, E., Iwai, T., Ikariyama, Y., and Aizawa, M. (1993) Bioluminescent immunoassay with a protein A-luciferase fusion protein. *Anal. Biochem.* 208, 300–305.
- (7) Marston, A. O. (1986) The purification of eukariotic polypeptides synthesized in *Escherichia coli*. *Biochem. J.* 240, 1–12.
- (8) Maina, C. V., Riggs, P. D., Grandea, A. G., III, Slatko, B. E., Moran, L. S., Taigiamonte, J. A., McReynolds, L. A., and Guan, C. (1988) An *Escherichia coli* vector to express and purify foreign proteins by fusion to and separation from maltose-binding protein. *Gene* 74, 365–373.
- (9) Smith, D. B., and Johnson, K. S. (1988) Single-step purification of polypeptides expressed in *Escherichia coli* as fusions with glutathione S-transferase. *Gene* 67, 31–40.
- (10) Weetall, H. H., and Suzuki, S., Eds. (1975) *Immobilized Enzyme Technology*, Plenum Press, New York.
- (11) Babu, Y. S., Sack, J. S., Greenhough, T. J., Bugg, C. E., Means, A. R., and Cook, W. J. (1985) Three-dimensional structure of calmodulin. *Nature* 315, 37–40.
- (12) Yoshino, H., Minari, O., Matsushima, N., Ueki, T., Miyake, Y., Matsuo, T., and Izumi, Y. (1989) Calcium-induced shape change of calmodulin with mastoparan studies by solution X-ray scattering. *J. Biol. Chem.* 264, 19706–19709.
- (13) Kakiuchi, S., Hidaka, H., and Means, A. R. (1982) *Calmodulin and Intracellular Ca^{2+} Receptors*, Plenum Press, New York.
- (14) Klee, C. B., and Vanaman, T. C. (1982) Calmodulin. *Adv. Protein Chem.* 35, 213–321.
- (15) Watterson, D. M., and Vanaman, T. C. (1976) Affinity chromatography purification of a cyclic nucleotide phosphodiesterase using immobilized molecular protein, a troponin c-like protein from brain. *Biochem. Biophys. Res. Commun.* 73, 40–46.
- (16) Klee, C. B., and Krinks, M. H. (1978) Purification of Cyclic 3',5'-Nucleotide Phosphodiesterase Inhibitory Protein by Affinity Chromatography on Activator Protein Coupled to Sepharose. *Biochemistry* 17, 120–126.
- (17) Kincaid, R. L., and Vaughan, M. (1983) Affinity Chromatography of Brain Nucleotide Phosphodiesterase Using 3-(2-Pyridyldithio)propionyl-Substituted Calmodulin Linked to Thiol-Sepharose. *Biochemistry* 22, 826–830.

- (18) Damrongchai, N., Kobatake, E., Haruyama, T., Ikariyama, Y., and Aizawa, M. (1995) Calcium Responsive Two-Dimensional Molecular Assembling of Lipid-Conjugated Calmodulin. *Bioconjugate Chem.* 6, 264–268.
- (19) Kato, S., Sekine, S., Oh, S.-W., Kim, N.-S., Umezawa, Y., Abe, N., Yokoyama-Kobayashi, M., and Aoki, T. (1994) Construction of a human full-length cDNA bank. *Gene* 150, 243–250.
- (20) Nagai, K., and Thogersen, H. C. (1984) Generation of β -globin by sequence-specific proteolysis of a hybrid protein produced in *Escherichia coli*. *Nature* 309, 810–812.
- (21) Kakiuchi, S., and Yamazaki, R. (1970) Calcium dependent phosphodiesterase activity and its activating factor (PAF) from brain: studies on cyclic 3',5'-nucleotide phosphodiesterase (III). *Biochem. Biophys. Res. Commun.* 41, 1104–1110.
- (22) Schiefer, S. (1985) In *Methods of Enzymatic Analysis*, Vol. 9, pp 317–331, VCH, Deerfield Beach, FL, Weinheim, Germany.

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A Hepatocyte-Targeted Conjugate Capable of Delivering Biologically Active Colchicine *in Vitro*

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A derivative of colchicine was synthesized, in a manner that preserved its important structural features, and conjugated to an asialoglycoprotein. The conjugate was characterized by ultraviolet–visible spectrophotometry and protein analysis. An average coupling ratio of 2 mol of colchicine per mole of asialoglycoprotein was achieved. The conjugate was stable to incubation in serum but was split into its separate components under chemically reducing conditions. Incubation with cells in culture revealed that the conjugate had antiproliferative activity similar to that of colchicine, but only in asialoglycoprotein receptor-containing cells. There was no effect at all on asialoglycoprotein receptor (–) cells. Furthermore, the antiproliferative effect of the conjugate on receptor (+) cells was blocked by addition of a large molar excess of free asialoglycoprotein. Immunofluorescence microscopy revealed disruption of microtubules in cell cultures that were pretreated with the conjugate. These results indicate that a colchicine conjugate that is taken up specifically into cells by asialoglycoprotein receptors and released intracellularly in a biologically active form can be prepared.

INTRODUCTION

Colchicine is an old drug that continues to find new uses (1). As early as the first century AD, extracts of the plant *Colchicum lingulatum* were recommended to alleviate the pain of arthritis. In the eighteenth century, a beneficial effect from the plant on gout was identified, and use of the plant's active component, colchicine, has continued for this purpose to the present day.

The ability of colchicine to bind tubulin and inhibit microtubule formation lies at the heart of its biological activity (2). Disruption of microtubule formation leads to the arrest of mitosis and interferes with the normal trafficking of endocytotic vesicles. In addition to clinical utility, these properties have rendered the drug useful as a mechanistic probe, for example, in the study of endocytosis (3, 4). However, applications of colchicine are limited by the drug's toxicity (5).

It has been shown that delivery of drugs via cell surface receptors can enhance their efficacy by 1 or more orders of magnitude (6, 7). Because receptor-mediated delivery could enhance the concentration of the drug in a particular target tissue, it might be possible to use less drug as a receptor-targeted conjugate and yet obtain a comparable therapeutic benefit. Toxicity would be mitigated. In the case of colchicine, from a chemical standpoint, functional groups that could be easily manipulated to couple colchicine to a glycoprotein are not present. Here,

we describe the synthesis of a conjugate of a colchicine derivative with an asialoglycoprotein and report a biological effect specific for asialoglycoprotein receptor (ASGPr)¹-bearing cells.

EXPERIMENTAL PROCEDURES

Chemistry. General. Ultraviolet–visible (UV–vis) spectra were acquired with samples in pure water on a Beckman DU70 spectrophotometer. NMR spectra were acquired on a Nicolet NT360 spectrometer. High-resolution mass spectra were obtained on a JEOL SX102A mass spectrometer with a JEOL FAB ion source. All reagents were of the highest quality commercially available.

Deacetylcolchicines. To a solution of trimethylcolchicinic acid (8) (0.85 g, 2.4 mmol) in chloroform (170 mL) were added methanol (17 mL) and (trimethylsilyl)diazomethane (2.0 M in hexane) (10 mL, 63 mmol). After the mixture was stirred for 3 h at 23 °C, volatiles were removed *in vacuo*. Chromatography on flash silica gel with 6% methanol/chloroform as eluent afforded the combined isomeric mixture (0.72 g, 80%): TLC (20% methanol/chloroform) R_f 0.67; UV–vis $\lambda_{\max}$ 351, 245 nm; UV–vis $\lambda_{\min}$ 291 nm.

N-[3-(2-Pyridyldithio)propionyl]deacetylcolchicines (5 and 6). To deacetylcolchicine (192 mg, 0.537 mmol) in dichloromethane (2 mL) were added SPDP (167 mg, 0.537 mmol) and DMAP (65.0 mg, 0.537 mmol). The solution was stirred for 2 h at 23 °C. The solution was then flash chromatographed on silica gel with 6% methanol/chloroform as eluent to yield **5** (119 mg, 40%) and **6** (136 mg, 46%): TLC (20% methanol/chloroform) **5** R_f 0.73, **6** R_f 0.81.

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¹ Abbreviations: ASGPr, asialoglycoprotein receptor; SPDP, N-succinimidyl 3-(2-pyridyldithio)propionate; DMAP, dimethylaminopyridine; ASOR, asialoorosomucoid; PDP-ASOR, N-3-(2-pyridyldithio)propionyl-ASOR; HEPES, N-(2-hydroxyethyl)piperazine-N'-2-ethanesulfonic acid; colchicine-SS-ASOR, conjugate of compound **7** disulfide linked to ASOR; PBS, phosphate-buffered saline [0.1 M phosphate (pH 7.2) and 0.15 M NaCl]; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol.

Analysis for **5**: $^1\text{H-NMR}$ (CDCl_3) δ 1.87, 2.30, 2.46, 2.53 (4m, 4H, $\text{H}_{5,5',6,6'}$), 2.67 (q, $J = 6.12$ Hz, 2H, NHCOCH_2), 3.03 (m, 2H, CH_2S), 3.65, 3.90, 3.94, 3.98 (4s, 12H, 4 $\times$ OCH_3), 4.68 (m, 1H, H_7), 6.53 (s, 1H, H_4), 6.80 (d, $J = 10.8$ Hz, 1H, H_{11}), 7.15 (m, 1H, pyridyl H_3), 7.30 (m, 1H, NH), 7.44 (s, 1H, H_8), 7.56 (d, $J = 8.2$ Hz, 1H, H_{12}), 7.63 (m, 2H, pyridyl $\text{H}_{4,5}$), 8.50 (d, $J = 4.4$ Hz, 1H, pyridyl H_6); $^{13}\text{C-NMR}$ (CDCl_3) δ 29.90, 35.34, 35.84, 37.07, 52.29, 56.08, 56.29, 61.39, 61.47, 107.28, 111.98, 120.57, 121.24, 125.69, 130.91, 133.99, 134.96, 135.94, 137.06, 141.65, 149.60, 150.54, 151.20, 153.38, 159.20, 163.97, 170.36, 179.38; FAB-HRMS calcd for $\text{C}_{28}\text{H}_{30}\text{N}_2\text{O}_6\text{S}_2$ [$\text{M} + \text{H}$] $^+$ 555.1624, found 555.1641.

Analysis for **6**: $^1\text{H-NMR}$ (CDCl_3) δ 2.17, 2.42, 2.43, 2.67 (4m, 4H, $\text{H}_{5,5',6,6'}$), 2.70 (m, 2H, NHCOCH_2), 3.03 (m, 2H, CH_2S), 3.67, 3.88, 3.90, 3.94 (4s, 12H, 4 $\times$ OCH_3), 4.68 (m, 1H, H_7), 6.57 (s, 1H, H_4), 7.08 (s, 1H, H_8), 7.16 (d, $J = 12.9$ Hz, 1H, H_{11}), 7.19 (m, 1H, pyridyl H_3), 7.42 (d, $J = 12.8$ Hz, 1H, H_{12}), 7.54 (m, 1H, NH), 7.63 (m, 2H, pyridyl $\text{H}_{4,5}$), 8.50 (d, $J = 3.96$ Hz, 1H, pyridyl H_6); FAB-HRMS calcd for $\text{C}_{28}\text{H}_{30}\text{N}_2\text{O}_6\text{S}_2$ [$\text{M} + \text{H}$] $^+$ 555.1624, found 555.1614.

N-[3-(2-Pyridyldithio)propionyl]-ASOR (PDP-ASOR). To 80 mg of ASOR in 1.0 mL of HEPES (0.1 M, pH 7.5) was added 50 mg of SPDP in 0.20 mL of dimethyl sulfoxide (DMSO). The mixture was stirred vigorously for 2 h at 0–4 °C and then microfuged at 3000 rpm for 10 min. The supernatant was chromatographed on a Sephadex G-25 column with sodium acetate and sodium chloride (0.1 M, pH 4.5). The macromolecular fraction was dialyzed against water (3 $\times$ 6 L) through Spectra/Por membranes with 12000–14000 MW exclusion limits and lyophilized.

Colchicine-SS-ASOR Conjugate. *Method 1.* Compound **5** (22 mg, 0.39 mmol) in methylene chloride (2 mL) was stirred with DTT (120 mg, 0.77 mmol) for 3 h at 23 °C. The resulting solution was chromatographed on flash silica gel with degassed 6% methanol/chloroform as eluent to afford a pale yellow solid (9 mg, 51%): TLC (6% methanol/chloroform) R_f 0.20; UV-vis λ_{max} 369, 292 nm; UV-vis λ_{min} 315 nm. This material (putative compound **7**) (9 mg) was dissolved in degassed PBS containing 1 mM EDTA and 0.02% sodium azide (0.2 mL) and added dropwise to a solution of the lyophilized PDP-ASOR (80 mg), in PBS (1.5 mL). The mixture was stirred initially at 0–4 °C and then at 23 °C for 17 h.

Method 2. The PDP-ASOR (80 mg) was redissolved in sodium acetate and sodium chloride (0.1 M, pH 4.5) (2.0 mL). DTT (48 mg) in 0.30 mL of the same buffer was added, and the solution was stirred for 30 min at 23 °C. The solution was chromatographed on a Sephadex G-25 column with degassed PBS containing 1 mM EDTA and 0.02% sodium azide. To the macromolecular fraction (10 mL) was slowly added **5** (20 mg) dissolved in 1.0 mL of DMSO. The mixture was stirred initially at 0–4 °C and then at 23 °C for 17 h. *N*-Ethylmaleimide (2 mg) was added, and the mixture was stirred for an additional 1 h.

For Both Methods. The mixture was then microfuged at 5000 rpm for 10 min and the supernatant chromatographed on a Sephadex G-25 column with PBS as eluent. The macromolecular fraction was dialyzed against water (3 $\times$ 6 L) through membranes with 12000–14000 MW exclusion limits and lyophilized. The resulting products were analyzed for colchicine by measuring the absorbance at 350 nm [$\epsilon_{350} = 1.7 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$; literature value (9), $\epsilon_{350} = 1.6 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$] and for protein with the BioRad protein assay. Purity was assessed by nonre-

ducing sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) on a 4 to 20% gradient gel, stained with Coomassie Blue.

Stability of the Conjugate. Silica gel TLC was used to monitor the conjugate for liberation of drug under various conditions. Samples were prepared by incubation of colchicine-SS-ASOR (33 mg/mL colchicine) in the appropriate buffer for 1 h at 25 °C. An aliquot (1 μL) of each solution was spotted onto a silica gel (Si250F, Baker) TLC plate. The plate was allowed to dry and then was eluted with 10% methanol/chloroform. Visualization was by UV absorbance.

Biology. Cells and Cell Culture. Human hepatoma, Huh7, asialoglycoprotein receptor (+) cells (10) and SK Hep1 receptor (–) cells (11) were grown in plastic dishes containing Dulbecco's modified Eagle's medium and 10% fetal calf serum (GIBCO/BRL, Gaithersburg, MD) at 37 °C under 5% CO_2 as described previously.

Proliferation Assays. To compare the antimetabolic effects of colchicine conjugate and free colchicine, Huh7 and SK Hep1 cells at a density of 1×10^5 cells/35 mm dish were incubated for 1 h in medium containing 0.25 μM free colchicine, or 0.25 μM colchicine-SS-ASOR at 37 °C, but without serum. At the end of the incubation, medium was removed and replaced with medium containing 5% fetal bovine serum. At 24 and 48 h, medium was removed from groups of dishes, and the cells were harvested by exposure to trypsin/EDTA solution (GIBCO/BRL). The number of viable cells per dish was determined by counting microscopically cells capable of excluding trypan blue. For all competition experiments, colchicine conjugate was mixed with an excess of free ASOR, 100-fold molar over the amount of ASOR component present in the conjugate. All assays were performed in triplicate and the results expressed as means $\pm$ standard deviation.

Imaging of Microtubules. For immunofluorescence studies, Huh7 cells were grown in Nunc Lab-Tek four-chamber tissue culture plastic plates in RPMI medium supplemented with 10% fetal calf serum, 5 $\mu\text{g/mL}$ insulin, 10^{-7} M dexamethasone, and 10 $\mu\text{g/mL}$ transferrin at 37 °C in a 5% CO_2 atmosphere. When the cells grew to subconfluence, the colchicine conjugate (dissolved in RPMI) was added to the media at 0 (control), 0.1, or 0.25 μM . After 3 h, the colchicine was removed by washing the cells three times with fresh media, and the cells were cultured for 16 h before immunofluorescence analysis as follows. The cells were washed twice with TRIS-buffered saline and permeabilized by exposure to 0.15% Triton X-100 for 15 min at 25 °C. The cells were then fixed with 3% formaldehyde for 10 min at 25 °C and rinsed with TRIS-buffered saline in 10% normal goat serum. The cells were treated with anti- β -tubulin (Sigma, St. Louis, MO), diluted to 1:200 for 30 min at 37 °C, followed by goat anti-mouse IgG coupled to the fluorescent marker Cy-3 (Biological Detection Systems, Pittsburgh, PA), and diluted to 1:100. The cells were examined with an Olympus fluorescence/phase microscope, provided with a rhodamine-type filter, using a 40 $\times$ objective.

RESULTS AND DISCUSSION

Colchicine (**1**) does not possess any molecular functionality that is easily amenable to elaboration into a protein conjugate. It has generally been found that the specific structural features of the A and C rings of colchicine are critical for retention of the drug's tubulin-binding and antimetabolic properties (12–17). Most sig-

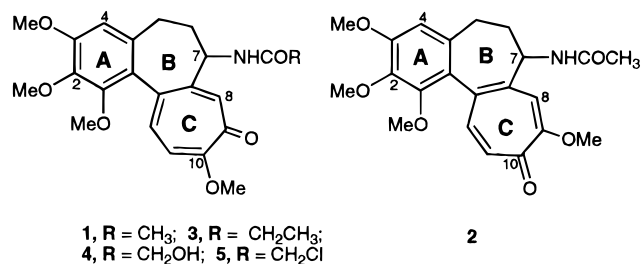


Figure 1. Structures of colchicine and derivatives.

nificantly, isocolchicine (**2**) is largely devoid of any biological activity (17). Substitution of the B ring is better tolerated, although the nature of substituents at C7 affects tubulin-binding kinetics (16). High-affinity binding (16) and toxicity (14) are favored by N7 acyl substituents, and those that possess electron-rich functionality maintain good bioactivity (13, 15). Steric bulk and aromatic substituents at N7 cause a reduction in tubulin-binding affinity and antiproliferative activity (15). Figure 1 shows several colchicine analogs which display potency comparable to that of colchicine. Assessment of this data suggested that a colchicine analog with structure **7** (Scheme 1) was likely to possess favorable tubulin-binding and antimitotic properties. In addition, **7** was amenable to formation of a protein conjugate using standard disulfide cross-linking chemistry (18).

Colchicine was hydrolyzed to trimethylcolchicinic acid following the procedure of Raffauf *et al.* (8) and remethylated with (trimethylsilyl)diazomethane (19) to obtain an intractable isomeric mixture of deacetylcolchicine/deacetyl-isocolchicine. This material could be reacylated to produce colchicine and isocolchicine in an approximate 1:1 ratio. Given that the isomeric ratio is determined at the methylation step, the use of (trimethylsilyl)diazomethane appears superior to use of diazomethane, which afforded a 25:75 ratio of deacetylcolchicine/isodeacetylcolchicine (20). The isomeric mixture was acylated (Scheme 1) with SPDP to afford the chromatographically separable isomers **5** (40%) and **6** (46%). The correct structure for each isomer was deduced by characteristic ¹H-NMR signals. The α -keto, H-8 proton of colchicine appears as a singlet at 7.48 ppm. For isocolchicine, this proton is α to the methoxy group and is shifted upfield to 6.96 ppm. By analogy, H-8 for **5** appears as a clearly distinguished singlet at 7.44 ppm, while H-8 for **6** appears at 7.08 ppm.

Two strategies (Scheme 1) for coupling the drug to ASOR were envisioned. In both approaches, ASOR was first derivatized with SPDP. In the first approach, the thiopyridyl group of **5** was to be reductively removed, giving the free sulfhydryl derivative **7**. This compound would combine with PDP-ASOR to form the disulfide-linked conjugate (colchicine-SS-ASOR) through displacement of the thiopyridyl group from PDP-ASOR. In the alternative approach, PDP-ASOR was treated with dithiothreitol to generate free sulfhydryl groups on the protein. This species was then combined with compound **5** to form colchicine-SS-ASOR by displacement of the thiopyridyl group from **5**.

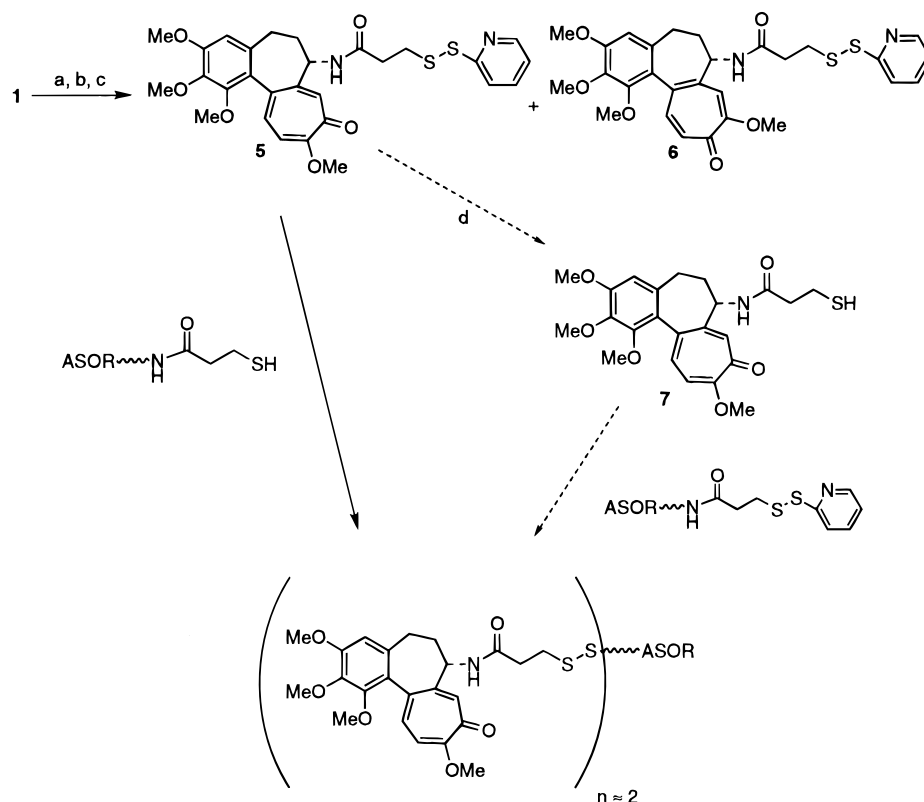
Figure 2 shows an overlay of the UV-vis absorption spectra of **1**, ASOR, and colchicine-SS-ASOR. The conjugate possesses absorption maxima that corresponded to those of both the protein (280 nm) and the drug (350 nm). Colchicine content was calculated from the absorbance at 350 nm. From the protein concentration assay, the molar coupling ratios of colchicine to protein were determined. The first procedure resulted

in less than 1 mol of colchicine per mole of ASOR. ¹H-NMR analysis of the product of reaction of **5** with DTT revealed a likely reason for the failure of the first approach. The silica gel-purified reaction product was contaminated with a significant amount of DTT. Neither changing the stoichiometry of DTT in the reaction nor the use of different solvent systems for chromatography cleanly afforded compound **7**. In light of these difficulties, this strategy was abandoned. The second procedure afforded conjugates with an average of 2 mol of colchicine per mole of ASOR. A concern with this procedure was that it might produce cross-linked protein in addition to the desired conjugate. However, nonreducing SDS-PAGE of conjugates prepared by this method revealed a single major band with an apparent mass of 40 kDa, indicating that oxidative cross-linking of the sulfhydryl-derivatized ASOR did not occur to any significant extent. This synthetic strategy was used for all subsequent preparations.

The conjugate was examined by silica gel TLC analysis for stability under various chemical and physiological conditions. Lane 6 of Figure 3 shows that free colchicine derivative (putative compound **7**), obtained by treatment of **5** with DTT, migrated with $R_f \approx 0.5$ on silica gel with 10% methanol/chloroform eluent. Exposure of conjugate to DTT (lane 4) resulted in the appearance of two spots: one which comigrated with compound **7** (see lane 5) and a second lower R_f spot corresponding to DTT, whose migration position is demonstrated in lane 7. Exposure of conjugate for 1 h to neutral conditions (lane 2), acidic conditions (lane 3), or serum (lane 1) failed to liberate any detectable colchicine derivative. Because ASOR has been shown to be essentially cleared from circulation by the ASGPr 15 min after iv injection (21), the latter result suggests that iv injection of conjugate *in vivo* is not likely to release the drug prior to internalization by hepatocytes. Liberation of **7** with DTT is consistent with the anticipated behavior of disulfide-linked bioconjugates (4, 22-24). This supports the expectation that reductive release of the species **7** within endosomes is a likely mechanism for delivery of a biologically active colchicine derivative.

To assess receptor specificity and establish biological efficacy, the ability of colchicine-SS-ASOR to inhibit cell proliferation was studied in a cell line, Huh7, which is asialoglycoprotein receptor (+), and a cell line, SK Hep1, which is asialoglycoprotein receptor (-). In Figure 4, panels A and B show that, at a concentration of 0.25 μ M free colchicine, there was no increase in the number of viable cells in either cell type. In contrast, panel A shows that conjugate had no effect on SK Hep1 cells as these cells increased in viable cell number from 3.7×10^3 to 1.2×10^4 over 48 h. On the other hand, unlike the results in SK Hep1 cells, panel B shows that Huh7 cells exposed to conjugate were inhibited in proliferation to nearly the same extent as free colchicine. The inhibitory effect was completely blocked by coaddition of excess ASOR during the incubation, indicating that the effects were mediated by the asialoglycoprotein receptor. Free colchicine inhibited proliferation of both cell lines in a similar dose-dependent manner, and addition of excess ASOR to free colchicine had no effect on cellular proliferation (data not shown).

The effect of colchicine-SS-ASOR on microtubules was examined by immunofluorescence. Control cells showed a delicate and extensive microtubular network extending to the cell periphery (Figure 5A). All cells in the field, as visualized by phase-contrast microscopy (A'),

Scheme 1. Synthesis of Colchicine–Asialoglycoprotein Conjugate^a

^a Key: (a) 25% H_2SO_4 , Δ ; (b) TMSCH_2N_2 , $\text{CHCl}_3/\text{MeOH}$; (c) SPDP, Et_3N , CH_2Cl_2 ; (d) DTT, CH_2Cl_2 .

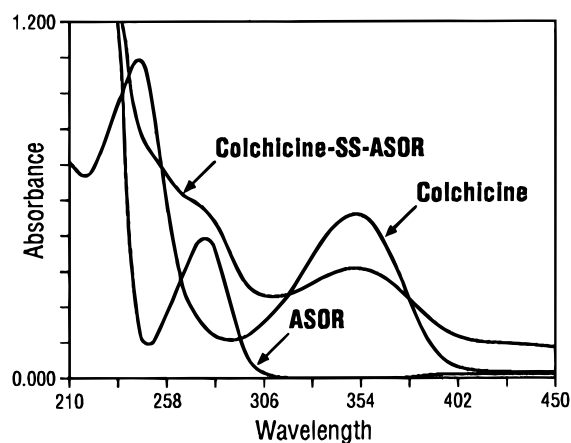


Figure 2. UV–vis spectra of colchicine, ASOR, and colchicine–SS–ASOR.

showed uniform immunofluorescence staining. Cells exposed to 0.1 μM conjugate (B and B') also exhibited positive staining in all cells, but the microtubular network was partly disrupted as indicated by larger spaces around the microtubular strands. The residual microtubules are seen radiating from the microtubule-organizing centers (centrosomes). Exposure to 0.25 μM colchicine–SS–ASOR (C and C') resulted in complete loss of tubulin immunofluorescence in many cells. In the remaining cells, microtubules were sparse and disorganized. This effect correlates with the antiproliferative activity of the conjugate and supports the hypothesis that a microtubule-disrupting mechanism, similar to that of free colchicine (2), is responsible for the biological activities of the conjugate.

Natural asialoglycoproteins such as asialofetuin and asialoorosomucoid as well as neoasialoglycoproteins such as lactosylated albumin and galactosylated polylysine

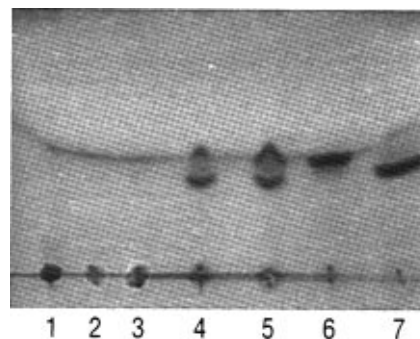


Figure 3. Stability of colchicine–SS–ASOR. A colchicine conjugate containing 2 molecules of colchicine per molecule of ASOR was incubated under various conditions and analyzed by TLC as described in Experimental Procedures: conjugate incubated in fresh mouse serum, lane 1; conjugate in 0.1 M HEPES (pH 7.0), lane 2; conjugate in 0.1 M sodium acetate and NaCl (pH 5.0), lane 3; conjugate plus 5 M DTT, lane 4; cospot (lanes 4 and 6), lane 5; compound 7, lane 6; and 5 M DTT alone, lane 7.

have been used previously as carriers for delivery of drug and other therapeutic agents (6, 25–28). It has become clear that consideration must be given to preserve receptor specificity and to present the therapeutic agent within the cell in an active form following endocytosis.

Studies on targeting adenine arabinoside and acyclovir to the liver have served to delineate some of the parameters necessary for a successful outcome of receptor-mediated drug delivery via asialoglycoproteins. Early investigations determined that the nature of the cross-linker had a significant effect on the potency of the conjugate. Fiume *et al.* prepared conjugates from nucleoside analog monophosphates. These conjugates displayed increased potency over conjugates prepared from nucleoside analog glutarates (29). Subsequently, it was shown that the nucleoside analog monophosphates were liber-

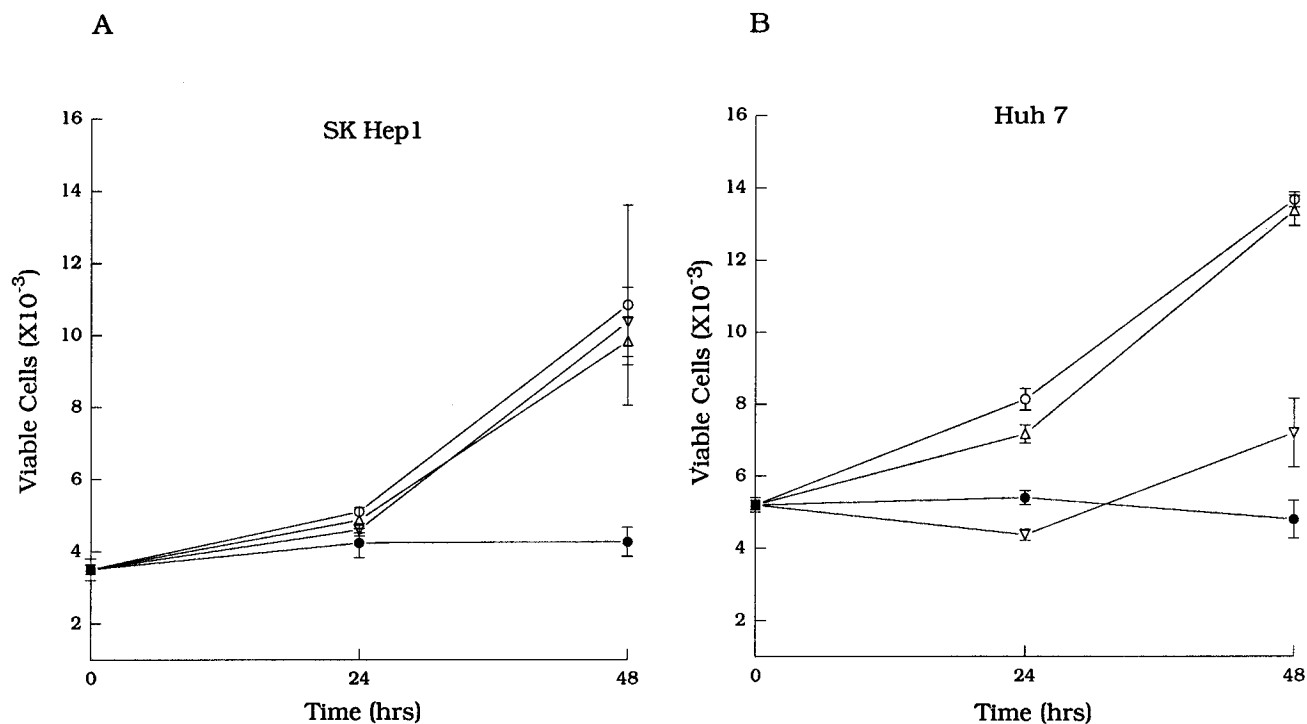


Figure 4. Antiproliferative activity of colchicine and colchicine-SS-ASOR conjugates *in vitro*. (A) SK Hep1, asialoglycoprotein receptor (−) cells; and (B) Huh 7, asialoglycoprotein receptor (+) cells. Untreated control (○), 0.25 μ M colchicine-SS-ASOR conjugate (▽), 0.25 μ M colchicine-SS-ASOR + 100 $\times$ ASOR (△), and 0.25 μ M colchicine alone (●).

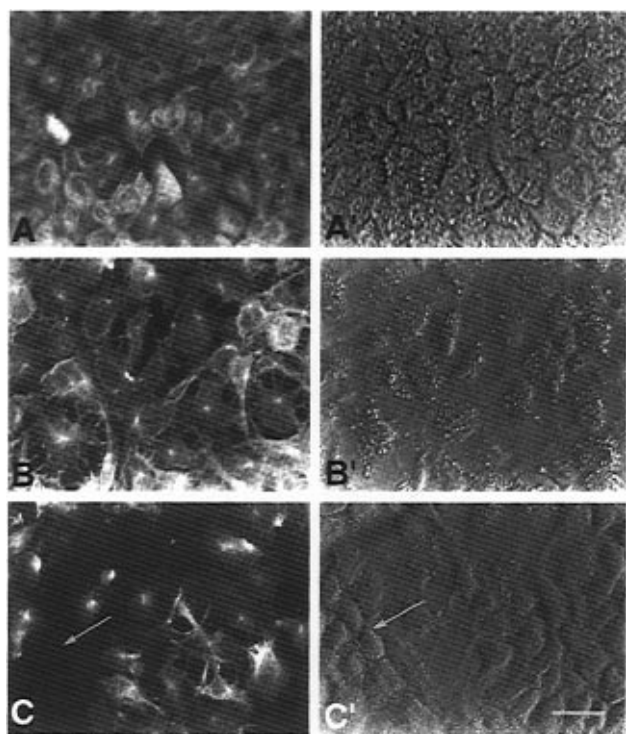


Figure 5. β -Tubulin immunofluorescence staining. HuH7 cells were exposed to 0 (A and A'), 0.1 (B and B'), or 0.25 μ M (C and C') colchicine-SS-ASOR for 3 h and then cultured for 16 h. Immunofluorescence staining was performed as described in Experimental Procedures. Panels A–C show immunofluorescence staining, and panels A'–C' show Nomarsky views of the corresponding fields. Arrows in panel C point to some cells which show no immunofluorescence. The white horizontal bar in panel C' indicates 40 μ m.

ated from the conjugate intracellularly (30) and were perhaps retained in the cell better than unphosphorylated drug. Furthermore, it was postulated that immediate intracellular availability of the nucleoside analog

phosphate resulted in more efficient conversion to the active species, bypassing the need for enzymatic phosphorylation. Conjugates that possess sulfur-derived cross-links have also been exploited for receptor-mediated delivery of drugs (22, 23) and toxins (4, 31). Although the exact mechanism of the process is disputable (23, 24), it is generally accepted that disulfides are reducible intracellularly and, therefore, capable of being split into two sulfhydryl-containing components of a conjugate. For example, Chang and Kullberg linked diphtheria toxin fragment A to ASOR through a reductively labile disulfide. The resulting conjugate was found to be 60 times more toxic than the fragment linked to ASOR through a stable thioether (4). Our data on the antiproliferative activity of the colchicine-SS-ASOR conjugate at concentrations similar to that of free colchicine suggest that the conjugate preserves the biological activity of colchicine. In addition, with the sensitivity of the conjugate's disulfide bond to chemical reduction, these data support our hypothesis that cleavage of the disulfide linkage to the carrier protein is efficient within the cell and that escape of 7 from endocytotic vesicles to the cytosolic compartment also occurs.

Another consideration is the number and type of drug residues coupled to the carrier. For asialoglycoproteins, this is important because excessive coupling ratios have been shown to alter the recognition of the resulting conjugate *in vivo* (32). Such alterations become significant issues when neoasialoglycoproteins are employed because the receptor affinity of these constructs is frequently lower than that of natural asialoglycoproteins (33). In addition, limitations of coupling ratios are important in drug targeting where the drug in the conjugate contributes significantly to biodistribution (34). This issue was a concern with colchicine because the free drug exhibits substantial extrahepatic localization when administered as the free drug (5). The substantial activity of colchicine-SS-ASOR conjugate in the asialoglycoprotein receptor (+) cell line, the lack of effect in

receptor (–) cells, and the blockade of the effect of the conjugate by addition of excess ASOR for competition demonstrate that addition of an average of 2 molecules of the rather hydrophobic colchicine derivative per molecule of ligand did not compromise the asialoglycoprotein receptor specificity of ASOR component for hepatocyte targeting. The synthesis of conjugates containing higher ratios of drug to ligand is in progress, and future studies will evaluate their receptor specificity. These data further indicate that, even with a modest coupling efficiency, the resulting conjugate has substantial effects *in vitro* and raise the possibility of targeting microtubule disruptive agents to obtain specific effects in hepatocytes *in vivo*.

Microtubule disruption mechanisms have been shown to be involved during liver regeneration following partial hepatectomy in rats. A practical result of this observation was achievement of persistence of expression of genes transfected into hepatocytes with asialoglycoprotein–polycation gene delivery (35, 36), in which partial hepatectomies were performed after the transfections *in vivo*. Theoretically, a similar effect could be obtained pharmacologically using colchicine or a comparable agent but would require large doses that could be toxic, especially to rapidly proliferating tissues. Thus, the ability to target colchicine as described in the current study may be particularly useful in extending the duration of targeted foreign gene expression without dose-limiting extrahepatic toxicity.

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LITERATURE CITED

- (1) Malkinson, F. D. (1982) Colchicine: New uses of an old, old drug. *Arch. Dermatol.* 118, 453–457.
- (2) Hastie, S. B. (1991) Interactions of colchicine with tubulin. *Pharmacol. Ther.* 51, 377–401.
- (3) Kolset, S. O., Tollashaug, H., and Berg, T. (1979) The effects of colchicine and cytochalasin B on the uptake and degradation of asialoglycoproteins in isolated rat hepatocytes. *Exp. Cell. Res.* 122, 159–167.
- (4) Chang, T.-M., and Kullberg, D. W. (1982) Studies of the mechanism of cell intoxication by diphtheria toxin fragment A-asialoorosomucoid hybrid toxins. *J. Biol. Chem.* 257, 12563–12572.
- (5) Levy, M., Spino, M., and Read, S. E. (1991) Colchicine: A state-of-the-art review. *Pharmacotherapy* 11, 196–211.
- (6) Fiume, L., Busi, C., Stefano, G. D., and Mattioli, A. (1994) Targeting of antiviral drugs to the liver using glycoprotein carriers. *Adv. Drug Delivery Rev.* 14, 51–65.
- (7) Plourde, R., Merwin, J. R., Ernst, M. F., Robinson, B. S., Suchoski, J. L., Spitalny, G. L., Findeis, M. A., and Carmichael, E. P. (1995) Acyclovir-asialoglycoprotein conjugates are potent inhibitors of hepatitis B virus replication. *Drug Delivery* 2, 136–143.
- (8) Raffauf, R. F., Farren, A. L., and Ulyot, G. E. (1953) Colchicine. Derivatives of Trimethylcolchicinic Acid. *J. Am. Chem. Soc.* 75, 5292–5294.
- (9) Bhattacharyya, B., and Wolff, J. (1974) Promotion of fluorescence upon binding of colchicine to tubulin. *Proc. Natl. Acad. Sci. U.S.A.* 71, 2627–2631.
- (10) Liang, T. J., Makdisi, W. J., Sun, S., Hasegawa, K., Zhang, Y., Wands, J. R., Wu, C. H., and Wu, G. Y. (1993) Targeted transfection and expression of hepatitis B viral DNA in human hepatoma cells. *J. Clin. Invest.* 91, 1241–1246.
- (11) Wu, G. Y., and Wu, C. H. (1988) Evidence for Targeted Gene Delivery to Hep G2 Cells in Vitro. *Biochemistry* 27, 887–892.
- (12) Fitzgerald, T. J. (1976) Molecular features of colchicine associated with antimetabolic activity and inhibition of tubulin polymerization. *Biochem. Pharmacol.* 25, 1383–1387.
- (13) Quinn, F. R., and Beisler, J. A. (1981) Quantitative Structure–Activity Relationships of Colchicines against P388 Leukemia in Mice. *J. Med. Chem.* 24, 251–256.
- (14) Quinn, F. R., Neiman, Z., and Beisler, J. A. (1981) Toxicity Quantitative Structure–Activity Relationships of Colchicines. *J. Med. Chem.* 24, 636–639.
- (15) Brossi, A., Sharma, P. N., Atwell, L., Jacobson, A. E., Iorio, M. A., Molinari, M., and Chignell, C. F. (1983) Biological Effects of Modified Colchicines. 2. Evaluation of Catecholic Colchicines, Colchifolines, Colchicide, and Novel *N*-Acyl- and *N*-Aroyldeacetylcolchicines. *J. Med. Chem.* 26, 1365–1369.
- (16) Bhattacharyya, B., Howard, R., Maity, S. N., Brossi, A., Sharma, P. N., and Wolff, J. (1986) B ring regulation of colchicine binding kinetics and fluorescence. *Proc. Natl. Acad. Sci. U.S.A.* 83, 2052–2055.
- (17) Staretz, M. E., and Hastie, S. B. (1993) Synthesis and Tubulin Binding of Novel C-10 Analogues of Colchicine. *J. Med. Chem.* 36, 758–764.
- (18) Carlsson, J., Drevin, H., and Axen, R. (1978) Protein thiolation and reversible protein-protein conjugation. *Biochem. J.* 173, 723–737.
- (19) Hashimoto, N., Aoyama, T., and Shioiri, T. (1981) New Methods and Reagents in Organic Synthesis. 14. A Simple Efficient Preparation of Methyl Esters with Trimethylsilyldiazomethane and Its Application to Gas Chromatographic Analysis of Fatty Acids. *Chem. Pharm. Bull.* 29, 1475–1478.
- (20) Wilson, L., and Friedkin, M. (1966) The Biochemical Events of Mitosis. 1. Synthesis and Properties of Colchicine Labelled with Tritium in Its Acetyl Moiety. *Biochemistry* 5, 2463–2468.
- (21) Ashwell, G., and Morell, A. G. (1974) The role of surface carbohydrates in the hepatic recognition and transport of circulating glycoproteins. *Adv. Enzymol. Relat. Areas Mol. Biol.* 41, 99–128.
- (22) Roche, A. C., Barzilay, M., Midoux, P., Junqua, S., Sharon, N., and Monsigny, M. (1983) Endocytosis of glycoconjugates by Lewis lung carcinoma cells. *J. Cell. Biochem.* 22, 131–140.
- (23) Shen, W.-C., Ryser, H. J.-P., and LaManna, L. (1985) Disulfide spacer between methotrexate and poly(D-lysine). *J. Biol. Chem.* 260, 10905–10908.
- (24) Ramakrishnan, S. (1993) Preparation and analysis of immunotoxins. *Cytotoxic Conjugates*, pp 27–43, R. G. Landes Co., Austin, TX.
- (25) Monsigny, M., Roche, A.-C., Midoux, P., and Mayer, R. (1994) Glycoconjugates as carriers for specific delivery of therapeutic drugs and genes. *Adv. Drug Delivery Rev.* 14, 1–24.
- (26) Seymour, L. W. (1994) Soluble polymers for lectin-mediated drug targeting. *Adv. Drug Delivery Rev.* 14, 89–111.
- (27) Frese, J. J., Wu, C. H., and Wu, G. Y. (1994) Targeting genes to the liver with glycoprotein carriers. *Adv. Drug Delivery Rev.* 14, 137–152.
- (28) Biessen, E. A. L., Beuting, D. M., Vietsch, H., Bijsterbosch, M. K., and Van Berkel, T. J. C. (1994) Specific targeting of the antiviral drug 5-iodo-2'-deoxyuridine to the parenchymal liver cell using lactosylated poly-L-lysine. *J. Hepatol.* 21, 806–815.
- (29) Fiume, L., Mattioli, A., Busi, C., Balboni, P. G., Barbanti-Brodano, G., De Vries, J., Altmann, R., and Wieland, T. (1980) Selective inhibition of Ectromelia Virus DNA synthesis in hepatocytes by adenine-9- β -D-arabinofuranoside (ARA-A) and adenine-9- β -D-arabinofuranoside 5'-monophosphate (ARA-

- AMP) conjugated to asialofetuin. *FEBS Lett.* 116, 185–188.
- (30) Fiume, L., Busi, C., Spinelli, C., and Spinosa, G. (1989) A conjugate of acyclovir monophosphate with lactosaminated albumin releases the phosphorylated drug in liver cells. *Naturwissenschaften* 76, 74–76.
- (31) Bodmer, J. L., and Dean, R. T. (1985) Carrier potential of glycoproteins. *Methods Enzymol.* 112, 298–306.
- (32) Van der Sluijs, P., Braakman, I., Meijer, D. K. F., and Groothuis, G. M. M. (1988) Heterogeneous acinar localization of the asialoglycoprotein internalization system in rat hepatocytes. *Hepatology* 8, 1521–1529.
- (33) Van der Sluijs, P., and Meijer, D. K. F. (1991) Limitations on the Specificity of Targeting Asialoglycoprotein-Drug Conjugates to Hepatocytes. In *Liver Diseases: Targeted Diagnosis and Therapy Using Specific Receptors and Ligands* (G. Wu and C. Wu, Eds.) pp 235–264, Marcel Dekker, Inc., New York.
- (34) Van der Sluijs, P., Bootsma, H. P., Postema, B., Moolenaar, F., and Meijer, D. K. F. (1986) Drug targeting to the liver with lactosylated albumins: Does the glycoprotein target the drug or is the drug targeting the glycoprotein? *Hepatology* 6, 723–728.
- (35) Wu, C. H., Wilson, J. M., and Wu, G. Y. (1989) Targeting genes: Delivery and persistent expression of a foreign gene driven by mammalian regulatory elements in vivo. *J. Biol. Chem.* 264, 16985–16987.
- (36) Bommineni, V. R., Roy Chowdhury, N., Wu, G. Y., Wu, C. H., Cammer, M., Franki, N., Hays, R. M., and Roy Chowdhury, J. (1994) Depolymerization of hepatocellular microtubules after partial hepatectomy. *J. Biol. Chem.* 269, 25200–25205.

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CORRECTIONS

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A HEPATOCYTE-TARGETED CONJUGATE
CAPABLE OF DELIVERING BIOLOGICALLY
ACTIVE COLCHICINE IN VITRO

Page 133. In the legend to Figure 1, compound **5**
should be compound **8**.

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Chiral Discrimination in the Binding of Tris(phenanthroline)ruthenium(II) to Calf Thymus DNA: An Electrochemical Study

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The binding of Δ -, Λ -, and *rac*-[Ru(phen)₃]²⁺ (phen = 1,10-phenanthroline) and Δ -, Λ -, and *rac*-[Ru(bpy)₃]²⁺ (bpy = 2,2'-bipyridyl) with calf thymus DNA has been examined by cyclic and differential pulse voltammetric techniques to obtain structural insight into the noncovalent binding of the enantiomers to DNA. The insignificant shift in Ru^{II}/Ru^{III} peak potentials on the addition of DNA suggests that both the oxidized and reduced forms bind to DNA to the same extent. Interestingly, DNA selectively decreases the peak currents of Δ -[Ru(phen)₃]²⁺ but not those of the Λ -enantiomer; *rac*-[Ru(phen)₃]²⁺ exhibits an intermediate behavior, thus suggesting that the Δ -form exhibits significant selectivity for B-DNA. The binding constants (K_{2+}) and binding site sizes (*s*) have been determined from the decrease in the peak currents. The binding constant (K_{2+}) of Δ -[Ru(phen)₃]²⁺ is on the order of 10⁴ M⁻¹ which is less than that for proven intercalators. In contrast, the electrochemical behavior of all three forms of [Ru(bpy)₃]²⁺ remains almost unaffected in the presence of DNA, suggesting that the complexes might reside on the hydrophilic coat of the DNA helix.

INTRODUCTION

The study of the consequences of incorporation of stereochemistry (chirality) into small inorganic complexes that bind to DNA has received enormous attention and exploitation. Chiral octahedral ruthenium complexes containing aromatic ligands like phenanthroline (phen) and bipyridyl (bpy) have the potential as possible non-radioactive probes of DNA secondary structure (1). Various spectroscopic and photophysical techniques and other methods have been employed to understand the mode of their binding to DNA (2–8). Such a knowledge would help to identify the features of DNA, which are being recognized, and is essential for the further development of compounds of practical and sensitive probes of DNA structure. Additional thermodynamic information is needed to determine how useful the ruthenium enantiomers are as probes of DNA structure.

Satyanarayana et al. have reviewed what is known concerning the DNA binding details of the most well-studied [Ru(phen)₃]²⁺ complex (9). On the basis of electric dichroism studies, Yamagishi et al. showed that Δ - and Λ -[Ru(phen)₃]²⁺ are oriented differently relative to the DNA helix axis and that the Δ -form is bound to DNA with one of its phen ligands intercalated between the base pairs (8) while the Λ -form is not intercalated but instead is bound by an electrostatic mechanism (10). On the basis of unwinding studies (11) using closed circular DNA, Barton et al. suggested that the Δ -enantiomer possesses greater affinity for the DNA duplex. They also showed that the hypochromic effect in the absorption spectra of bound Δ - and Λ -enantiomers is small compared to those of other known intercalators and that the increase in fluorescence lifetime in the presence of DNA is higher for the Δ - than for the Λ -enantiomer. However, equilibrium dialysis and fluorescence lifetime measurements (4) showed that both intercalation and surface binding were used by both enantiomers to bind to DNA.

The recent NMR experiments (6, 12) on the interaction of ruthenium enantiomers with the oligonucleotides d(GTGCAC)₂ and d(CGCGCG)₂ led to the conclusion that the Δ -form prefers intercalation while the Λ -form prefers surface binding in the minor groove. In contrast, linear dichroism (7) and two-dimensional (2D) NMR studies (13) revealed that both enantiomers employ nonintercalative modes to interact with DNA within the minor groove.

Very recently, Chaires et al. made a reinvestigation of the interaction of [Ru(phen)₃]²⁺ with DNA (9) using absorbance and fluorescence methods, circular dichroism, viscosity, and competition dialysis and showed that both Δ - and Λ -[Ru(phen)₃]²⁺ weakly bind to DNA by a single mode. However, the two isomers had different effects on the hydrodynamic properties of DNA as measured by viscosity which revealed that they differ in their individual binding modes. The binding of both enantiomers to DNA was found to be very weak in comparison to that of proven intercalators, with binding constants of 10⁴ M⁻¹. Further, both of them have been shown to bind only weakly to DNA by a predominantly electrostatic, entropically driven binding mode. The observation of modest changes in optical properties upon binding led to the conclusion that stereoselectivity is modest.

Thus, there is controversy surrounding the DNA binding mechanism of Δ - and Λ -[Ru(phen)₃]²⁺, and so our recent interest in the quantitative evaluation of binding of copper complexes (14) to DNA using electrochemical techniques prompted us to employ this technique to understand the binding of [Ru(phen)₃]²⁺ to DNA. Voltammetric techniques have so far not been exploited for noncovalent labeling of enantioselective interaction of chiral metal complexes with DNA, though they have been successfully used recently to probe the interaction of metal chelates (15–18) and a viologen-derived compound (19) with DNA. In fact, DNA-binding complexes and ligands that possess redox activity are scarce. We report here for the first time the results of a voltammetric investigation of the stereoselective interaction of enantiomeric and racemic [Ru(phen)₃]²⁺ and [Ru(bpy)₃]²⁺ with calf thymus DNA.

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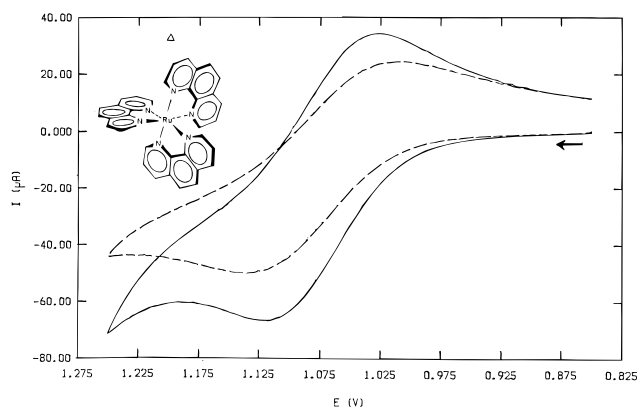


Figure 1. Cyclic voltammograms of 5.0×10^{-4} M Δ -Ru(phen) $_3^{2+}$ in the absence (—) and in the presence (---) of 12.5 mM NP: supporting electrolyte, 50 mM NaCl + 5 mM Tris-HCl (pH 7.1); and scan rate, 100 mV/s.

EXPERIMENTAL PROCEDURES

Materials. Disodium salt of calf thymus DNA (SIGMA) was stored at 4 °C and used as received. Solutions of DNA in 50 mM NaCl/5 mM Tris-HCl (pH 7.1) (buffer 1) gave a ratio of UV absorbance at 260 and 280 nm, A_{260}/A_{280} , of ≈ 1.9 , indicating that the DNA was sufficiently pure and free of protein (20). Concentrated stock solutions of DNA were prepared in 50 mM NaCl/5 mM Tris-HCl (pH 7.1), and the concentration of nucleotide phosphate was determined by UV absorbance at 260 nm on 1:100 dilutions. The extinction coefficient, ϵ_{260} , was taken as $6600 \text{ M}^{-1} \text{ cm}^{-1}$ (21). Stock solutions were stored at 4 °C and used after no more than 4 days.

Tris(phenanthroline)ruthenium(II) diperchlorate ([Ru(phen) $_3$](ClO $_4$) $_2$) (22) and tris(bipyridyl)ruthenium(II) diperchlorate ([Ru(bpy) $_3$](ClO $_4$) $_2$) (23) were synthesized and enantiomers separated (24) as described previously.

Methods and Instrumentation. All cyclic (CV) and differential pulse (DPV) voltammetry experiments were performed in a single compartment cell with a three-electrode configuration on an EG&G PAR 273 potentiostat/galvanostat equipped with an IBM PS/2 computer and a glassy carbon disk, and the reference electrode was a saturated calomel electrode. A platinum plate was used as the counter electrode. The supporting electrolyte was buffer 1. Solutions were deoxygenated by purging with nitrogen gas for 15 min prior to the measurements; during the measurements, a stream of N $_2$ was passed over the solution. All experiments were carried out at 25 ± 0.2 °C, maintained by a Haake D8-G circulating bath.

RESULTS AND DISCUSSION

Electrochemical Behavior of [Ru(phen) $_3$] $^{2+}$ and [Ru(bpy) $_3$] $^{2+}$. Typical CV behaviors of Δ - and Λ -[Ru(phen) $_3$] $^{2+}$ in buffer 1 as the supporting electrolyte are shown in Figures 1 and 2. A summary of the voltammetric results is given in Table 1. The formal potentials of the Ru(II)/Ru(III) couple, E° (or voltammetric $E_{1/2}$), taken as the average of the anodic peak potential, E_{pa} , and the cathodic peak potential, E_{pc} , determined from CV, are in good agreement with those from DPV. In DPV, the $E_{1/2}$ was determined from the peak potential, E_p , using the relationship (25a)

$$E_{1/2} = E_p + \Delta E/2 \quad (\Delta E \text{ is the pulse amplitude})$$

The enantiomers as well as the racemic form exhibited the same $E_{1/2}$ (1.074 V vs SCE) values. The CV peak potentials and ΔE_p ($E_{pa} - E_{pc}$) values were independent of scan rate over the range 0–0.5 V/s, and the ΔE_p°

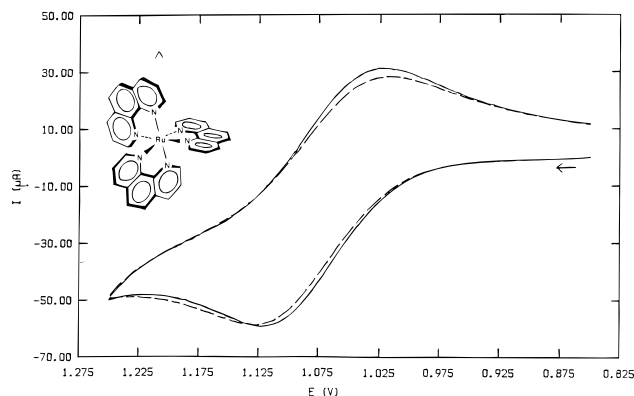


Figure 2. Cyclic voltammograms of 5.0×10^{-4} M Λ -Ru(phen) $_3^{2+}$ in the absence (—) and in the presence (---) of 12.5 mM NP: supporting electrolyte, 50 mM NaCl + 5 mM Tris-HCl (pH 7.1); and scan rate 100 mV/s.

Table 1. Voltammetric Behavior of Ru(phen) $_3^{2+}$ Enantiomers in the Presence of DNA

complex	ν (V s $^{-1}$)	R	E_{pc} (V)	E_{pa} (V)	ΔE_p (mV)	$E_{1/2}$ (V)	i_{pc}/i_{pa}	i_{pa}/i_{pa} ($R=0$)
Δ -Ru(phen) $_3^{2+}$	0.01	0	1.032	1.118	86	1.075	1.1	1
		25	1.030	1.118	88	1.074	1.0	0.72
	0.05	0	1.030	1.120	90	1.075	1.1	1
		25	1.028	1.118	90	1.073	1.0	0.73
	0.10	0	1.030	1.118	88	1.074	1.1	1
		25	1.032	1.120	88	1.076	1.0	0.71
Λ -Ru(phen) $_3^{2+}$	0.01	0	1.028	1.116	88	1.072	1.0	1
		25	1.028	1.118	90	1.073	1.0	0.98
	0.05	0	1.028	1.120	92	1.074	1.1	1
		25	1.026	1.120	94	1.073	1.0	0.98
	0.10	0	1.028	1.118	90	1.073	1.1	1
		25	1.030	1.122	88	1.076	1.0	0.96
<i>rac</i> -Ru(phen) $_3^{2+}$	0.01	0	1.028	1.118	90	1.074	1.1	1
		25	1.030	1.122	92	1.076	1.0	0.87
	0.05	0	1.032	1.122	90	1.076	1.1	1
		25	1.028	1.120	92	1.079	1.1	0.89
	0.10	0	1.032	1.120	92	1.076	1.1	1
		25	1.030	1.120	90	1.075	1.0	0.86

Table 2. Voltammetric Behavior of Ru(bpy) $_3^{2+}$ Enantiomers in the Presence of DNA

complex	ν (V s $^{-1}$)	R	E_{pc} (V)	E_{pa} (V)	ΔE_p (mV)	$E_{1/2}$ (V)	i_{pc}/i_{pa}	i_{pa}/i_{pa} ($R=0$)
Δ -Ru(bpy) $_3^{2+}$	0.01	0	1.022	1.100	78	1.061	1.04	1
		25	1.022	1.100	78	1.061	1.10	0.98
	0.05	0	1.020	1.102	82	1.061	1.09	1
		25	1.020	1.100	80	1.060	1.18	0.96
	0.10	0	1.024	1.102	78	1.063	1.17	1
		25	1.022	1.104	82	1.063	1.19	0.95
Λ -Ru(bpy) $_3^{2+}$	0.01	0	1.020	1.100	80	1.060	1.07	1
		25	1.024	1.102	78	1.063	1.09	0.94
	0.05	0	1.020	1.102	82	1.061	1.09	1
		25	1.020	1.100	80	1.060	1.10	0.96
	0.10	0	1.022	1.100	78	1.060	1.13	1
		25	1.026	1.102	76	1.064	1.16	0.97
<i>rac</i> -Ru(bpy) $_3^{2+}$	0.01	0	1.022	1.102	80	1.062	1.06	1
		25	1.022	1.100	78	1.061	1.08	0.95
	0.05	0	1.020	1.100	80	1.060	1.09	1
		25	1.024	1.104	80	1.064	1.12	0.94
	0.10	0	1.026	1.104	78	1.065	1.13	1
		25	1.024	1.102	78	1.063	1.14	0.97

(obtained by the extrapolation of ΔE_p to a zero scan rate) values are almost the same (84–88 mV) (aqueous 0.1 M NaClO $_4$ solution of 1 mM (hydroxymethyl)ferrocene under identical experimental conditions gave the ΔE_p° value of 73 mV with $E_{1/2}$ 0.176 V vs SCE).

The voltammetric results for Δ -, Λ -, and *rac*-[Ru(bpy) $_3$] $^{2+}$ are collected in Table 2. Both the enantiomers and the racemic form exhibited the same $E_{1/2}$ (1.060 V vs SCE) and ΔE_p° (76 mV) values.

The i_{pa} vs $\nu^{1/2}$ plots for all the present complexes give straight lines passing through the origin, and the ratio

of the cathodic to anodic peak currents, i_{pc}/i_{pa} , is nearly unity, suggesting that the redox systems undergo reversible one-electron transfer (25b) under the present conditions. The closeness of the latter ratio to 1 under all our experimental conditions indicates that both the 2+ and 3+ species are stable on the time scale of the CV measurements.

Redox Behavior of [Ru(phen)₃]²⁺ and [Ru(bpy)₃]²⁺ in the Presence of Calf Thymus DNA. On the addition of DNA ($R = [NP]/[Ru] = 25$) to all three forms of [Ru(phen)₃]²⁺, ΔE_p remains almost unaffected, i_{pc}/i_{pa} is nearly unity, and the shift in $E_{1/2}$ is less than 5 mV. At higher scan rates, however, a slight increase in ΔE_p is observed, possibly due to the onset of kinetic complications. The electrochemical responses of all three forms of [Ru(bpy)₃]²⁺ are also unaffected by the addition of DNA. These changes are reminiscent of small changes (9) in the magnitudes of optical properties of bound Δ - and Λ -enantiomers compared to those of other known intercalators. Further, [Ru(bpy)₃]²⁺ has been found (26) to bind to DNA but at a low salt concentration. Beyond $R = 25$, the solution became highly viscous, rendering the location of CV peaks difficult.

Analogous to the treatment of the association of small molecules with micelles (27), the net shift in $E_{1/2}$ can be used to estimate the ratio of equilibrium constants for the binding of the Ru(II) and Ru(III) complexes to DNA using the equation

$$E_b^{\circ'} - E_f^{\circ'} = 0.059 \log(K_{2+}/K_{3+})$$

where $E_b^{\circ'}$ and $E_f^{\circ'}$ are the formal potentials of the 3+/2+ couple in the free and bound forms, respectively, and K_{2+} and K_{3+} are the corresponding binding constants for the binding of 2+ and 3+ species to DNA, respectively. The ratio of binding constants of Ru(II) and Ru(III) species estimated is nearly 1 for all three forms of phen and bpy complexes, suggesting that both Ru(II) and Ru(III) forms interact with DNA to the same extent. This is in contrast to the observation (19) that viol^{2+} species is bound ca. 70 times more strongly than the viol^{+} species to DNA, indicating the importance of the electrostatic component in the interaction of the bis-9-acridinyl derivative containing a viologen linker chain with DNA.

Changes in Peak Current Values in the Presence of DNA and Determination of Binding Constants.

The addition of DNA, though it causes no change in $E_{1/2}$ and ΔE_p , diminishes the peak currents appreciably for the Ru(II)/Ru(III) couple of Δ -[Ru(phen)₃]²⁺ (Figure 1). The plot of the anodic peak current, i_{pa} , vs $\nu^{1/2}$ in the presence of DNA is linear, with the y intercept equal to zero, within the error of the measurements, as expected for a reversible electron transfer (25b). The slope of this plot decreases with an increase in R , indicating a reduction in the apparent diffusion coefficient of Δ -[Ru(phen)₃]²⁺. Thus, the slope of line A of Figure 3a ($R = 0$) gives the diffusion coefficient of free Δ -[Ru(phen)₃]²⁺, D_f , of $(3.08 \pm 0.40) \times 10^{-5} \text{ cm}^2/\text{s}$, while line B corresponds to an apparent D value of $(1.75 \pm 0.20) \times 10^{-5} \text{ cm}^2/\text{s}$ ($R = 25$). In contrast, no significant change in peak currents is observed for the Λ -isomer (Figure 2). For the racemic one, as expected, a decrease lesser than that for the Δ -isomer is observed (Figure 3b). Thus, the apparent reduction in anodic peak current for the addition of excess DNA ($R = 25$) is 26% for the Δ -isomer, while it is only 12% for the racemic one. The decrease in current upon the addition of DNA may be interpreted in terms of diffusion of an equilibrium mixture of free and DNA-bound ruthenium complex to the electrode surface. This suggests that Δ -[Ru(phen)₃]²⁺ binds to DNA more strongly

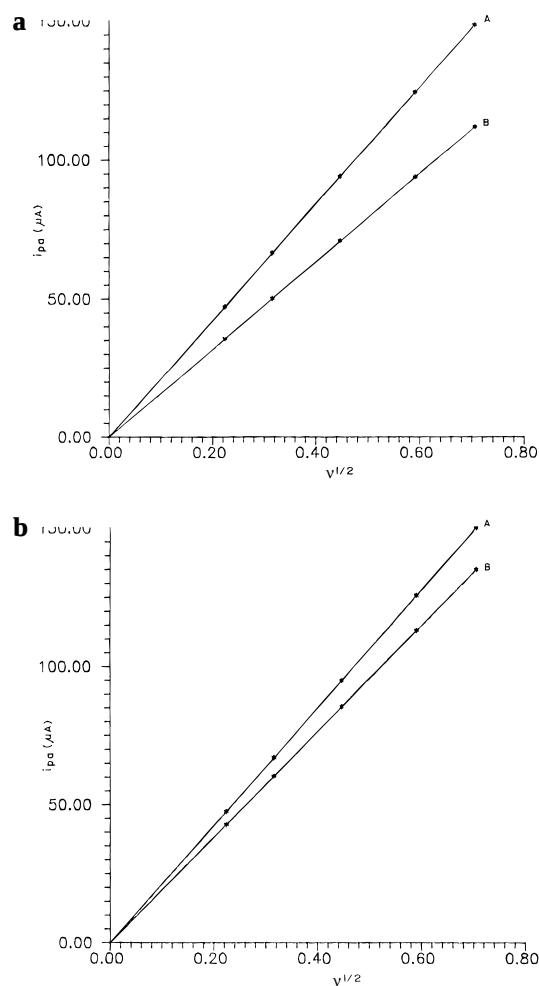


Figure 3. Effect of DNA on the diffusion of (a) Δ -Ru(phen)₃²⁺ at (A) $R = 0$ and (B) $R = 25$ and (b) $\text{rac-Ru(phen)}_3^{2+}$ at (A) $R = 0$ and (B) $R = 25$.

than the Λ -isomer. The observation of enantioselectivity is consistent with that by Barton et al. (28); for DNA-mediated long range electron transfer from photoexcited [Ru(phen)₃]²⁺ enantiomers to isostructural complexes of Co(III), Rh(III), and Cr(III) bound along the DNA strand, the rate constant for the Λ -enantiomer is from 1.5 to 2 times greater than that for the Δ -enantiomer, suggesting the preferential overall binding of the latter to DNA.

From the dependence of peak currents on the value of R (Figure 4), the binding parameters for binding of ruthenium species to DNA have been calculated (Table 3), assuming both static and mobile equilibria using the treatment given by Bard et al. (16) by

$$i_t = B(C_f D_f^{1/2} + C_b D_b^{1/2}) \quad (\text{static equilibria}) \quad (1)$$

$$i_t = B C_t (X_f D_f + X_b D_b)^{1/2} \quad (\text{mobile equilibria}) \quad (2)$$

$$B = (2.69 \times 10^5) A n^{3/2} \nu^{1/2}$$

$$C_b = [b - (b^2 - 2K^2[NP]C_t/s)^{1/2}]/2K \quad (3)$$

$$b = 1 + C_t K + K[NP]/2s$$

fitting the experimental data into eqs 1–3 by a nonlinear regression analysis, where i_t is the total anodic peak current, A is the area of the working electrode, n is the number of electrons involved in the redox reaction, ν is the scan rate, C_b is the concentration of the bound

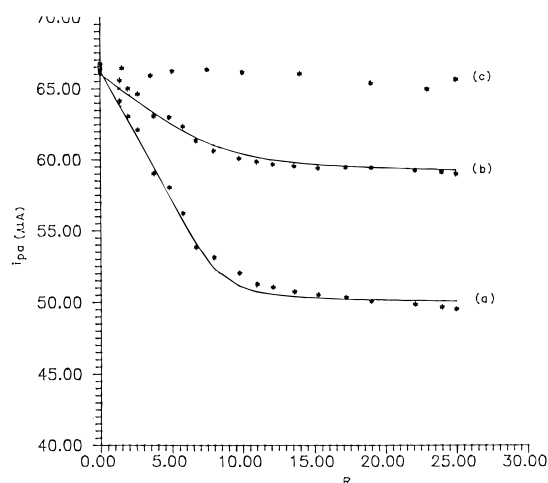


Figure 4. Voltammetric titration [measurement of i_{pa} at different R ($=[NP]/[Ru]$) values] of (a) 5×10^{-4} M Δ -Ru(phen) $_3^{2+}$, (b) 5×10^{-4} M *rac*-Ru(phen) $_3^{2+}$, and (c) 5×10^{-4} M Δ -Ru(phen) $_3^{2+}$ with CT DNA. The points represent experimental data while the solid lines the best fit for the mobile equilibrium-limiting case.

complex, C_f is the concentration of the free complex, C_t is the total concentration of the complex, $[NP]$ is the total concentration of nucleotide phosphate, and K is the binding constant for the interaction of $[Ru(phen)_3]^{2+}$ with DNA. The insignificant changes in peak current for Δ - $[Ru(phen)_3]^{2+}$ and for all the forms of $[Ru(bpy)_3]^{2+}$ did not permit the calculation of the binding parameters.

Binding Mechanism for Ruthenium Enantiomers; Δ - $[Ru(phen)_3]^{2+}$ Is a Nonclassical Intercalator. In the original work of Barton et al. utilizing luminescence and NMR methods, two major DNA binding modes are described (4, 6): one intercalative favored by Δ - $[Ru(phen)_3]^{2+}$ and the other a "surface bound" mode favored by Δ - $[Ru(phen)_3]^{2+}$. The novel EPR and time-resolved luminescence studies (29) on nitroxide-labeled Ru(II) polypyridyl complexes reveal, in addition to these two independently different modes of binding, the presence of unbound complex species in the atmospheric collar of ions, which is not entirely independent of or is loosely associated with the polyanionic DNA.

In the present study, the magnitude of the binding constant, K_{2+} , determined for the Δ -isomer is found to be greater than that for the racemic form, suggesting the preferential enantioselective interaction of the former with B-DNA. The interaction may be primarily intercalative, involving the hydrophobic interior of the DNA molecule, and it is difficult to rule out the other two modes of interaction involving the outer anionic coat of DNA. The intercalation may be partial as suggested earlier (11, 26, 30), stacking one phen in between the adjacent base pairs in the DNA helix and the remaining two phen ligands pointing outward and aligned along the right-handed major groove. Interestingly, the same binding site size ($s = 4$) is obtained for both Δ - and *rac*-forms,

supporting the involvement of the same binding mode. The values of K and s are in reasonable agreement with those obtained by optical spectroscopy ($K = 5.5 \times 10^3$ M $^{-1}$; $s = 5$ bp) (5), fluorescence measurements ($K = 4.9 \times 10^4$ M $^{-1}$) (9), electrochemiluminescence investigation ($K = 8.1 \times 10^3$ M $^{-1}$; $s = 4$ bp) (31), and DNA-melting studies ($K = 5.5 \times 10^4$ M $^{-1}$) (9) and are in the same range as those determined (16) for the corresponding Co(III) ($K = 2.6 \times 10^4$ M $^{-1}$; $s = 5$ bp) and Fe(II) ($K = 1.5 \times 10^4$ M $^{-1}$; $s = 4$ bp) complexes.

Further, the binding constants obtained here are lower than those obtained for the classical intercalators, e.g., ethidium and proflavin, compounds with proven DNA binding mode where complete insertion of the planar molecules between base pairs is possible. For example, K_+ for ethidium is 7×10^7 M $^{-1}$ in 40 mM Tris-HCl buffer (pH 7.9) (32a) and 1.4×10^6 M $^{-1}$ in 40 mM NaCl/25 mM Tris-HCl (32b), and proflavin binds with $K = 4.1 \times 10^5$ M $^{-1}$ (*Escherichia coli* DNA, 50% GC content) in 0.1 M Tris-HCl (33). The overall nonplanar structure of the present tris(phen) complexes, which contrasts with the fused ring aromatic structures of intercalating agents, make classical intercalation or groove binding either impossible or extremely unlikely. Hence, for steric reasons, these compounds cannot fully intercalate into DNA, and so binding is expected to occur by the partial insertion of a portion of the compound between the adjacent DNA base pairs. This is consistent with the molecular-modeling and energy minimization calculations (34). Thus, the present phen complexes may be considered to fall among a third class (9) of partially intercalating or nonclassical DNA binding agents.

The noncovalent interaction of one of the phen ligands with the DNA base pairs and the concomitant disposition of the remaining ligands along the groove brings the Δ - $[Ru(phen)_3]^{2+}$ complex into close contact with an environment that is hydrophobic compared to the region of the charged sugar-phosphate backbone. The interplay between electrostatic and hydrophobic (intercalative) interactions therefore can be important in the overall binding of a charged species which possesses a planar, aromatic moiety like a phen ring. Generally, there is an interplay between electrostatic and hydrophobic interactions, even in systems in which intercalation is evident. The degree to which hydrophobic interactions predominate over electrostatic ones is likely to be dictated by structural, geometric, and charge considerations for the binding molecule. Since there is no preference for either the oxidized or the reduced form of Δ - and *rac*- $[Ru(phen)_3]^{2+}$ species for DNA, charge appears to be less important and the intercalative component of binding the most important.

It is worth noting that the i_{pa} vs R plot (Figure 4) for both *rac*- and Δ - $[Ru(phen)_3]^{2+}$ levels off even at low R (≈ 10) values. This is interesting because, for the corresponding Fe(II) and Co(III) complexes, the leveling off takes place at a higher R (≈ 30) value (16). This is similar to the decrease in the hydrodynamic property (viscosity)

Table 3. Cyclic Voltammetric Titration of $Ru(phen)_3^{2+}$ with DNA^a

complex	model	experiment ^b		regression ^c			
		$10^5 D_f$ (cm ^{2} s $^{-1}$)	$10^5 D_b$ (cm ^{2} s $^{-1}$)	$10^5 D_f$ (cm ^{2} s $^{-1}$)	$10^5 D_b$ (cm ^{2} s $^{-1}$)	$10^{-4} K_{2+}$ (M $^{-1}$)	s (bp)
<i>rac</i> ⁻	static	3.11 (0.4)	2.52 (0.5)	3.05	2.41	1.3 (0.8)	4
	mobile	3.11 (0.4)	2.52 (0.5)	3.05	2.41	1.8 (1.4)	4
Δ ⁻	static	3.08 (0.4)	1.75 (0.2)	3.03	1.73	6.1 (0.6)	4
	mobile	3.08 (0.4)	1.75 (0.2)	3.03	1.73	10.4 (1.0)	4

^a D_f = free diffusion coefficient. D_b = bound diffusion coefficient. K = binding constant. s = binding site size in base pairs (bp).

^b Numbers in parentheses are standard deviations of experimental measurements. ^c Numbers in parentheses represent the 95% confidence interval of the parameter estimate from nonlinear regression.

of DNA at a low binding ratio, which is consistent with the bending or kinking of the DNA helix upon binding of the Δ - or *rac*-form to DNA by partial intercalation (9). So the decrease in peak current may be due to the involvement of the Δ -enantiomer in the formation of kinks in the DNA helix. As more kinks are formed with increased binding, a rodlike superhelical structure would result since the kinks would not be phased (35); this leads to the formation of a plateau, a stage beyond which further binding of complex is restricted. It is interesting to note that the Δ -form has been reported (9) not to encourage kinking. However, it should be pointed out that there is no direct evidence for the metal-promoted kink formation.

The Electrochemical Technique Is More Sensitive than Optical Methods for the Study of the Interaction of Redox Species with DNA. The DNA strand may be considered a local "solvent" environment as far as bound metal complex is concerned. Since the nature of the solvent can have substantial effects on electron transfer thermodynamics and since the Δ -enantiomer bound via partial intercalation differs in dielectric constant and charge distribution from the corresponding complex in bulk medium, the binding could be very easily detected by the decrease in peak currents which depend on the concentration and diffusion coefficient of the redox active species. Thus both Δ - and Λ -[Ru(phen)₃]²⁺ bind to DNA by different binding modes which consequently manifest themselves in peak currents rather than in redox potentials. On the other hand, optical changes of [Ru(phen)₃]²⁺ enantiomers upon DNA binding are unlike those observed for proven intercalators. Unlike the case of single chromophore intercalating ligands (such as ethidium bromide and daunomycin), assigning the binding mode from alterations in the absorption spectra for a compound which has three symmetry equivalent chromophores, only one of which can undergo a significant perturbation in the presence of DNA, is a much more difficult task (9). Thus, the peak currents but not the peak potentials are better estimates of the enantioselective interaction of redox active tris-chelated species than the spectroscopic techniques. Hence, the differing effect of DNA on the CV peak currents of [Ru(phen)₃]²⁺ enantiomers provides a simple and direct method to monitor binding events. Further, one would be optimistic that electrochemical techniques would be useful in studying site or base specificity; Δ -[Ru(phen)₃]²⁺ would prefer GC bp while the Λ -enantiomer AT bp, leading to a substantial decrease in peak currents. Thus, though some AT preference with the latter enantiomer has been described (4), a high level of base specificity is not expected nor has been observed so far for the weak overall binding by tris(phen)metal complexes. Further, these enantiomers are expected to discriminate against radically different conformations of DNA (i.e., B- vs Z-DNA); Δ -[Ru(phen)₃]²⁺ would selectively bind to B-DNA as in the present case while Λ -enantiomer to Z DNA, leading to appreciable decrease in peak currents; however, no evidence for enantioselective binding of the Λ -form to Z-DNA has been obtained so far.

Binding Mechanism for Ruthenium Enantiomers; Both Δ - and Λ -[Ru(bpy)₃]²⁺ Bind Electrostatically. Our observation that the peak currents of neither the racemic nor the enantiomeric forms of [Ru(bpy)₃]²⁺ are affected significantly by the addition of DNA is consistent with the findings that they do not unwind poly[d(G-C)] or poly[d(A-T)] (5) and that their excited state luminescence is rapidly quenched by ferrocyanide in the presence of DNA, as opposed to that of [Ru(phen)₃]²⁺ which is protected from quenching while intercalated into DNA

(3). Further, it has been shown that the tris-chelated metal complexes possessing bpy ligands reside primarily at the outer hydrophilic coat of DNA, predominantly via electrostatic interaction with the negatively charged deoxyribose-phosphate backbone (26, 36). The smaller size and higher nonplanarity of the bpy compared to the phen ligand preclude effective intercalation between the adjacent base pairs of DNA.

It is really surprising to see that [Ru(bpy)₃]²⁺ is not strongly bound to DNA at 50 mM NaCl; a similar observation has been made previously (3). However, at 10 mM phosphate buffer, some weak binding has been observed (26) but not precisely quantitated. Further, at an ionic strength less than 5 mM, the K_{2+} for [Ru(bpy)₃]²⁺ has been reported to be $2 \times 10^5 \text{ M}^{-1}$ (37). Other workers have reported the K_{2+} to be $3 \times 10^6 \text{ M}^{-1}$ at 1 mM NaCl and $1.4 \times 10^6 \text{ M}^{-1}$ at 10 mM NaCl (38). Similarly, [Fe(bpy)₃]²⁺ binds at 10 mM NaCl but does not at 50 mM NaCl (16). Barton et al. have also observed that at high NaCl concentrations the binding of the Ru(II)-phen complex is diminished (29). Thus, it appears that an increase in ionic strength causes a decrease in the extent of binding. The effective attraction between the oppositely charged species *viz* polyanionic DNA and the Ru(II) complex cation is known to be reduced at higher ionic strengths (39). Haq et al. have also very recently shown (40) the salt dependence of binding of [Ru(phen)₂dppz]²⁺ to DNA, the binding constant decreasing with an increase in NaCl concentration. They have used polyelectrolyte theory (41) to illustrate that the counterion release arising from an increased phosphate spacing resulting from the intercalation of the complex becomes unfavorable at high salt concentrations.

At 10 mM NaCl, the binding constant K_{2+} follows the trend [Ru(bpy)₃]²⁺ ($1.4 \times 10^6 \text{ M}^{-1}$) (38) $\gg$ [Os(bpy)₃]²⁺ ($7.3 \times 10^3 \text{ M}^{-1}$) (17) $>$ [Fe(bpy)₃]²⁺ ($1.4 \times 10^3 \text{ M}^{-1}$) (16), illustrating that the Ru(II) complex appears to exhibit selective binding with DNA. Similarly, at 50 mM NaCl, [Co(bpy)₃]³⁺ (K_{3+} , $1.4 \times 10^4 \text{ M}^{-1}$) (16) binds strongly while Ru(II) and Fe(II) complexes do not (26, 16). This is obviously because of the higher charge on and the smaller size of the Co(III) complex and illustrates the importance of the electrostatic component of binding. Since 1 equiv of a divalent ion is known to have a greater influence on DNA binding than 2 equiv of a monovalent ion (39), it is expected that the trivalent Co(III) complex would be more strongly bound than the divalent Ru(II) complex.

CONCLUSION

Our studies show that Δ -[Ru(phen)₃]²⁺ prefers to bind to DNA relatively strongly by partial rather than classical intercalation and consequently decreases the currents of redox peaks, while Λ -[Ru(phen)₃]²⁺ binds weakly by a predominantly electrostatic mode with no effect on current response. The extent of binding of *rac*-[Ru(phen)₃]²⁺ is intermediate between those of the enantiomers. In contrast, the enantiomers of [Ru(bpy)₃]²⁺ bind weakly at 50 mM NaCl but do not show any selectivity, suggesting that the middle ring in phen has a role to play possibly by facilitating partial intercalation. So we are tempted to place Δ -[Ru(phen)₃]²⁺ among the nonclassical DNA binding agents. Thus, the present study shows that it is possible to use the potentially sensitive and versatile electrochemical method as a new tool to study the enantioselective interaction of enantiomers of [Ru(phen)₃]²⁺ with DNA and hence to examine the subtle features of the polynucleotide structure.

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LITERATURE CITED

- (1) Barton, J. K. (1986) *Science* 233, 727.
- (2) Barton, J. K., Basile, L. A., Danishefsky, A., and Alexandrescu, A. (1984) *Proc. Natl. Acad. Sci. U.S.A.* 81, 1961.
- (3) Kumar, C. V., Barton, J. K., and Turro, N. J. (1985) *J. Am. Chem. Soc.* 107, 5518.
- (4) Barton, J. K., Goldberg, J. M., Kumar, C. V., and Turro, N. J. (1986) *J. Am. Chem. Soc.* 108, 2081.
- (5) Pyle, A. M., Rehmann, J. P., Meshoyrer, R., Kumar, C. V., Turro, N. J., and Barton, J. K. (1989) *J. Am. Chem. Soc.* 111, 3051.
- (6) Rehmann, J. P., and Barton, J. K. (1990) *Biochemistry* 29, 1701.
- (7) Hiort, C., Norden, B., and Rodger, A. (1990) *J. Am. Chem. Soc.* 112, 1971.
- (8) Yamagishi, A. (1983) *J. Chem. Soc., Chem. Commun.*, 572.
- (9) Satyanarayana, S., Dabrowiak, J. C., and Chaires, J. B. (1993) *Biochemistry* 32, 2573.
- (10) Yamagishi, A. (1984) *J. Phys. Chem.* 88, 5709.
- (11) Barton, J. K., Danishefsky, A. T., and Goldberg, J. M. (1984) *J. Am. Chem. Soc.* 106, 2172.
- (12) Rehmann, J. P., and Barton, J. K. (1990) *Biochemistry* 29, 1710.
- (13) Eriksson, M., Leijon, M., Hiort, C., Norden, B., and Graslund, A. (1992) *J. Am. Chem. Soc.* 114, 4933.
- (14) Mahadeven, S., and Palaniandavar, M. (manuscript under preparation).
- (15) Carter, M. T., and Bard, A. J. (1987) *J. Am. Chem. Soc.* 109, 7528.
- (16) Carter, M. T., Rodriguez, M., and Bard, A. J. (1989) *J. Am. Chem. Soc.* 111, 8901.
- (17) Rodriguez, M., and Bard, A. J. (1990) *Anal. Chem.* 62, 2658.
- (18) Rodriguez, M., Kodadek, T., Torres, M., and Bard, A. J. (1990) *Bioconjugate Chem.* 1, 123.
- (19) Takenaka, S., Ihara, T., and Takagi, M. (1990) *J. Chem. Soc., Chem. Commun.*, 1485.
- (20) Marmur, J. (1961) *J. Mol. Biol.* 3, 208.
- (21) Reichmann, M. E., Rice, S. A., Thomas, C. A., and Doty, P. (1954) *J. Am. Chem. Soc.* 76, 3047.
- (22) Dwyer, F. P., Humpoletz, J. E., and Nyholm, R. S. (1947) *J. Proc. R. Soc. N.S.W.* 81, 212.
- (23) Dwyer, F. P. (1949) *J. Proc. R. Soc. N.S.W.* 83, 134.
- (24) Gillard, R. D., and Hill, R. E. E. (1974) *J. Chem. Soc., Dalton Trans.*, 1217.
- (25) Bard, A. J., and Faulkner, L. R. (1980) *Electrochemical Methods*, (a) p 194, (b) p 219, Wiley, New York.
- (26) Kelley, J. M., Tossi, A. B., McConnell, D. J., and OhUigin, C. (1985) *Nucleic Acids Res.* 13, 6017.
- (27) Kaifer, A. E., and Bard, A. J. (1985) *J. Phys. Chem.* 89, 4876.
- (28) Purugganan, M. D., Kumar, C. V., Turro, N. J., and Barton, J. K. (1988) *Science* 241, 1645.
- (29) Ottaviani, M. F., Ghatlia, N. D., Bossmann, S. H., Barton, J. K., Durr, H., and Turro, N. J. (1992) *J. Am. Chem. Soc.* 114, 8946.
- (30) Gorner, H., Tossi, A. B., Stradowski, C., and Schulte-Frohlinde, D. (1988) *Photochem. Photobiol.* 2B, 67.
- (31) Carter, M., and Bard, A. J. (1990) *Bioconjugate Chem.* 1, 257.
- (32) (a) Waring M. (1965) *J. Mol. Biol.* 13, 269. (b) LePecq, J.-B., and Paoletti, C. (1967) *J. Mol. Biol.* 27, 87.
- (33) Baba, Y., Beatty, C. L., and Kagemoto, A. (1982) in *Biological Activities of Polymers* (C. E. Carreher, Jr., and C. G. Gebelein, Eds.) Chapter 14, pp 177–189, ACS Symposium Series 186, American Chemical Society, Washington, DC.
- (34) Haworth, I. S., Elcock, A. H., Freeman, J., Rodger, A., and Richards, W. G. (1991) *J. Biomol. Struct. Dyn.* 9, 23.
- (35) Dattagupta, N., Hogan, M., and Crothers, D. M. (1978) *Proc. Natl. Acad. Sci. U.S.A.* 75, 4286.
- (36) Stradowski, C., Gorner, H., Currell, L. J., and Schulte-Frohlinde, D. (1987) *Biopolymers* 26, 189.
- (37) Howe-Grant, M., and Lipaprd, S. J. (1979) *Biochemistry* 18, 5762.
- (38) Hard, T., and Norden, B. (1986) *Biopolymers* 25, 1209.
- (39) Manning, G. S. (1979) *Acc. Chem. Res.* 12, 443.
- (40) Haq, I., Lincoln, P., Suh, D., Norden, B., Chowdhry, B. Z., and Chaires, B. (1995) *J. Am. Chem. Soc.* 117, 4788.
- (41) Record, M. T., Anderson, C. F., and Lohman, T. M. (1978) *Q. Rev. Biophys.* 11, 103.

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An Adduct of *cis*-Diamminedichloroplatinum(II) and Poly(ethylene glycol)poly(L-lysine)–Succinate: Synthesis and Cytotoxic Properties

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A noncovalent adduct of the antineoplastic drug *cis*-diamminedichloroplatinum (cDDP) and a biocompatible graft copolymer of poly(L-lysine) and methylpoly(ethylene glycol) succinate is described. Upon incubation of cDDP with [O-methylpoly(ethylene glycol)-O'-succinyl]-N- ϵ -poly(L-lysine)_n-N- ϵ -succinate, $n = 250$ – 270 , highly soluble, long circulating adducts were formed which contained 4.3% of platinum by weight. Approximately 60% of the polymer-associated drug was released during dialysis against saline or serum albumin containing saline, with a half-time of release of 63 h. The adducts showed a pronounced antineoplastic effect in BT-20 human adenocarcinoma cell cultures. In cell proliferation assays, the concentration of half-inhibition of [³H]thymidine uptake was $0.9 \pm 0.2 \mu\text{M}$ for the drug-copolymer adduct compared to $0.3 \pm 0.1 \mu\text{M}$ for free cDDP. The adduct showed a long blood half-life (ca. 14 h in rats) and accumulated in experimental mammary adenocarcinomas at 2.5–3.5% injected dose per gram of tissue. A control adduct of cDDP with the backbone portion of the copolymer, poly(L-lysine)-N- ϵ -succinate, had a short half-life in the bloodstream (ca. 30 min) and low accumulation (0.5% injected dose per gram) in tumor. A dual therapeutical effect of methylpoly(ethylene glycol)succinylpoly(L-lysine)-succinate as a carrier of cDDP is suggested: (1) as a carrier for systemic release of the active drug from the macromolecule while it circulates in the bloodstream and (2) as a carrier for on-site delivery which results from the release of the drug in the tumor as a consequence of accumulation of the copolymer in the tumor.

INTRODUCTION

The chemotherapeutic agent *cis*-diamminedichloroplatinum(II) (cisplatin, cDDP)¹ (1) is widely used in the treatment of testicular, ovarian, and head and neck tumors (1, 2). It is especially effective in combined drug therapy against squamous cell carcinoma, transitional cell carcinoma, and small cell lung cancer (2). The antineoplastic effect of cDDP results from cross-linking of predominantly N-7 guanine atoms in DNA, resulting in intra- and interstrand cross-links. The drug is therefore most active against proliferating cells entering the G1 phase of the cell cycle (2, 3), but is also capable of inducing non-phase-specific apoptosis (4). Free drug after iv administration is cleared rapidly by binding to plasma proteins and tissue uptake and by kidney filtration. One to two hours after injection, only 10–20% of the injected drug remains in circulation. This portion of the dose is irreversibly bound to plasma proteins and, therefore, therapeutically ineffective (2, 5). Although highly efficient against many neoplasms, the therapeutic window of the drug is limited by its harmful systemic effects. The free drug fraction that is eliminated by the kidneys is nephrotoxic, and this is the main factor limiting the clinical use of cDDP (6, 7).

Attempts to reduce the toxicity of platinum(II) compounds have focused on either alteration of the structure of the drug itself (8) or combination of the unaltered drug with a carrier to reduce peak levels of the drug and to sustain its release (8, 9). Attachment of cDDP to a carrier is thought to preserve elevated local levels of cDDP while minimizing systemic toxicity (9). Various carrier macromolecules and colloids, including carboxymethylated dextrans (10–12), anionic poly(amino acids) (13), tumor-specific antibodies (14–16), microspheres (17–19), micelle-forming block copolymers (20, 21), and liposomes (22, 23), as well as local implantations of drug/carrier complexes (24) have been studied for these purposes. Unfortunately, many of the reported carriers exhibit a "burst" effect wherein the drug dissociates rapidly from the carrier and the resulting systemic toxicity is similar to that of the free drug. The release of cDDP from the drug carrier at the tumor site is essential, however, because only free platinum complexes are therapeutically effective (10). An ideal carrier would therefore be a site-directed and/or long-circulating agent capable of drug release within the tumor.

We have previously developed a long-circulating biocompatible graft copolymer and used it as a carrier for chelated gadolinium(III) for magnetic resonance imaging (25). This polymeric chelate was shown to aid in the visualization of tumor blood supply and to accumulate at 3–5% of the injected dose in tumor tissue (26, 27). We subsequently hypothesized that attachment of carboxyl groups to the carrier would promote the formation of an adduct between cDDP and the copolymer. Here we report the synthesis, purification, and characterization of the antineoplastic effects of the drug-copolymer adduct *in vitro* as well as some preliminary data *in vivo*.

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¹ Abbreviations: cDDP, cisplatin, *cis*-diamminedichloroplatinum(II); DTPA(A), cyclic diethylenetriaminepentaacetic acid (anhydride); EDC, 1-ethyl-3-(3,3-diaminopropyl)carbodiimide; MPEGs, O-methyl(polyethylene glycol) O'-succinate; PL, poly(L-lysine); BSA, bovine serum albumin; TNBS, trinitrobenzenesulfonic acid.

MATERIALS AND METHODS

Materials. 1-Ethyl-3-(3,3-diaminopropyl)carbodiimide, poly(L-lysine), bovine serum albumin fraction V, and Sephadex G-25 were obtained from Sigma Chemical Co., St. Louis, MO. Poly(ethylene glycol) 5000 monomethyl ether was purchased from Polysciences, Inc., Warrington, PA. *N*-Hydroxy(sulfo)succinimide and diethylenetriaminepentaacetic acid cyclic anhydride (DTPAA) were acquired from Pierce Chemicals, Rockford, IL. All other chemicals were obtained from Aldrich Chemical Co., Milwaukee, WI.

Copolymer Synthesis and Characterization. *O*-Methylpoly(ethylene glycol) *O'*-succinate (**I**) (Figure 1) was prepared by acylation of 75 g (15 mmol) of poly(ethylene glycol) 5000 monomethyl ether with 7.9 g (75 mmol) of succinic anhydride in the presence of 1.9 g (15 mmol) of 4-(dimethylamino)pyridine in 400 mL of dioxane at 90 °C under nitrogen for 16 h. The solvent was removed by rotary evaporation, 200 mL of chloroform was added, and the precipitate was recovered with a glass filter. Chloroform was removed, and the residue was kept for 1 h at 60 °C with a slurry of 3 g of activated charcoal in 300 mL of ethanol. Product (**I**) was recovered from the filtrate by precipitation with 500 mL of ethyl acetate, redissolved in 800 mL of ethanol, and passed through 100 g of Bio-Rad AG50 W-X8 resin to remove the catalyst. The final purification of **I** was achieved by crystallization from ethanol/ethyl acetate (2:3) at -20 °C. The product was 98% homogeneous by size-exclusion high-performance liquid chromatography (HPLC). Yield: 57%, theor. Anal. Calcd for C₂₃₁H₄₆₀O₁₁₇: C, 54.34; O, 37.09; H, 9.07. Found: C, 53.78; O, 36.7; H, 9.02.

Graft Copolymer of **I and Poly(L-lysine).** *O*-Methylpoly(ethylene glycol)-*O'*-succinyl-*N*-ε-poly(L-lysine) (**III**) was obtained by covalent conjugation of *O*-methylpoly(ethylene glycol)-*O'*-succinyl-*N*-hydroxy(sulfo)succinimide (**II**) to poly(L-lysine). Intermediate product **II** was obtained by treating **I** in the presence of water soluble carbodiimide (EDC) and *N*-hydroxy(sulfo)succinimide (25, 28). Poly-L-lysine (1 g) was dissolved in 175 mL of 0.1 M Na₂CO₃ (pH 8.7). An aliquot was removed for NH₂ group determination by TNBS titration (final concentration of NH₂ groups, 25 mM). **I** (9.6 g, 1.9 mmol) was dissolved in 25 mL of water and degassed, and *N*-hydroxy(sulfo)succinimide (500 mg, 2.3 mmol) was added, followed by 1 g (5 mmol) of EDC in 2 mL of water. The solution was incubated for 10 min at room temperature and added dropwise to the solution of poly(L-lysine), with a final pH of 7.7. The mixture was incubated overnight. The product **III** was used without purification.

[*O*-Methylpoly(ethylene glycol)-*O'*-succinyl]-*N*-ε-poly(L-lysine)_{*n*}-*N*-ε-succinate (**IV**) was obtained by addition of a 5-fold molar excess of succinic anhydride (1.13 g, 13 mmol) in 30 mL of DMSO to a solution of **III**, maintaining pH at 8.0 by addition of 2 N NaOH. After overnight incubation, no free NH₂ groups were detected. The product **IV** was purified by diafiltration. For labeling of copolymers with ¹¹¹In, approximately 1% of ε-amino groups of poly(L-lysine) were substituted by diethylenetriaminepentaacetic acid before the addition of an excess of succinic anhydride. The product was filtered through a 0.2 μm nylon filter and purified using diafiltration (YM 50 membrane, Amicon, Beverly, MA). The duration of diafiltration was determined by subjection of an aliquot of purified copolymer to size-exclusion HPLC analysis (Hydropore-5-SEC, 5 μm particle size, 4.6 mm ID × 25 cm L, Rainin Instrument Co., Inc., Woburn, MA) and eluted with 50 mM sodium phosphate (pH 6.8) at 0.5 mL/min. Yield: 1.8 g, 40% theor. Elemental analysis of **IV**: C, 52.77; N, 2.36; H, 8.94; O, 35.45.

Copolymer/Diamminedichloroplatinum(II) Adduction and Characterization. Drug carrier adducts were prepared by incubation of cDDP, (12 mg/mL DMF) at a 1:1 (v/v) ratio with a water solution of **IV** (40 mg/mL) (final concentrations, 6 and 20 mg/mL, respectively) for 24 h at 37 °C. Free cDDP was removed on 10 mL Sephadex G-25 spin columns equilibrated with water. Adduct binding studies were carried out by varying cDDP concentration and by varying the time of incubation at 10 mg of copolymer/mL. Samples were applied to size-exclusion HPLC and the first peak fractions combined, lyophilized, and measured for cDDP content. To investigate dissociation kinetics, we incubated cDDP and copolymer (6 and 20 mg/mL, respectively) for 24 h at 37 °C, adjusted the pH to 6.0 with sodium citrate solution, and further incubated the preparation with 55 μCi ¹¹¹In^{III}Cl (NEN Dupont, North Billerica, MA) for 1 h. Free cDDP and free ¹¹¹In was removed using a Sephadex G-25 spin column. The double-labeled preparation (1 mL) was dialyzed (12 kDa pore and 2.5 cm tubing, Spectrum Medical, Los Angeles, CA) in two separate portions, in one against 1 L of 0.15 M NaCl and in the other against 10 mg/mL BSA (fraction V, Calbiochem, San Diego, CA) in 0.15 M NaCl. Aliquots were withdrawn from the dialysis bag and assayed for cDDP content. Correction for the dilution effect was performed by ¹¹¹In radioactivity estimation using a γ-counter (Compugamma, LKB, Gaithersburg, MD).

Cell Culture and Cytotoxicity Studies. Human BT-20 adenocarcinoma cells (ATCC, Rockville, MD) were grown in Dulbecco's modified Eagle's medium containing 10% fetal calf serum. Free cDDP, cDDP-poly(L-lysine)-*N*-ε-succinate, or cDDP-**IV** were serially diluted with the medium and incubated with cells overnight. Cytotoxicity was determined by [³H]thymidine (ICN Radiochemicals, Irvine, CA) incorporation assay.

Animal Experiments. Rat mammary carcinomas R3230AC and 13762NF were obtained from Biomeasure Inc. (Milford, MA). Biodistribution experiments were carried out in female Fisher rats (150 g) (Charles River Laboratories, Wilmington, MA) bearing 0.4–0.6 g tumors. Animals (*n* = 3/time point) were injected intravenously with 60 μCi of ¹¹¹In-labeled cDDP-MPEGs-PL-succinate or cDDP-PL-succinate at the dose of 40 mg of polymer/kg. Animals were sedated by pentobarbital injection i.p. and subjected to nuclear imaging to visualize the accumulation of the agent in tumors 48 h after injection (γ-camera Technicare 410, Technicare, Solon, OH). Biodistribution of the copolymer was determined at 24, 48, and/or 96 h after injection by determining percent dose/g accumulated in major organs.

Analytical Procedures. The content of **I** by weight in the copolymer was determined as in Baleux (29). The platinum content of the copolymer-drug complex was determined as in Schechter et al. (10) using *o*-phenylenediamine as a ligand for Pt(II). Elemental analysis was performed by Galbraith Laboratories, Inc., Knoxville, TN. ¹H NMR study was performed on a MSL 400 spectrometer (Bruker Instruments, Billerica, MA) at 9.4 T (proton resonance frequency of 400.13 MHz). The spectra were acquired at ambient temperature using a Bruker 5 mm probe. The pulse sequence was a single read pulse (tip angle of 20°) followed by acquisition at a recycle delay of 10 s (20 scans averaged). All chemical shifts were referenced externally to tetramethylsilane (TMS).

RESULTS AND DISCUSSION

Polyanions bearing carboxyl groups, such as polyglutamate or polyaspartate, form metastable, predominantly noncovalent complexes with bifunctional aquated

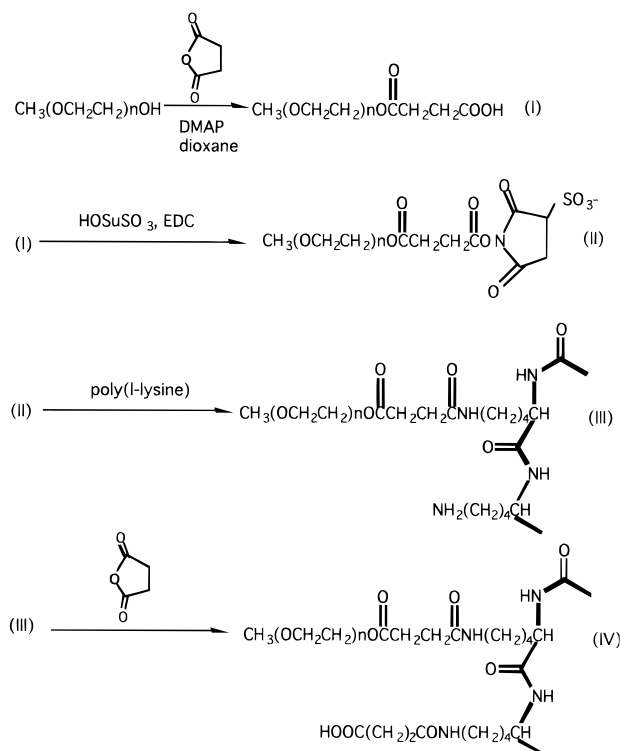


Figure 1. Scheme of graft copolymer synthesis.

coordination compounds of Pt(II). Such complexes have previously been shown to exhibit lower toxicity *in vivo* after intraperitoneal injections and an increase in the life span of mice bearing F9 teratocarcinomas in the peritoneal cavity (11, 13). The pharmacological activity of polymer-bound cDDP correlates well with the ability of cDDP to form low-affinity complexes with the polymeric drug carrier (10, 11, 13). It should be noted that accumulation of the cDDP-polymer complex in tumor tissue is necessary for a maximal antineoplastic effect, while uptake in other organs must be minimized. The graft copolymer of methylpoly(ethylene glycol) succinate (molecular weight of 5100) and poly(L-lysine), prepared by N- ϵ -acylation (IV), is nontoxic and has a lasting blood circulation (half-lives, 14 h in rats and 72 h in rabbits) (25, 30). The copolymer has been shown to accumulate efficiently in tumors, presumably due to an increased permeability of the vasculature and a reduced rate of clearance from the interstitium as a consequence of a lack of lymphatic drainage (31, 32).

We hypothesized that, for intravenous applications, a complex of cDDP with the copolymer (IV) may have advantages over complexes of cDDP with linear polyanions since the latter typically have short blood half-lives. Anticipated was a dual function of long-circulating adducts. First, they may function as a long-circulating depot slowly releasing the drug into circulation. Second, these adducts may also accumulate in tumors because of the high permeability of tumor neovascularity. We expected that together these two effects would facilitate antitumor activity of platinum(II) compounds with a simultaneous decrease of their systemic toxicity if administered intravenously. In this study, we examined cDDP-polymer binding and release, and cytotoxic properties and biodistribution (specifically tumor and kidney accumulation) of the adduct.

The synthesis of IV (Figure 1) yielded a compound with an apparent hydrodynamic radius corresponding to a protein with a molecular mass of 365 kDa by HPLC (Figure 2). The compound contained 55% I by weight.

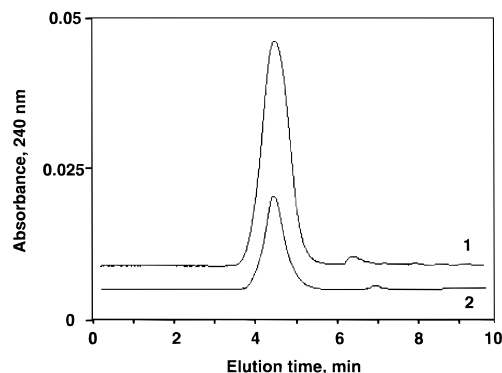


Figure 2. Size-exclusion HPLC [SEC-5, 0.4×25 cm, Rainin Inst. Co., Inc.; 50 mM sodium phosphate (pH 6.8), 0.5 mL/min] of 20 μg of cDDP adduct with the copolymer IV (trace 1) or copolymer IV alone (trace 2): detection, absorbance at 240 nm.

This indicated that a modification degree of poly(L-lysine) backbone at ϵ - NH_2 positions with methylpoly(ethylene glycol) succinate was approximately 28%. This result was confirmed by TNBS titration of available amino groups. The remainder of ϵ - NH_2 groups were covalently modified with anhydrides of DTPA (1% of amino groups) and succinic acid (remainder of available groups). Since Pt(II) is known to form complexes with carboxyl residues of polycarboxylic acids (13), we hypothesized that succinyl residues attached to the backbone of IV will similarly bind the aquated platinum(II)-diamine complex. We found that, upon simple coinubation of cDDP and the copolymer IV in a DMF/water mixture, an adduct between the copolymer and platinum was formed which could be isolated by fast size-exclusion chromatography. Elemental analysis showed that the adduct contained approximately 4.3% platinum by weight. We thus estimated that a mean of 68 platinum atoms were bound per molecule of copolymer IV. Assuming that there is no interaction of cDDP with MPEGs side chains, there are 95 theoretical binding sites for cDDP on the average which correspond to the association of Pt(II) with a pair of carboxyl residues of succinic acid of the copolymer. Therefore, the drug-carrier complex obtained is saturated by the drug by 70%.

Structural analysis of both IV and cDDP-IV by proton NMR showed two major peaks with similar chemical shifts at 4.75 ± 0.05 and 3.60 ± 0.01 ppm. These shifts correspond to MPEG-bound water and CH_2 protons of MPEG chains, respectively. A considerable broadening of both resonance peaks detected in the case of cDDP-IV (not shown) indicates intrabackbone cross-linking of polylysine succinate by the bifunctional platinum complex. An intra- rather than an inter-cross-linking pattern was confirmed by a parallel HPLC study that showed no substantial changes in the hydrodynamic radius of the drug-carrier complex when compared to the polymeric carrier alone (Figure 2).

The capacity of IV to bind cDDP was studied in two experiments to determine optimum time and concentration of cDDP during incubation. This was done by determining concentration of reversibly bound cDDP which can form colored complexes with *o*-phenylenediamine (10). Incubation of 0.6 mg of cDDP/mg of polymer for 20 versus 40 h at 37 $^\circ\text{C}$ yielded no significant difference in binding efficiency. At 20 h, approximately 37 molecules of cDDP per molecule of polymer were reversibly bound and available for the *o*-phenylenediamine reaction (5.5% of initial drug load). At 40 h, 33 molecules of drug were reversibly bound to each molecule of polymer (4.9% of initial drug load). In an experiment that varied incubation concentrations of cDDP for 20 h

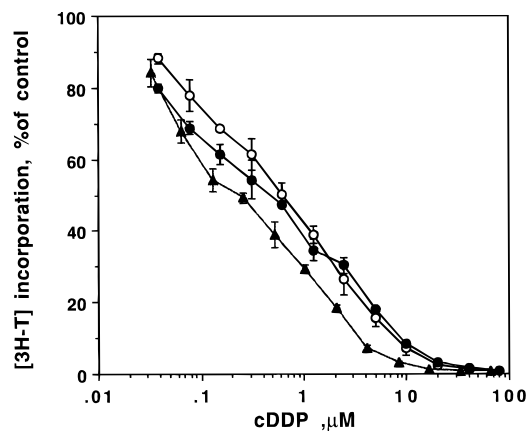


Figure 3. Comparative cytotoxicity of cDDP (triangles), cDDP-IV (closed circles), and cDDP-PL-succinate (open circles) determined as percent of $[^3\text{H}]$ thymidine incorporation in BT-20 human adenocarcinoma cells.

at 37 °C, the lowest and highest incubation concentrations (240 and 600 μg of cDDP/mg of polymer) yielded, respectively, 27 and 37 reversibly bound molecules of drug per molecule of polymer. We concluded that cDDP-IV saturated with cDDP could be prepared by incubation of 600 μg of cDDP/mg of IV for 24 h at 37 °C. Our results further indicate that approximately 55% of the platinum associated with IV can be released in the presence of *o*-phenylenediamine, and therefore is bioavailable without degradation of the carrier.

A study of dissociation kinetics of the adduct showed that platinum complex was slowly released from the adduct with IV upon dialysis in saline. Using monoexponential modeling, we estimated a half-time of release of 63 h. At the same experimental setting but in the presence of an excess of serum albumin in the dialysate, 20% of the drug was released from the polymer within 2 h. The major fraction of the drug, however, was released slowly with a rate similar to that of protein-free saline. Our results indicate the presence of a subpopulation of cDDP binding centers of the polymer that bind the drug with lower affinity than the major fraction of cDDP binding centers. Since this subfraction represents only 20% of potentially releasable cDDP, we assumed that systemic administration of cDDP-IV will result in substantially prolonged cDDP release.

To determine the cytotoxic effect of cDDP-IV, we compared it to free cDDP in an inhibitory cell proliferation assay. These experiments were carried out using BT-20 human mammary adenocarcinoma since preliminary experiments showed that proliferation of this cell line assessed by $[^3\text{H}]$ thymidine incorporation is highly sensitive to cDDP and thus represents a convenient model for drug cytotoxicity studies.

Both cDDP-IV and free drug showed pronounced cytotoxic effects by inhibiting DNA synthesis in human mammary adenocarcinoma cells after 16 h of incubation (Figure 3). Concentrations resulting in 50% inhibition of cell proliferation were, in a typical experiment, $0.3 \pm 0.1 \mu\text{M}$ for the free drug, $0.9 \pm 0.2 \mu\text{M}$ for both cDDP-poly(L-lysine)-*N*- ϵ -succinate and cDDP-IV. Since both graft copolymer and linear complexes of cDDP resulted in very similar concentration dependencies of $[^3\text{H}]$ thymidine incorporation in BT-20 cells, we assumed that cDDP dissociation from polyanionic backbone present in both polymeric complexes is largely responsible for the cytotoxic effects observed. However, at concentrations of cDDP lower than $0.5 \mu\text{M}$, cDDP-IV adduct showed a higher cytotoxicity than an adduct obtained with succi-

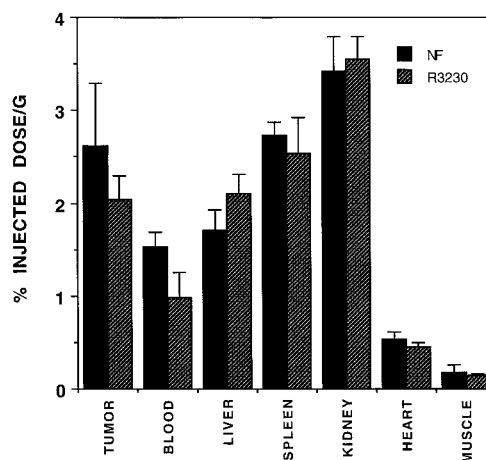


Figure 4. Biodistribution of cDDP-MPEGs-PL-succinate (20 mg of polymer adduct/animal, 0.5 mg of cDDP/animal) in female Fisher rats with NF13762 adenocarcinoma (solid bars) or R3230 adenocarcinoma (hatched bars) implanted subcutaneously. Data presented as mean $\pm$ standard deviation ($n = 3$ animals). Results obtained $[^{111}\text{In}]$ DTPA-labeled polymers 48 h after iv injection.

nylated poly(L-lysine), (PL-*N*- ϵ -succinate), i.e. with a polymeric carrier devoid of protective chains. This effect may have been due to a fraction of loosely bound drug in cDDP-IV.

The *in vivo* behavior of the polymer was studied in rats using an ^{111}In -radiolabeled IV which contained approximately 1% of *N*- ϵ -lysine groups modified with DTPA. cDDP-IV adduct exhibited a long circulation in the bloodstream, whereas cDDP-PL-succinate did not. Twenty-four hours after iv injection of the DTPA-labeled copolymer, 25% of the dose was found in the blood, whereas only 1% of the dose of the MPEG free adduct remained in circulation. After 48 h, more than 80% of MPEGs-PL-succinate-cDDP adduct had been removed from circulation. MPEG free adducts accumulated predominantly in kidneys ($15.0 \pm 1.2\%$ dose/g), whereas accumulation of MPEGs-PL-succinate-cDDP in kidney was almost 5-fold lower ($3.5 \pm 0.5\%$ dose/g). Assuming cDDP dissociation kinetics are similar for both adducts, there is a considerably lower opportunity for nephrotoxicity in the case of the long-circulating form of cDDP than in the MPEGs free polymer adduct.

Accumulation of MPEG-containing adduct in highly vascularized rat 13762NF mammary adenocarcinomas was approximately 5-fold higher ($2.6 \pm 0.6\%$ dose/g) than that of cDDP-PL-succinate, a conjugate devoid of MPEGs protective chains ($0.5 \pm 0.1\%$ dose/g). Biodistribution experiments in R3230 adenocarcinoma-bearing rats also showed an increased cDDP-IV accumulation in tumor. After 48 h in circulation, $2.05 \pm 0.25\%$ dose/g accumulated in the tumor (Figure 4). The difference between accumulation of polymeric adducts in 13762NF and R3230 tumors may arise as a result of a difference in vascularization and/or vascular permeability as the major factor affecting the extent of polymeric drug uptake at the tumor site.

Ultimately, γ -camera imaging of tumor-bearing animals was performed. No tumor accumulation was observed in the case of cDDP-PL-succinate (Figure 5A). Radioactivity was largely associated with kidney region. On the contrary, imaging results of tumor-bearing rats injected with radiolabeled cDDP-MPEGs-PL-succinate are consistent with biodistribution findings and show high regional activity in tumors (Figure 5B).

In conclusion, we established that MPEGs-PL-succinate forms soluble complexes (adducts) with cDDP at

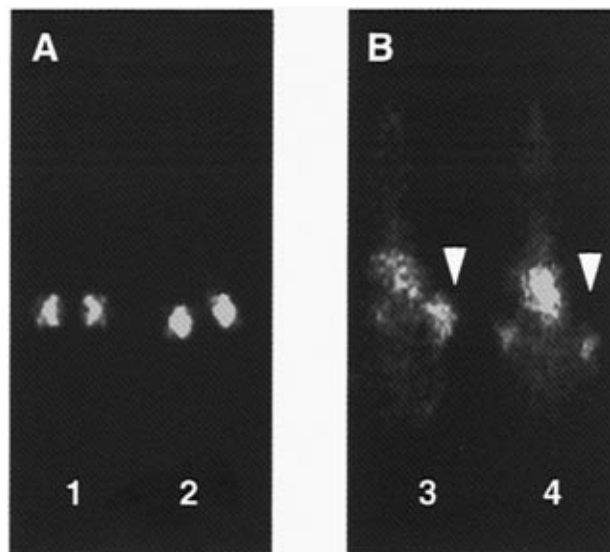


Figure 5. (A) γ -Camera imaging of the accumulation of cDDP-PL-succinate (rats 1 and 2) in NF13762 adenocarcinoma-bearing Fisher rats. Note that no visible accumulation in the tumor can be detected, and most of the compound remains in the kidneys. (B) Accumulation of cDDP-IV in NF13762 adenocarcinoma-bearing Fisher rats. Images obtained by γ -camera using ^{111}In -labeled polymers (20 mg/animal) 48 h after iv injection. Tumor location is indicated by an arrowhead.

high cDDP:succinate ratios. At least 80% of copolymer-bound cDDP is released from the carrier at a slow rate in the presence of plasma protein. Therefore, MPEG-poly(L-lysine)-succinate can serve as a high-capacity carrier for sustained release of cDDP. In addition, cDDP-MPEGs-poly(L-lysine)-succinate has a pronounced inhibitory effect on adenocarcinoma cell proliferation *in vitro*. Results from the biodistribution studies indicated high accumulation of cDDP-IV in mammary adenocarcinomas, probably due to the increased permeability of neoplastic vessels.

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LITERATURE CITED

- (1) Rozenzweig, M., Von Hoff, D. D., Slovik, M., et al. (1977) Cis-diamminedichloroplatinum (II), a new anticancer drug. *Ann. Intern. Med.* 86, 803–812.
- (2) *American Cancer Society textbook of clinical oncology* (1991) (A. I. Holleb, D. J. Fink, and G. P. Murphy, Eds.) p 691, American Cancer Society, Inc., Atlanta.
- (3) Sherman, S. E., and Lippard, S. J. (1987) Structural Aspects of Platinum Anticancer Drug Interactions with DNA. *Chem. Rev.* 87, 1153–1181.
- (4) Meyn, R. E., Stephens, L. C., Hunter, N. R., and Milas, L. (1995) Kinetics of cisplatin-induced apoptosis in murine mammary and ovarian adenocarcinomas. *Int. J. Cancer* 60, 725–729.
- (5) Muggia, F. M., Rozenzweig, M., and Penta, J. (1980) Clinical implications of cisplatin pharmacology. *Recent Results Cancer Res.* 74, 132–138.
- (6) Dentino, M., Luft, F. C., Yum, M. N., Williams, S. D., and Einhorn, L. H. (1978) Long-term effect of cis-diamminedichloride platinum (CDDP) on renal function in man. *Cancer* 41, 1274–1281.
- (7) Groth, S., Nielson, H., Sorenson, J. B., Christensen, A. B., Pedersen, A. G., and Rorth, M. (1986) Acute and long-term nephrotoxicity of cis-platinum in man. *Cancer Chemother. Pharmacol.* 17, 191–196.
- (8) Weiss, R. B., and Christian, M. C. (1993) New cisplatin analogues in development. A review. *Drugs* 46, 360–377.
- (9) Noteborn, H. P. J. M., and Verrijck, R. (1989) Drug delivery systems. *Curr. Opin. Oncol.* 1, 222–230.
- (10) Schechter, B., Pauzner, R., Arnon, R., and Wilchek, M. (1986) cis-Platinum(II) Complexes of Carboxymethyl-Dextran as Potential Antitumor Agents. I. Preparation and Characterization. *Cancer Biochem. Biophys.* 8, 277–287.
- (11) Schechter, B., Pauzner, R., Arnon, R., and Wilchek, M. (1986) cis-Platinum(II) complexes of carboxymethyl-dextran as potential antitumor agents. II. *In Vitro* and *In Vivo* Activity. *Cancer Biochem. Biophys.* 8, 289–298.
- (12) Schechter, B., Rosing, M. A., Wilchek, M., and Arnon, R. (1989) Blood levels and serum protein binding of cis-Platinum(II) complexed to carboxymethyl-dextran. *Cancer Chemother. Pharmacol.* 24, 161–166.
- (13) Schechter, B., Wilchek, M., and Arnon, R. (1987) Increased therapeutic efficacy of cis-platinum complexes of poly-L-glutamic acid against a murine carcinoma. *Int. J. Cancer* 39, 409–413.
- (14) Schechter, B., Pauzner, R., Arnon, R., Haimovich, J., and Wilchek, M. (1987) Selective cytotoxicity against tumor cells by cisplatin complexed to antitumor antibodies via carboxymethyl dextran. *Cancer Immunol. Immunother.* 25, 225–230.
- (15) Schechter, B., Arnon, R., Wilchek, M., Schlessinger, J., Hurwitz, E., Aboud-Pirak, E., and Sela, M. (1991) Indirect Immunotargeting of cis-Pt to Human Epidermoid Carcinoma KB using the Avidin-Biotin System. *Int. J. Cancer* 48, 167–172.
- (16) Arnon, R., Schechter, B., and Wilchek, M. (1991) Complexes and Conjugates of cis-Pt for Immunotargeted Chemotherapy. *Adv. Exp. Med. Biol.* 303, 79–90.
- (17) Verrijck, R., Smolders, I. J. H., Bosnie, N., and Begg, A. C. (1992) Reduction of Systemic Exposure and Toxicity of Cisplatin by Encapsulation in Poly-lactide-co-glycolide. *Cancer Res.* 52, 6653–6656.
- (18) Splenhauer, G., Vert, M., Benoit, J. P., and Boddaert, A. (1989) *In Vitro* and *In Vivo* degradation of poly(D,L-lactide/glycolide) type microspheres made by solvent evaporation method. *Biomaterials* 10, 557–563.
- (19) Ike, O., Shimizu, Y., Wada, R., Hyon, S.-H., and Ikada, Y. (1992) Controlled cisplatin delivery system using poly(D,L-lactic acid). *Biomaterials* 13, 230–234.
- (20) Yokoyama, M., Okano, T., Sakurai, Y., Ekimoto, H., Shibasaki, C., and Kataoka, K. (1991) Toxicity and antitumor activity against solid tumors of micelle-forming polymeric anticancer drug and its extremely long circulation in blood. *Cancer Res.* 51, 3229–3236.
- (21) Yokoyama, M. (1995) Polymeric micelles for drug delivery: their strategy and perspective. *Proceedings of 7th International Symposium on Recent Advances in Drug Delivery*, pp 99–102, Center for Controlled Chemical Delivery, Salt Lake City, UT.
- (22) Perez-Soler, R., Lopez-Berestein, G., and Lautersztain, J. (1989) Phase I clinical and pharmacological study of liposome-entrapped cis-bis-neodecanoato-trans-R,R-1,2-diaminocyclohexane platinum(II). *Cancer Res.* 50, 4254–4259.
- (23) Gondal, J. A., Preuss, H. G., Swartz, R., and Rahman, A. (1993) Comparative pharmacological, toxicological and antitumoral evaluation of free and liposome-encapsulated cisplatin in rodents. *Eur. J. Cancer* 19A, 1536–1542.
- (24) Yolles, S., Leafe, T. D., and Meyer, F. J. (1975) Timed-Release Depot for Anticancer Agents. *J. Pharm. Sci.* 64, 115–116.
- (25) Bogdanov, A. A., Jr., Weissleder, R., Frank, H., Bogdanova, A., Nossiff, N., Schaffer, B., Tsai, E., Papisov, M., and Brady, T. J. (1993) A new macromolecule as a contrast agent for MR angiography: preparation, properties and animal studies. *Radiology* 187, 701–706.
- (26) Bogdanov, A. A., Jr., Weissleder, R., Tsai, E., Schaffer, B., Bogdanova, A., Nossiff, N., Papisov, M., and Brady, T. (1993) Tumor neovascularity imaging by contrast-enhanced MR imaging. *Proceedings of the Society of Magnetic Resonance in Medicine, 12th Meeting*, New York, p 577, Society of Magnetic Resonance, Berkeley, CA.

- (27) Bogdanov, A. A., Jr., Weissleder, R., and Brady, T. J. (1995) Protected angiopolymers for vascular imaging. *Handbook of targeted delivery of imaging agents* (V. P. Torchilin, Ed.) pp 501–522, CRC Press, Boca Raton, FL.
- (28) Staros, J. V., Wright, R. W., and Swingle, D. M. (1986) Enhancement by N-hydroxysulfosuccinimide of water-soluble carbodiimide-mediated coupling reactions. *Anal. Biochem.* 156, 220–222.
- (29) Baleux, B. (1972) Dosage colorimetrique d'agente de surface non ioniques polyoxyethylenes a l'aide d'une solution iodo-iodaide. *C. R. Acad. Sci., Ser. III* 274, 1617–1620.
- (30) Bogdanov, A. A., Jr., Callahan, R. J., Wilkinson, R. A., Martin, C., Cameron, J. A., Fischman, A. J., Brady, T. J., and Weissleder, R. (1994) Synthetic Copolymer Kit for Radionuclide Blood-Pool Imaging. *J. Nucl. Med.* 35, 1880–1886.
- (31) Jain, R. K. (1988) Transvascular and interstitial transport in tumors. *Vascular endothelium in health and disease* (S. Chien, Ed.) pp 215–220, Elsevier, Amsterdam.
- (32) Jain, R. K. (1989) Delivery of novel therapeutic agents in tumors: physiological barriers and strategies. *J. Natl. Cancer Inst.* 81, 570–576.

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Dextran Modification of a Fab'– β -Lactamase Conjugate Modulated by Variable Pretreatment of Fab' with Amine-Blocking Reagents

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The physical and pharmacological properties of proteins can be altered by chemical modification with polymers. Preliminary studies showed that attachment of oxidized dextran to the bacterial protein, β -lactamase (β L) effectively reduced *in vivo* immunogenicity in mice with no loss of enzymatic activity. This report describes a general method for differentially dextran modifying the Fab' component of a Fab'– β -lactamase conjugate by the use of amine-blocking reagents. Methyl acetimidate (MeAcm) and the *N*-succinimidyl derivative of (methylsulfonyl)ethyl carbonate (NHS-Msc), reagents which can reversibly block primary amines, were used in model studies to modulate the level of available reactive amines on the F(ab')₂ fragments of both the anti-carcinoembryonic antigen antibody, ZCE025, and the antitumor-associated glycoprotein-72 antibody, CC49. MeAcm had little or no effect on immunoreactivity and was maximally effective in modulating dextran attachment, while NHS-Msc was much less effective. A comparison of NHS-Msc and MeAcm is described. Treatment of F(ab')₂ with 5–300 mM MeAcm prior to dextran treatment showed a proportional decline in the level of dextran attachment as well as intramolecular cross-linking of the protein by the dextran polymers (6 kDa or 33-mer). A conjugate of β L coupled to MeAcm-treated ZCE025 Fab' [reduced F(ab')₂] was constructed under standard conditions using sulfo-succinimidyl *N*-(4-carboxycyclohexyl)methylmaleimide. After dextran modification, this conjugate maintained good immunoreactivity and enzymatic activity. Biodistribution studies in tumor-bearing nude mice of dextranated and nondextranated conjugate showed comparable overall distribution profiles except that the clearance of the dextranated conjugate from both blood and tumor was delayed about 48–72 h.

INTRODUCTION

In previous work, bioconjugates of the bacterial protein β -lactamase coupled to antibody Fab' fragments of ZCE025 and CC49 were shown to be efficacious in the tumor-specific delivery of oncolytic drugs (1). While effective in the mouse model studies, extension of this work in human clinical trials required consideration of the immunogenic response to such conjugates, especially since multiple injection protocols would likely be necessary to achieve maximum efficacy (1–3). The immune response to protein-based injectables, while somewhat variable, is a significant problem for most *in vivo* applications (2–4).

In the case of antibody-based bioconjugates, multiple strategies have been devised to minimize immunogenic response, including chimeric or humanized expression of antibodies (5–7) or the expression of small Fv fragments (8, 9). Because such strategies are ongoing in our laboratory as well, the primary focus of the current study has been directed towards β L,¹ a bacterial protein for which no good recombinant expression alternatives are currently available for amelioration of the immunogenic response.

Chemical methods of reducing the immunogenicity of proteins have generally focused on the modification of

the protein surface with polymers of poly(ethylene glycol) or oxidized dextran. While both techniques involve covalent attachment to the reactive free amines on proteins, the fundamental difference between these techniques is that PEG is generally only attached through a single "activated" terminus of methyl-PEG (10–12) while an oxidized form of the dextran polymer is used which permits attachment to the protein at multiple sites throughout the polymeric dextran chain (13, 14). Dextran polymers have been shown in other work to be immunogenic (15), though even this is markedly reduced at polymer molecular masses of less than 50 kDa (16). The oxidized form of the 6 kDa dextran used in the current procedure no longer contained the intact ring structure of native dextran and has been shown previously to be nonimmunogenic (13).

The primary goal of the current project was to study the effect of oxidized dextran attachment on the Fab'– β L conjugates we have employed in previous studies (1, 17, 18). Previous work showed that dextran modification had no effect on the immunoreactivity of the T101 antibody (13) but was inhibitory to the anti-CEA antibody intended for use in this work (19). β L activity was

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¹ Abbreviations: PEG, poly(ethylene glycol); MeAcm, methyl acetimidate; NHS-Msc, *N*-succinimidyl (methylsulfonyl)ethyl carbonate; *M*_r, relative molecular weight; β L, β -lactamase; Fab'– β L, covalent conjugate of Fab' with β L; anti-CEA, anticarcinoembryonic antigen; anti-TAG-72, antitumor-associated glycoprotein-72; NEM, *N*-ethylmaleimide; DMSO, dimethyl sulfoxide; HIC, hydrophobic interaction chromatography; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; RT, room temperature; sulfo-SMCC, sulfo-succinimidyl *N*-(4-carboxycyclohexyl)methylmaleimide; TNBS, trinitrobenzenesulfonic acid; SEC, size exclusion chromatography; ELISA, enzyme-linked immunoabsorbent assay.

unaffected by dextran treatment. Therefore, the objective of the first phase of this investigation was 2-fold: (1) to examine the ability of amine-blocking reagents to modulate the level of dextran attachment on the Fab's to determine if conditions could be found which would preserve the immunoreactivity and (2) to use the existing murine Fab's as model proteins to establish the parameters necessary to create a conjugate with fully dextranated β L but with modulated levels of dextran on the Fab'.

Thus, the current study involves the development of general methods for modulation of the dextran attachment in a protein-protein conjugate where the individual protein components may be differentially affected by the extent of polymeric attachment. Since both the protein-protein coupling procedures (20) as well as the dextran attachment (13) utilized the same lysine ϵ -amino groups, it was not feasible simply to dextranate the β L prior to coupling to the Fab'. Without the availability of specialized, protected linkers, it was necessary to form the Fab'- β L conjugate prior to dextran treatment.

The reversible amine-blocking reagents methyl acetimidate (MeAcM) (21-23) and the amino-reactive *N*-succinimidyl derivative of (methylsulfonyl)ethyl carbonate (NHS-Msc) (24) have been widely used for blocking amines on polypeptide chains. NHS-Msc and MeAcM were tested both for their direct effects on Fab' immunoreactivity and for their ability to selectively block key lysine residues and thus modulate the subsequent attachment of oxidized dextran. The results of this work demonstrate the feasibility of construction of a protein-protein conjugate with modulated availability of amino groups for subsequent modifications.

EXPERIMENTAL PROCEDURES

Reaction of MeAcM and NHS-Msc with F(ab')₂. F(ab')₂ fragments of ZCE025 antibody were prepared by incubation of intact antibody with 3% pepsin (w/w) at pH 3.5 in 100 mM citrate buffer at 37 °C for 90 min (25, 26). CC49 fragments were obtained by incubation in the same buffer at pH 3.9 for 4 h. F(ab')₂ fragments in both cases were purified to homogeneity by cation exchange chromatography using Pharmacia S Sepharose Fast Flow resin. Buffer A was 170 mM sodium acetate (pH 4.5) and buffer B the same with 0.5 M NaCl. With a linear gradient, F(ab')₂ fragments elute at about 50% buffer B. Concentrations of F(ab')₂ used in these experiments were from 7-10 mg/mL using an $\epsilon_{280}^{0.1\%}$ of 1.8.

A 2 M solution of MeAcM was freshly prepared by dissolving solid methyl acetimidate hydrochloride (Pierce Chemical Co., Rockford, IL) in 100 mM borate buffer (pH 10), on ice, and adjusting the pH to about pH 9 with 6 N NaOH (as determined with pH paper). All reactions with MeAcM and protein were conducted at 4 °C (on ice). An aliquot of this MeAcM solution was then immediately added to F(ab')₂ which had been dialyzed versus 100 mM borate buffer (pH 10) (pH 9 in some experiments, see Results) to make a final concentration from 5 to 300 mM MeAcM. In a typical experiment, 77 mg of MeAcM HCL was added to 225 mL of borate buffer (pH 10) plus 57 mL of 6 N NaOH to yield about 350 mL of 2 M MeAcM pH (~9). Dilutions of the 2 M MeAcM were sufficiently large so as not to affect the pH of the final F(ab')₂ solution. The samples were allowed to react for 2 h on ice and then dialyzed versus 160 mM sodium phosphate buffer at 4 °C to remove small molecules. Determination of reactive amines after treatment with MeAcM was determined with TNBS as described by Habeeb (27).

The NHS-Msc was dissolved in DMSO at a concentration of 1 M. Typical F(ab')₂ samples were dialyzed versus

100 mM borate buffer (pH 8) (pH 7.5 in some experiments, see Results). Aliquots of this DMSO solution were added directly to F(ab')₂ solutions and the samples incubated for 2 h at RT. Samples were then dialyzed in phosphate buffer as above.

Deprotection conditions for the MeAcM-treated F(ab')₂ consisted of addition of 200 mL of the sample above to 500 mL of 15:1 concentrated ammonia/glacial acetic acid (21, 23) which was allowed to incubate at RT for 90 min. Samples were then dialyzed versus 160 mM phosphate buffer (pH 6.7) at 4 °C in preparation for dextran treatment. NHS-Msc-treated samples were deprotected by incubation at pH 10 in borate buffer for 4 h at RT prior to dialysis in phosphate buffer.

Dextran Attachment to F(ab')₂ and Fab'- β L Conjugate. Oxidized, lyophilized dextran (average molecular weight of 6000 $\pm$ 500), prepared by oxidation with sodium periodate as described previously (13), was freshly prepared by dissolving solid oxidized dextran in 160 mM phosphate buffer at 600 mg/mL. An aliquot of this solution was added to the protein at a concentration of 12 mg of oxidized dextran per milligram of protein. Conjugate concentrations were calculated using an $\epsilon_{280}^{0.1\%}$ of 2.0. Samples were incubated at RT for 15 min, and then a freshly prepared solution of 1 M NaBH₃CN was added in 10-fold molar excess over the dextran and the sample incubated for an additional 2 h at RT. To remove excess dextran and other small molecules, samples were then applied to an FPLC Pharmacia Superose 12 HR10/30 column equilibrated in 100 mM phosphate (pH 7.0), and the main protein peak was collected. Typically about 10% of the A₂₈₀ eluted at a retention time consistent with protein dimer (confirmed by SDS-PAGE), the result of intermolecular dextran cross-linking, and this peak was discarded. The flow rate was 0.7 mL/min.

After peaks were collected from the Superose column, the samples were immediately cooled on ice and an aliquot of freshly prepared 2 M NaBH₄ was added to a final concentration of 100 mM and the sample allowed to set at 4 °C overnight. Samples were then dialyzed versus PBS prior to further analysis.

The size exclusion chromatography data shown in Results was performed with 2 Zorbax G-250 columns in series, eluted with 100 mM phosphate buffer (pH 7) at a flow rate of 1 mL/min. SDS-PAGE was performed on the Pharmacia PhastSystem with 4 to 15% gradient gels under reducing or nonreducing conditions as described in Results. Native PAGE was also performed on the PhastSystem utilizing native buffer strips.

Immunoreactivity and Biodistribution. The immunoreactivity of (Fab')₂ and conjugate samples was determined using a competitive assay format. Intact ZCE025 antibody was iodinated to a specific activity of 3.8×10^6 cpm/ μ g by the solid state Iodobead (Pierce) procedure according to the method of Markwell (28). Serial dilutions of the samples from 2.5 to 0.125 μ M were combined with 100 000 cpm of ¹²⁵I-labeled ZCE025 and diluted to a total volume of 25 μ L in PBS plus 10% γ -globulin-free horse serum (AHS) (Gibco) which was then added to duplicate wells of microtiter plates containing dried LS174T cells. After incubation for 60 min at room temperature, the cells were washed with PBS plus 10% AHS and bound counts per minute in the wells was determined by γ -counting.

In addition, the immunoreactivity of conjugate samples was tested by solid-phase RIA against antigen(+) LS174T and antigen(-) M14 tumor cells. A specific activity of 1.3×10^7 cpm/ μ g was obtained for the MeAcM control, while a specific activity of 1×10^7 cpm/ μ g was reached for the dextranated conjugate. ¹²⁵I-labeled conjugate was

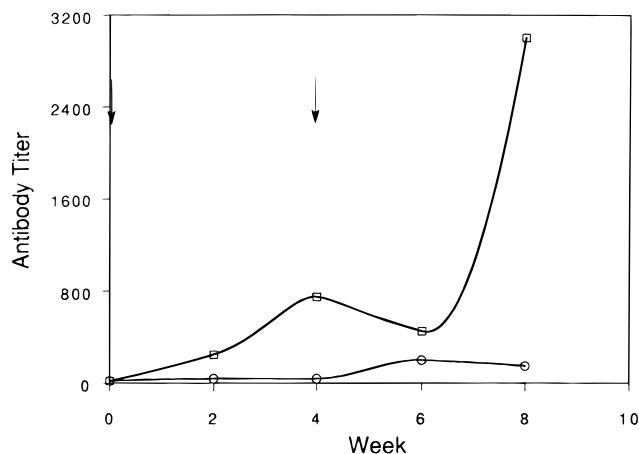


Figure 1. Immune response in mice to β L with and without attached dextran. β L (20 mg) was injected into six mice at time 0 and at 4 weeks, as indicated by the arrows on the graph. Mouse serum titers were determined on pooled samples at each time point using microtiter plate ELISA assays as described in Experimental Procedures. Titers were defined as the dilution of sera resulting in 50% of the maximum response against unmodified β L. Open squares are the serum titers of mice injected with unmodified β L, and open circles are the titers for dextran-modified β L.

diluted in PBS plus AHS and added to duplicate wells at serial dilutions from 4×10^5 to 5×10^4 cpm in 25 μ L aliquots. After incubation for 60 min at RT, samples were washed and counted as above.

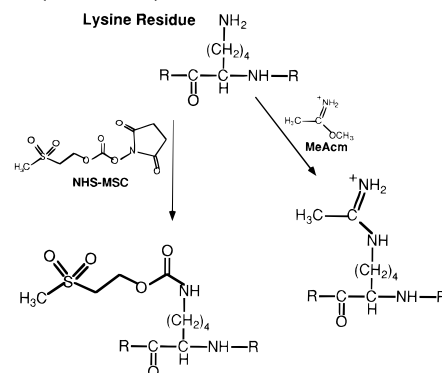
Evaluation of Immunogenicity. The immunogenicity of β L was evaluated by iv injection of 20 mg of dextranated and nondextranated β L in groups of 6 mice each and measurement of the resulting anti-mouse response in pooled serum samples, using a modification of the ELISA assay previously reported (13). Briefly, microtiter plate wells were coated by exposure to 0.25 mg of unmodified β L in 10 mM sodium phosphate buffer (pH 7.0) for 24 h and then washed thoroughly. Nonspecific binding sites were blocked with a 1 mg/mL solution of BSA in the same buffer. Wells were incubated with serial dilutions of mouse sera for 24 h and washed thoroughly. Wells were then exposed to goat anti-mouse immunoglobulins conjugated to HRP (TAGO Inc., Burlington, CA), and color was developed with the addition of 1 mg/mL *o*-phenylenediamine in 0.1% (v/v) hydrogen peroxide. Color development was quantified by measuring the absorbance at 490 nm with an ELISA reader. Titers were defined as the reciprocal of the highest dilution of mouse sera resulting in 50% of the maximum response.

RESULTS

In Vivo Immune Response to β L $\pm$ Dextran Modification. *In vivo* studies in mice were performed to confirm that the reduction in immunogenic response to dextran-modified β L was comparable to that seen for other proteins (13, 14). Figure 1 shows that the immunogenic response to the strongly antigenic bacterial protein was virtually eliminated after dextran modification. Approximately 4 mol of dextran was attached per mole of β L as determined by size exclusion chromatography, SDS-PAGE, and the anthrone assay (29). This represents the maximum level of dextran attachment under the conditions used in this work.

Effect of MeAcM on ZCE025 F(ab')₂ Dextran Attachment. Scheme 1 shows that the reaction product of MeAcM with the ϵ -amino group of lysine resembles arginine in structure and has a pK similar to that of

Scheme 1. Reaction of Protein Lysine Residues with Amine Reactive Reagents, Methyl Acetimidate (MeAcM) and *N*-Succinimidyl (Methylsulfonyl)ethyl Carbonate (NHS-Msc)^a



R = the remainder of the polypeptide chain on the protein

^a R = the remainder of the polypeptide chain on the protein.

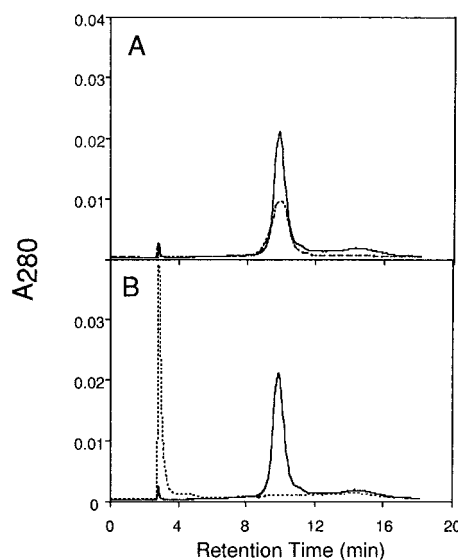


Figure 2. Cation exchange chromatography of ZCE025 F(ab')₂ samples. (A) Solid line, native F(ab')₂; dashed line, F(ab')₂ treated with 300 mM MeAcM. (B) Solid line, native F(ab')₂; dotted line, F(ab')₂ treated with 100 mM NHS-Msc. The native F(ab')₂ was well retained on the column, while the sample treated with NHS-Msc eluted in the void volume, indicating that the protein had insufficient positive charges to be retained on the column.

arginine at 12.5 (21, 23). As such, the pK is 2 orders of magnitude higher than that of lysine, and the product is essentially nonreactive with lysine-reactive reagents at pHs of 8 and below. Figure 2 shows that the retention time of F(ab')₂ by cation exchange chromatography was unaffected by reaction with MeAcM, indicating that the overall charge of the protein was not substantially altered under the column buffer conditions at pH 4.5. Native PAGE of F(ab')₂ treated with 300 mM MeAcM was, however, able to discriminate the slight charge difference from native F(ab')₂ in that such samples had somewhat slower mobility on the gel, indicating a more positively charged molecule than native F(ab')₂ (gel not shown).

The immunoreactivity of MeAcM-treated samples was not appreciably different from that of native F(ab')₂ (Figure 3, curve 6). Subsequent treatment of this sample under standard dextranation procedures (see Experimental Procedures) showed no reduction in the immunoreactivity (curve 8), whereas dextran attachment to native F(ab')₂ showed almost complete loss of immunore-

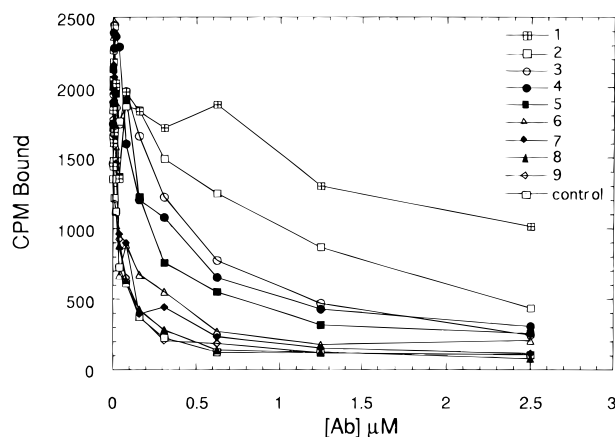


Figure 3. Immunoreactivity by competitive assay of $F(ab')_2$ samples treated with 300 mM MeAcM or 100 mM NHS-Msc, with and without subsequent dextran attachment. $F(ab')_2$ samples indicated in the plot legend show increasing immunoreactivity in approximately the following order: 1, native $F(ab')_2$ treated with dextran; 2, NHS-Msc at pH 8; 3, NHS-Msc at pH 7.5; 4, NHS-Msc at pH 7.5 followed by dextran treatment; 5, NHS-Msc at pH 8 plus dextran; 6, MeAcM at pH 10; 7, 300 mM MeAcM at pH 9; 8, 300 mM MeAcM pH 10 plus dextran; 9, 300 mM MeAcM at pH 9 plus dextran; 10, $F(ab')_2$ control with no treatment. The immunoreactivities fell into three main categories. (1) Control dextran-modified with dextran was clearly most inhibited, while the immunoreactivity after NHS-Msc treatment alone at pH 8 was also severely impaired. (2) Intermediate levels were obtained with NHS-Msc at pH 7.5 and after dextran modification of samples after treatment with NHS-Msc at either pH 7.5 or 8. (3) Near control values were obtained with all MeAcM-treated samples.

activity (curve 1). Partial removal of the acetimidyl groups by limited treatment with ammonium hydroxide/acetic acid (pH 11) (21, 23) for 90 min at RT showed no adverse effect on the immunoreactivity of the $F(ab')_2$ and no change in the ion exchange retention time shown in Figure 2A. Subsequent dextran treatment of these samples did, however, restore about half the dextran attachment as compared to native $F(ab')_2$.

In addition to the standard MeAcM reaction conditions at pH 10, reaction at pH 9 was also performed in order to test the pH sensitivity and selectivity of this reagent at a lower pH. Figure 3 shows no appreciable difference in immunoreactivity after dextranation whether the acetimidylation pretreatment was performed at pH 9 or 10 (curves 8 and 9, respectively). A slight retention time and peak width increase observed by size exclusion chromatography did, however, suggest that MeAcM derivatization at pH 10 was somewhat more effective in blocking dextran attachment (data not shown).

In addition to the $F(ab')_2$ of ZCE025 described above, the immunoreactivity of the $F(ab')_2$ fragment of CC49 was also investigated and showed almost identical results under the same reaction conditions (data not shown).

Treatment of ZCE025 $F(ab')_2$ with Variable Levels of MeAcM. The effect of different concentrations of MeAcM pretreatment on subsequent dextran attachment was studied as a means of determining optimal reaction conditions and the effect of different levels of attached dextran on the immunoreactivity. Figure 4 shows the increase in retention time by SEC, indicating a decrease in M_r , as the level of MeAcM pretreatment was increased prior to dextran modification. Table 1 shows the calculated values for the moles of dextran attached per mole of $F(ab')_2$ based on the retention times in Figure 4. In addition, Table 1 shows the number of reactive amines available after MeAcM treatment as determined by TNBS. An average of 3–4 amines per mole of oxidized

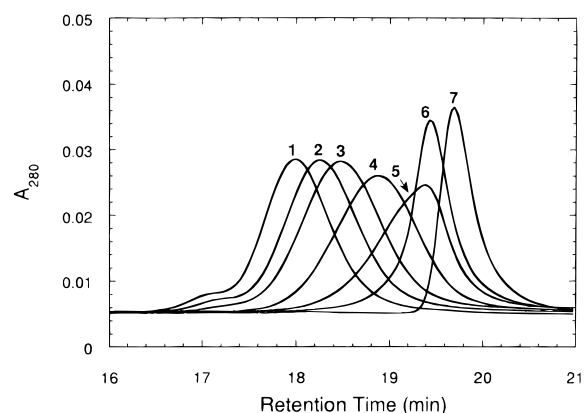


Figure 4. Size exclusion chromatography (SEC) overlay of $F(ab')_2$ samples treated with increasing levels of MeAcM prior to dextran modification. Increased retention times indicate a lower apparent M_r . Peak 7 shows native $F(ab')_2$ with no MeAcM or dextran treatment. MeAcM treatment did not affect the retention time of native $F(ab')_2$. Peaks 1–6 show the retention times of samples modified with dextran subsequent to the following pretreatment with MeAcM: 1, native $F(ab')_2$ with no pretreatment; 2, 5 mM; 3, 10 mM; 4, 20 mM; 5, 50 mM; 6, 300 mM. The 100 mM peak was not plotted for clarity but had the same retention time as the 300 mM sample, though with a slightly wider peak width at base line. Peak 1 represents $F(ab')_2$ with approximately 10 mol of dextran attached per $F(ab')_2$.

Table 1

MeAcM treatment (mM)	reactive amines per $F(ab')_2$	dextran chains per $F(ab')_2$	Amines per dextran
0	39	9.2	4.2
5	25	7.3	3.4
10	17	6.2	2.7
20	10	3.8	2.6
50	5	1	5
100	3.4	0.5	6.8
300	1.4	0.5	2.8

dextran was available for attachment within the range of 0–20 mM MeAcM treatment. Levels of 100 and 300 mM MeAcM resulted in identical retention times as seen in Figure 4, indicative of minimal or no dextran attachment.

The same general trend in gradually decreasing molecular weight was also seen in SDS–PAGE of the same samples (Figure 5). When run under reducing conditions (Figure 5A), the SDS–PAGE demonstrated another aspect of dextran attachment, intramolecular cross-linking. A single $F(ab')_2$ molecule is composed of two identical Fab' molecules held together by three disulfide bonds at the hinge region (25, 26). Each Fab' molecule is in turn composed of a heavy and light chain held together by a single disulfide bond. Thus, each 100 kDa $F(ab')_2$ molecule is composed of 4 mol of individual polypeptide chains, visible as two poorly resolved bands of approximately 24 and 26 kDa when run denatured and reduced on SDS–PAGE (Figure 5A, lane 8).

Regardless of whether the sample was run under reducing conditions, the dextran-modified $F(ab')_2$ had the same mobility on SDS–PAGE which indicated that the polypeptide chains of dextranated $F(ab')_2$ were fully crosslinked (Figure 5, lanes A1 and B1). The Fab'–Fab' as well as the Fab' heavy–light chains remained covalently attached despite reduction of the disulfide bonds in lane B1.

However, after pretreatment with 5 mM MeAcM, four broad and heterogeneous bands of protein were discernible from the reduced gel, lane A2, consistent with the four possible forms of covalent attachment: intact $F(ab')_2$

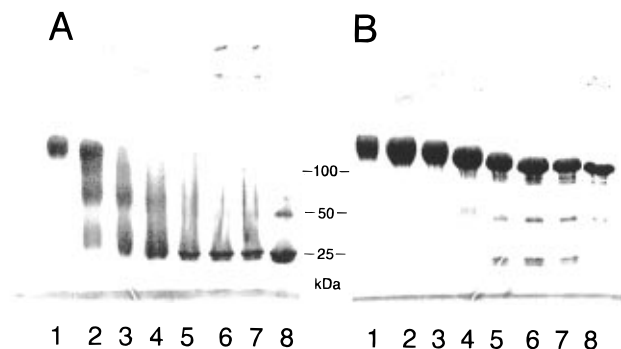


Figure 5. Gradient PhastGel (4 to 15%) of $F(ab')_2$ samples pretreated with increasing levels of MeAcM prior to dextran modification. Gel A was run under reducing conditions with 5% 2-mercaptoethanol, while gel B was run under nonreducing conditions. Lane 8 shows native $F(ab')_2$ with no treatment with MeAcM or dextran. Identical samples were applied to lanes 1–8 on each gel: lane 1, native $F(ab')_2$ with no MeAcM pretreatment; lane 2, 5 mM; lane 3, 10 mM; lane 4, 20 mM; lane 5, 50 mM; lane 6, 100 mM; lane 7, 300 mM; lane 8, native $F(ab')_2$. Gel A demonstrated dextran intramolecular cross-linking of the $F(ab')_2$ molecule under conditions which reduced the disulfide bonds holding its fragment parts together. Lanes A1 and B1 are identical, indicating that the $F(ab')_2$ was fully cross-linked by the dextran. Lanes A2–A7 showed a decreasing percentage of cross-linking as the level of MeAcM pretreatment was increased. Gel B shows the increased mobility and less heterogeneous bands of $F(ab')_2$ as the level of dextran attachment decreased.

as in lane A1, two lower bands consistent with the loss of one and then two of the four component fragment parts, and finally the free heavy and light chains as the fastest running bands. Pretreatment with 10 mM MeAcM (lane A3) showed only the dimeric and single chains remaining. With the pretreatment levels of MeAcM increased to 20 and 50 mM MeAcM (lanes A4 and A5), the level of dextran cross-linking of the $F(ab')_2$ polypeptide chains reached minimal levels, though some apparent dextran attachment on the individual polypeptides was still evident since the bands ran slightly slower and were more heterogeneous than those of native heavy–light chains seen in lane A8. This is supported by the values in Table 1, where calculated dextran attachment dropped significantly after treatment with 50 mM MeAcM.

The immunoreactivity of these samples appeared to be directly proportional to the level of dextran modification as modulated by pretreatment with MeAcM (Figure 6). The higher levels of MeAcM pretreatment which resulted in lowered dextran attachment also showed the highest immunoreactivity. At MeAcM concentrations of 50 mM, where all significant intramolecular cross-linking was blocked, the immunoreactivity approached that of native $F(ab')_2$.

Effect of NHS-Msc on Dextran Attachment to ZCE025 $F(ab')_2$. Scheme 1 shows the product formed when NHS-Msc reacts with the ϵ -amine of lysine. Aside from blocking the ϵ -amino group of the lysine, the main effect of the Msc derivative on the protein is neutralization of the positive charge which is normally present on the lysine at typical physiological pHs. This effect was demonstrated by cation exchange chromatography of ZCE025 $F(ab')_2$ before and after treatment with 100 mM NHS-Msc (Figure 2B). Native $F(ab')_2$ bound well to the column matrix, but after NHS-Msc treatment, the derivatized $F(ab')_2$ did not bind to the column, instead eluting in the void volume. Samples on native PAGE also showed greater mobility after NHS-Msc treatment, indicating a more negatively charged protein (gel not shown).

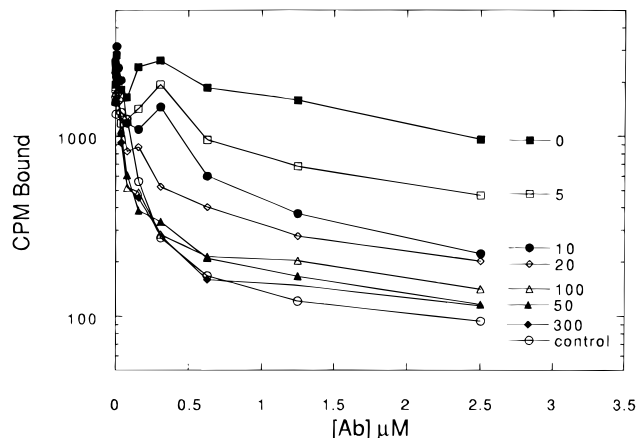


Figure 6. Competitive assay showing the increase in immunoreactivity as the level of MeAcM pretreatment was increased prior to dextran modification. These were the same samples shown in Figures 4 and 5. Pretreatment levels from 0 to 20 mM MeAcM showed clearly impaired immunoreactivity after dextran modification, whereas dextran-modified samples pretreated with 50–300 mM MeAcM showed values comparable to those of native $F(ab')_2$.

However, NHS-Msc treatment under standard conditions at pH 8 had an adverse effect on the immunoreactivity of $F(ab')_2$ (Figure 3, curve 2). Reaction with NHS-Msc was also tested at a lower pH, pH 7.5 (curve 3), to monitor reagent sensitivity and selectivity at a lower pH. While cation exchange chromatography and native PAGE gels both suggested comparably charged protein with NHS-Msc reaction at either pH 7.5 or 8 (comparative data not shown), NHS-Msc treatment at pH 8 was more inhibitory to the immunoreactivity (Figure 3) which, by inference, suggested higher levels of neutralized lysines. Curiously, dextran attachment after pH 8 NHS-Msc treatment showed slightly improved immunoreactivity over the nondextranated NHS-Msc control (curve 4 versus 2). Another aspect of working with Msc-derivatized $F(ab')_2$ was anomalously high retention times by size exclusion chromatography on the Zorbax columns and conditions used in this work. While likely due to column matrix effects (since slightly denatured proteins would be expected to have decreased retention times by SEC), the observation was not investigated further.

The overall ability of NHS-Msc to block dextran attachment was lower than that attained with MeAcM. After 100 mM NHS-Msc pretreatment, and subsequent dextran attachment, the $F(ab')_2$ on reduced SDS–PAGE looked much like the 10 mM MeAcM pretreatment seen in lane 3 of Figure 5A (NHS-Msc gel not shown). NHS-Msc in higher levels was not soluble. Attempts to use the *p*-nitrophenyl derivative of Msc as a means to increase the reaction with amines resulted only in precipitation of the protein.

$F(ab')_2$ was also reacted with NHS-Mac at lower concentrations to test the possibility that a particularly reactive amine was one of the key sites responsible for the reduced immunoreactivity. Reaction of $F(ab')_2$ with 2, 5, and 10 mM NHS-Msc clearly blocked some amines, as indicated by increased mobility on native PAGE, but the immunoreactivity of these samples both before and after dextran attachment was not discernibly different from that of native $F(ab')_2$ controls. Incubation at pH 10 in borate buffer for 4 h at RT removed about half of the Msc groups as indicated by intermediate binding on the cation exchange column as well as increased dextran attachment, but such treatment failed to restore the immunoreactivity (data not shown).

F(ab')₂ fragments of CC49 were also reacted with NHS-Msc under the above conditions and showed somewhat greater inhibition of immunoreactivity than observed for ZCE025.

Biodistribution of Fab'- β L Conjugate $\pm$ Dextran Treatment. β L and ZCE025 Fab' were coupled using sulfo-SMCC as described previously (17) except that the F(ab')₂ was pretreated with 100 mM MeAcM prior to reduction to Fab' with 20 mM cysteine. Dextran modification of this conjugate was performed at the same mass ratio of oxidized dextran to conjugate as used for F(ab')₂, i.e., 12 mg of dextran per milligram of conjugate protein. Since the Fab' half of the conjugate was essentially unreactive with dextran, the ratio of dextran mass to β L protein mass was approximately 2 times higher than that used in the model studies with F(ab')₂. The β L activity of the dextran-modified conjugate was identical to that of native β L on a molar basis. SDS-PAGE of the dextranated conjugate showed a single band under nonreducing conditions which was similar in heterogeneous appearance to the broad band of F(ab')₂ seen in lane 1 of Figure 5B. Band mobility on SDS-PAGE suggested an average of approximately 4 mol of dextran per conjugate, consistent with the nominal value expected for β L alone.

The immunoreactivity of this Fab'-blocked, β L-dextranated conjugate was the same as that of the Fab' used to make the conjugate, as determined by the competition assay formats seen in Figures 3 and 6. To serve as a single-arm control for immunoreactivity studies, an aliquot of the MeAcM-treated Fab' was removed prior to coupling to β L, and the sulfhydryls were capped with NEM.

In addition to the sample used for biodistribution studies above, another sample of the Fab'- β L conjugate was treated at a 10 times higher level of dextran, i.e., 120 mg per milligram of conjugate protein. The observed immunoreactivity, β L activity, and SDS-PAGE mobility for this sample were not discernibly different than those of samples treated at 12 mg of dextran per milligram protein.

Prior to biodistribution studies, both dextranated and nondextranated conjugate were also tested versus antigen-positive and antigen-negative cells. In Figure 7, the conjugates show good binding to antigen-positive cells, though the dextranated conjugate was clearly lower than the MeAcM-treated Fab' or Fab' control. No nonspecific binding to antigen-negative cells was observed with dextranated or nondextranated conjugate.

The biodistribution studies of the dextranated conjugate showed a longer half-life in all tissues, including the blood and the tumor, compared to the MeAcM-treated, nondextranated control conjugate (Figure 8). Typical protocols with Fab'- β L conjugate wait 72–96 h after injection before administration of prodrug to allow conjugate clearance from the blood in order to increase the tumor to blood ratio (1). As expected from other work (14, 30), attachment of polymers to proteins increased the overall circulating half-life. From Figure 8, it can be seen that the clearance of the dextranated conjugate from the blood and tissues is generally delayed about 48–72 h over that of the nondextranated control conjugate. The tumor to blood ratio for the dextranated conjugate was lower than for nondextranated conjugate at all time points.

DISCUSSION

This work demonstrates the feasibility of construction of Fab'-enzyme bioconjugates with differential levels of polymeric protection. In this case, it was considered

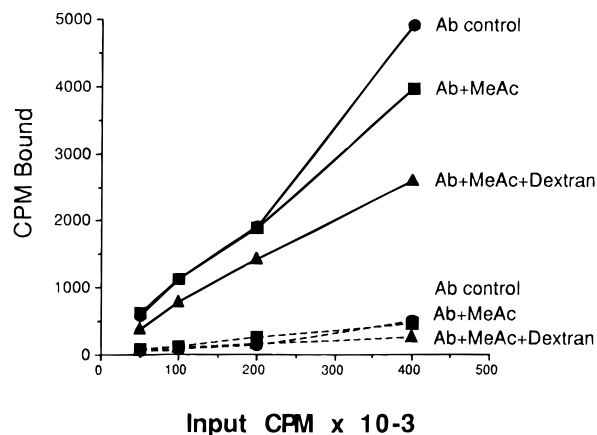


Figure 7. Direct measure of the binding of ¹²⁵I-labeled Fab'- β L conjugate to antigen-positive (LS174T) and antigen-negative (M14) cells. Samples are as follows. Circles are untreated Fab' which serves as a single-arm binding control. This sample was treated with NEM to block hinge region sulfhydryls. Squares are conjugate prepared with Fab' treated with 100 mM MeAcM prior to coupling to the β L. Triangles are conjugate prepared with 100 mM MeAcM pretreatment of the Fab' and subsequent dextran modification of the entire conjugate. The solid lines show binding to LS174T cells, and the dotted lines show binding to M14 cells. Negligible nonspecific binding to antigen-negative cells was observed. While reduced binding to antigen-positive cells was seen under these conditions, competitive assays performed as seen in Figures 3 and 6 showed no significant differences between the control, MeAcM-treated, and MeAcM-treated dextranated conjugate.

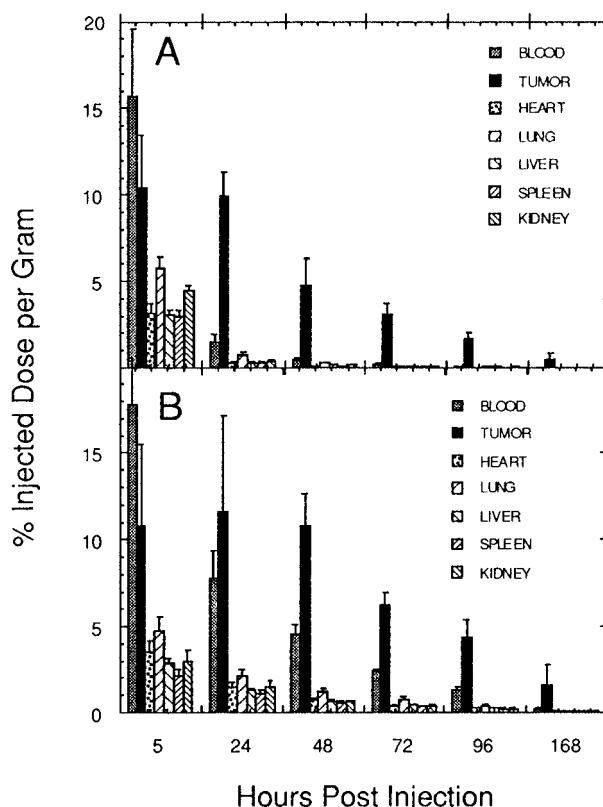


Figure 8. Biodistribution in tumor-bearing nude mice of ¹²⁵I-labeled conjugate. Direct binding assay of these samples is shown in Figure 7. (A) Dextran-modified conjugate prepared from Fab' pretreated with 100 mM MeAcM prior to coupling to β L. (B) Conjugate prepared as above except with no dextran modification. Dextranated conjugate showed longer clearance times from the blood and tissues compared to the control conjugate without dextran.

essential that the β L portion of the conjugate be adequately dextran-modified since there are no currently

available methods for amelioration of the immunogenic response to this bacterial protein by recombinant technology as exists for antibodies. Dextran modification of the β L was, by itself, an effective method of reducing the immunogenic response as seen in Figure 1.

While the two Fab's tested in this work were of murine origin, and would thus be expected to be unsatisfactory for human trials, a number of humanized fragments are under development in this laboratory as potential candidates for the final conjugate. It was deemed appropriate, however, to establish the parameters and limits on the modulation of dextran attachment in model studies since such methodology should, in principle, be applicable to most proteins of future interest. It was also of some interest to determine if conditions could be found which would allow partial dextran modification while preserving immunoreactivity, thereby opening new possibilities for otherwise unsuitable murine antibody fragments. F(ab')₂ was used to study the amine-blocking reagents since this molecule is stable and can be easily reduced to Fab' for conjugation to β L. Reagent treatment and dextranation of Fab' was performed in some experiments and showed no discernible differences compared to that of F(ab')₂.

The effect of the amine-blocking reagents was evaluated by measuring the changes in dextran attachment, in effect, a measure of available amines with regard to both the moles of dextran per F(ab')₂, as determined by size exclusion chromatography in Figure 4, and the degree of dextran intramolecular cross-linking as indicated in Figure 5. TNBS was used to provide approximate values for the number of reactive amines before and after MeAcM treatment. Though subject to interference when used for absolute quantitation (22), the number of amines determined as being available by this technique gave good proportionality to the number of dextrans attached to the protein (Table 1). The trend showing availability/attachment of 3–4 amines per mole of dextran in the MeAcM treatment range of 0–20 mM seen in Table 1 generally reflects the cross-linking trend seen in Figure 5. After treatment with 50 mM or higher MeAcM, the few remaining amines may have been sterically hindered, though the 50 mM MeAcM sample itself clearly had at least one dextran attached as indicated in Figure 4. The 50 mM sample was also the most heterogeneous as indicated by irregular peak shape and increased peak width in Figure 5 which reflects its transitional status between samples with cross-linked dextran attachment and those samples with essentially no dextran attachment.

Of the two amine-blocking reagents tested, MeAcM and NHS-Msc, MeAcM appeared to be the reagent of choice. MeAcM had little effect on the activity of the Fab' binding by itself, possibly due to the similarity in size and charge of the reaction product to lysine (Scheme 1). Pretreatment of the F(ab')₂ with increasing levels of MeAcM was able to facilitate a relatively orderly decrease in the number of dextrans attached in subsequent dextranation steps (Figure 4). An examination of Figures 4–6 suggests that there may be a level of MeAcM pretreatment at about 20–50 mM which could yield a conjugate with sufficient immunoreactivity but still with some protective dextran on the Fab'. Since even humanized antibodies and fragments can elicit anti-idiotypic immune response (2, 3), it may be desirable in some cases to maximize the level of dextran attachment even to sensitive antibodies/fragments which may have already been engineered for reduced immunogenicity. Clearly the reaction conditions will be different for different proteins, but this work

establishes a framework within which to determine those parameters.

The exact reasons for loss of protein activity, in this case the loss of Fab' immunoreactivity, after treatment with dextran is not clear. It is speculated that the loss of immunoreactivity after dextranation was likely due, at least in part, to steric hindrance. The antigens for both ZCE025 and CC49, CEA (31) and TAG-72 (32), respectively, are large proteins and as such might be expected to be more sensitive to steric problems in the proximal association with the antibody binding site. Such a relationship with substrate size was demonstrated with chymotrypsin, an enzyme which exhibited differential activity toward substrates of different sizes after it had been modified with poly(ethylene glycol) (33). The ability of the PEG-modified chymotrypsin to act on a denatured form of BSA but not on native BSA suggested that conformation of the substrate played a role as well. This may explain why dextran-modified T101 antibody maintained good immunoreactivity with its antigen (13) even though the antigen is a large cell surface protein (34).

Evidence from the current work supports the idea of steric hindrance to some extent in that the immunoreactivity approached native levels only at MeAcM concentrations where intramolecular cross-linking by the dextran was largely blocked (Figure 5). On the other hand, loss of immunoreactivity after treatment with NHS-Msc alone might suggest that reduced immunoreactivity resulted from loss of positive charge, also a consequence of amine reaction with dextran. Disruption of protein conformation or simple charge repulsion of the antigen could also account for loss of immunoreactivity after NHS-Msc treatment. Given that treatment with NHS-Msc levels as high as 10 mM had no effect on the immunoreactivity even though the net charge on the protein became more negative, it is at least evident that there was no particularly reactive or sensitive amino group with influence on the binding kinetics.

NHS-Msc may have application for limited blocking of key lysines or to deliberately increase anionic character of the protein for purposes of altered biodistribution, but its effectiveness was too limited for the current objectives. Though the main drawback to using NHS-Msc was loss of immunoreactivity, this reagent also showed a more limited ability to block dextran attachment. Levels of NHS-Msc of 100 mM were able to prevent dextran cross-linking only to the level achieved with about 10 mM MeAcM as determined by SDS-PAGE under reducing conditions (data not shown). Given the clear excess of reagent, it would appear that reaction with additional NHS-Msc was sufficiently unfavorable as to preclude further reaction. In fact, attempts to force further reaction with the *p*-nitrophenyl derivative of Msc simply precipitated the protein. Even with the NHS-Msc reagent, the occasional failure to recover the expected levels of protein suggested that protein stability was at issue.

The results from the immunoreactivity and biodistribution studies in Figures 3 and 8, respectively, suggest that removal of the acetimidyl groups on the Fab' portion of the conjugate may not be necessary to achieve an effective conjugate. From other studies with both dextranated (13, 14) and PEG-modified proteins (12, 30), it was anticipated that the dextran-modified version of the Fab'- β L conjugate would have an increased half-life in the blood compared to nondextranated conjugate. This may be due in part to the increased stability of such conjugates as measured by their resistance to proteases and denaturants (30, 35). Figure 8 shows the clearance half-life of the dextranated conjugate was prolonged

about 48–72 h over that of the nondextranated control. For some applications, this approach has been used deliberately to increase blood residence of proteins which would otherwise be cleared too rapidly (14).

There is often a relationship between higher circulating levels of antibody and the levels found localized at the tumor site (36), a correlation also observed in Figure 8. At the 48 and 72 h time points, the control levels in the tumor and blood have begun to drop appreciably while the level of dextranated conjugate remained much higher. For therapeutic applications, however, a high tumor to blood ratio is more important than the overall level in the tumor. For the enzymatic, site-specific activation of prodrugs, a higher tumor to blood ratio is desirable for minimization of nonspecific activation of prodrug by circulating conjugate. It is evident from Figure 8 that the tumor to blood ratio is higher for the nondextranated conjugate. While this is not the desired trend, the pharmacological variables are sufficiently complex that no conclusions can be drawn without the therapeutic studies to evaluate the efficacy of this conjugate after increased blood clearance protocols have been implemented. If blood clearance ultimately proves to be a limiting factor, another approach is the addition of agents to artificially remove circulating conjugate from the bloodstream as has been used successfully by others (37, 38).

Since dextran modification has been shown in this and previous work to be an effective method of reducing β L immunogenicity, future studies will focus on the immunogenic response to the various forms of conjugates suggested by this investigation, including (1) the current conjugate with partially dextran-modified murine Fab', (2) conjugate containing humanized Fab' with and without dextran modification, and (3) conjugates with intermediate levels of dextran attachment on both the β L and the Fab' portion of the conjugate.

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LITERATURE CITED

- Meyer, D. L., Jungheim, L. N., Law, K. L., Mikolajczyk, S. D., Shepherd, T. A., Mackensen, D. G., Briggs, S. L., and Starling, J. J. (1993) Site-Specific Prodrug Activation by Antibody- β -Lactamase Conjugates: Regression and Long-Term Growth Inhibition of Human Colon Carcinoma Xenograft Models. *Cancer Res.* 53, 3956–3963.
- Hinman, L. M., and Yarranton, G. (1993) *Annu. Rep. Med. Chem.* 28, 237–246.
- Shawler, D. L., Bartholomew, R. M., Smith, L. M., and Dilman, R. O. (1985) Human Immune Response to Multiple Injections of Murine Monoclonal IgG. *J. Immunol.* 135, 1530–1535.
- Khazaeli, M. B., Saleh, M. N., Wheeler, R. H., Hustler, W. J., Holden, H., Carrano, R., and LoBuglio, A. F. (1988) Phase I Trial of Multiple Large Doses of Murine Monoclonal Antibody CO17-1A. II. Pharmacokinetics and Immune Response. *J. Natl. Cancer Inst.* 80, 937–942.
- Khazaeli, M. B., Saleh, M. N., Liu, T. P., Meredith, R. F., Wheeler, R. H., Baker, T. S., King, D., Secher, D., Allen, L., Rogers, K., Colcher, D., Schlom, J., Shochat, D., and LoBuglio, A. F. (1991) Pharmacokinetics and Immune Response of 131I-Chimeric Mouse/Human B72.3 (Human g4) Monoclonal Antibody in Humans. *Cancer Res.* 51, 5461–5466.
- Phelps, J. E., Beidler, D. E., Jue, R. A., Unger, B. W., and Johnson, M. W. (1990) Expression and Characterization of a Chimeric Antibody with Therapeutic Applications. *J. Immunol.* 145, 1200–1204.
- Beidler, C. B., Ludwig, J. R., Cardenas, J., Phelps, J., Papworth, C. G., Melcher, E., Sierzega, M., Myers, L. J., Unger, B. W., Fisher, M., David, G. S., and Johnson, M. J. (1988) Cloning and High Level Expression of a Chimeric Antibody with Specificity for Human Carcinoembryonic Antigen. *J. Immunol.* 141, 4053–4060.
- Yokota, T., Milenic, D. E., Whitlow, M., and Schlom, J. (1992) Rapid Tumor Penetration of a Single-Chain Fv and Comparison with Other Immunoglobulin Forms. *Cancer Res.* 52, 3402–3408.
- Milenic, D. E., Yokota, T., Filpula, D. R., Finkelman, M. A. J., Dodd, S. W., Wood, J. F., Whitlow, M., Snoy, P., and Schlom, J. (1991) Construction, binding properties, metabolism and tumor targeting of a single-chain Fv derived from the pancreatic carcinoma monoclonal antibody CC49. *Cancer Res.* 51, 6363–6371.
- Use of functionalized polyethylene glycols for modifications of polypeptides*, pp 347–370, Zalipsky, S., and Lee, C., Eds. (1992) Plenum Press, New York.
- Delgado, C., Francis, G. E., and Fisher, D. (1992) The Uses and Properties of PEG-Linked Proteins. *Crit. Rev. Ther. Drug Carrier Syst.* 9, 249–304.
- Beauchamp, C. O., Gonias, S. L., Menapace, D. P., and Pizzo, S. V. (1983) A New Procedure for the Synthesis of Polyethylene Glycol-Protein Adducts, Effects on Function, Receptor Recognition, and Clearance of Superoxide Dismutase, Lactoferrin, and α -2-Macroglobulin. *Anal. Biochem.* 131, 25–33.
- Fagnani, R., Hogan, M. S., and Bartholomew, R. (1990) Reduction of Immunogenicity by Covalent Modification of Murine and Rabbit Immunoglobulins with Oxidized Dextran of Low Molecular Weight. *Cancer Res.* 50, 3638–3645.
- Wileman, T. E., Foster, R. L., and Elliott, P. N. C. (1986) Soluble asparaginase-dextran conjugates show increased circulatory persistence and lowered antigen reactivity. *J. Pharm. Pharmacol.* 38, 264–271.
- Richter, W., and Kagedal, L. (1972) Preparation of Dextran-Protein Conjugates and Studies of their immunogenicity. *Int. Arch. Allergy Immunol.* 42, 885–902.
- Kabat, E. A., and Bezer, A. E. (1958) The effect of variation in molecular weight on the antigenicity of dextran in man. *Arch. Biochem. Biophys.* 78, 306–318.
- Meyer, D. L., Jungheim, L. N., Mikolajczyk, S. D., Shepherd, T. A., Starling, J. J., and Ahlem, C. N. (1992) Preparation and Characterization of a β -Lactamase-Fab' Conjugates for the Site-Specific Activation of Oncolytic Agents. *Bioconjugate Chem.* 3, 42–48.
- Mikolajczyk, S. D., Meyer, D. L., Starling, J. J., Law, K. L., Rose, K., Dufour, B., and Offord, R. E. (1994) High Yield, Site-Specific Coupling of N-Terminally Modified β -Lactamase to a Proteolytically Derived Single-Sulphydryl Murine Fab'. *Bioconjugate Chem.* 5, 636–646.
- Fagnani, R., Halpern, S., and Hagan, M. (1995) Altered pharmacokinetic and tumour localization properties of Fab' fragments of a murine monoclonal anti-CEA antibody by covalent modification with low molecular weight dextran. *Nucl. Med. Commun.* 16, 362–369.
- Yoshitake, S., Yamada, Y., Ishikawa, E., and Masseyeff, R. (1979) Conjugation of Glucose Oxidase from *Aspergillus niger* and Rabbit Antibodies Using N-Hydroxysuccinimide Ester of N-(4-Carboxycyclohexylmethyl)-Maleimide. *Eur. J. Biochem.* 101, 395–399.
- Ludwig, M. L., Hunter, and M. J. (1967) Amidation. *Methods Enzymol.* 11, 595–605.
- Reynolds, J. H. (1968) Acetimidation of Bovine Pancreatic Ribonuclease A. *Biochemistry* 7, 3131–3135.
- Wallace, C. J. A., and Harris, D. E. (1984) The preparation of fully N- ϵ -acetimidylated cytochrome c. *Biochem. J.* 217, 589–594.
- Tesser, G. I., and Balvert-Geers, I. C. (1975) The methylsulfonyl ethoxycarbonyl group, a new and versatile amino protective function. *Int. J. Pept. Protein Res.* 7, 295–305.
- Lamoyl, E. (1986) Preparation of F(ab)2 Fragments from Mouse IgG of Various Subclasses. *Methods Enzymol.* 121, 652–663.

- (26) Parham, P. (1983) On the Fragmentation of Monoclonal IgG1, IgG2a, and IgG2b from Balb/c Mice. *J. Immunol.* 131, 2895–2902.
- (27) Habeeb, A. F. S. A. (1966) Determination of Free Amino Groups in Proteins by Trinitrobenzenesulfonic acid. *Anal. Biochem.* 14, 328–336.
- (28) Markwell, M. A. K. (1982) A new solid-state reagent to iodinate proteins. *Anal. Biochem.* 125, 427–432.
- (29) Viles, F. J., Jr., and Silverman, L. (1949) Determination of Starch and Cellulose with Anthrone. *Anal. Chem.* 21, 950–953.
- (30) Abuchowski, A., McCoy, J. R., Palczuk, N. C., Es, T. v., and Davis, F. F. (1976) Effect of Covalent Attachment of Polyethylene Glycol on Immunogenicity and Circulating Life of Bovine Liver Catalase. *J. Biol. Chem.* 252, 3582–3586.
- (31) Kuroki, M., Fernstein, P. D., Wunderlich, D., Colcher, D., Simpson, J. F., Poole, D. J., and Schlom, J. (1990) Serological mapping of the TAG-72 tumor-associated antigen using 19 distinct monoclonal antibodies. *Cancer Res.* 50, 4872–4879.
- (32) Johnson, V. G., Schlom, J., Peterson, A. J., Bennett, J., Magnani, J. L., and Colcher, D. (1986) Analysis of a human tumor-associated glycoprotein (TAG-72) identified by monoclonal antibody B72.3. *Cancer Res.* 46, 850–857.
- (33) Chiu, H.-C., Zalipsky, S., Kopeckova, P., and Kopecek, J. (1993) Enzymatic Activity of Chymotrypsin and Its Poly(ethylene glycol) Conjugates toward Low and High Molecular Weight Substrates. *Bioconjugate Chem.* 4, 290–295.
- (34) Royston, I., Majda, J. A., Baird, S. M., Meserve, B. L., and Griffiths, J. C. (1980) Human T cell antigens defined by monoclonal antibodies: the 65,000-dalton antigen of T cells (T65) is also found on chronic lymphocytic leukemia cells bearing surface immunoglobulins. *J. Immunol.* 125, 725–731.
- (35) Liliberte, M., Gayet, J.-C., and Fortier, G. (1994) Surface modification of horseradish peroxidase with poly(ethylene glycol)s of various relative molecular masses (M_r): relationship between the M_r of polyethylene glycol and the stability of horseradish peroxidase-poly(ethylene glycol) adducts under various denaturing conditions. *Biotechnol. Appl. Biochem.* 20, 397–413.
- (36) Boxer, G. M., Begent, R. H. J., Kelly, A. M. B., Southall, P. J., Blair, S. B., Theodorau, N. A., Dawson, P. M., and Ledermann, J. A. (1992) Factors influencing variability of localization of antibodies to carcinoembryonic antigen (CEA) in patients with colorectal carcinoma—implications for radioimmunotherapy. *Br. J. Cancer* 65, 825–831.
- (37) Kerr, D. E., Garrigues, U. S., Wallace, P. M., Hellstrom, K. E., Hellstrom, I., and Senter, P. D. (1993) Application of Monoclonal Antibodies against Cytosine Deaminase for the in Vivo Clearance of a Cytosine Deaminase Immunoconjugate. *Bioconjugate Chem.* 4, 353–357.
- (38) Sharma, S. K., Bagshawe, K. D., Burke, P. J., Boden, R. W., and Rogers, G. T. (1990) Inactivation and Clearance of an Anti-CEA Carboxypeptidase G2 Conjugate in Blood After Localisation in a Xenograft model. *Br. J. Cancer* 61, 659–662.

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TECHNICAL NOTES

A Biotinylated Undecylthiophene Copolymer Bioconjugate for Surface Immobilization: Creating an Alkaline Phosphatase Chemiluminescence-Based Biosensor

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Methodology is described for the creation of a molecular assembly consisting of the enzyme alkaline phosphatase immobilized onto a glass surface using a biotinylated conjugated copolymer, poly(3-undecylthiophene-co-3-thiophenecarboxaldehyde) 6-biotinamidohexanohydrazone. The biotinylated polymer is attached to the inside walls of a silanized glass capillary via hydrophobic interactions, and a streptavidin-conjugated alkaline phosphatase is interfaced with the polymer through the classical biotin–streptavidin interaction. Utilizing a simple optical setup, we can detect the activity of as little as approximately 0.1 fmol of alkaline phosphatase with this molecular assembly. The assembly is mechanically robust and retains the majority of bound enzyme activity for up to 30 days. We have utilized this molecular assembly for the detection of organophosphorus-based pesticides. Both paraoxon and methyl parathion inhibit the enzyme-mediated generation of chemiluminescence signal. We are able to detect paraoxon and methyl parathion concentrations down to 500–700 ppb.

INTRODUCTION

We are pursuing the creation of monolayer and thin film materials that incorporate biological macromolecules and conjugated polymer for applications in optoelectronics and biosensors (1). The development of such practical devices is dependent upon devising appropriate procedures for the immobilization and stabilization of the biological component. Over the last few years, our aim has been to interface biomolecules with conducting polymers which possess unusual optical and electronic properties. Besides being potentially useful in signal transduction, these polymers offer ruggedness and stability.

Streptavidin, a tetrameric protein, has the ability to bind four molecules of biotin and derivatized forms of biotin with exceptionally high affinity (2, 3). This property has led to its wide application in bioanalytical and biomedical research (4, 5). Previous studies undertaken by our group have involved creation of molecular assemblies using streptavidin as a coupling component between a polymer support and biotinylated macromolecules. These assemblies included a biotinylated lipid monolayer cassette system (6) and biotinylated derivatives of conjugated polymers (7) for the creation of a more robust cassette system.

Derivatives of polythiophenes are a widely investigated

class of conjugated polymers because of their stability and processability (8–10). We recently reported the synthesis of a biotinylated copolymer of a 3-alkylthiophene and 3-(hydroxymethyl)thiophene. The copolymer was used to orient and spatially organize protein molecular assemblies utilizing the Langmuir technique (11).

In the present report, we modify this system by employing a longer spacer arm between the polymer and the biotin group. The copolymer synthesized was poly(3-undecylthiophene-co-3-thiophenecarboxaldehyde) 6-biotinamidohexanohydrazone (B-PUHT). Longer spacers are known to enhance biotin binding to the deep set biotin binding cleft of the streptavidin subunits (12). In addition to enhancing thermal stability, the alkyl chain side groups on B-PUHT impart hydrophobicity for van der Waals interactions with hydrophobic surfaces.

We describe the development of a molecular assembly created by the attachment of B-PUHT to the inside walls of a silanized glass capillary via hydrophobic interactions. Streptavidin-conjugated alkaline phosphatase was immobilized on the polymer matrix. Alkaline phosphatase catalyzes the dephosphorylation of organophosphorus (OP) compounds. An interesting substrate for this enzyme is the chemiluminescent 3-(4-methoxyspiro[1,2-dioxetane-3,2'-tricyclo[3.3.1.1]decan]-4-yl)phenyl chlorophosphate (CSPD) (13). The utilization of chemiluminescence, the production of light as a result of chemical reactions, has gained favor as a method of choice for molecule detection. Its major advantages over fluorescence-based techniques are the lack of the requirement of an external light source for excitation energy and the avoidance of background buildup as is the case for fluorescent substrates. Dephosphorylation of CSPD results in the formation of a product in an excited state. Upon de-excitation, a chemiluminescence signal is spontaneously generated. Detection is based on chemilumi-

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nescence measurements by a simple optical setup. We also describe the utilization of the molecular assembly for the detection of OP-based pesticides. These pesticides act as inhibitors to the alkaline phosphatase-catalyzed dephosphorylation of CSPD. Without optimization of this system, we can detect paraoxon and methyl parathion around 500–700 ppb. We discuss this immobilization strategy as a generic approach for a number of different sensing applications.

EXPERIMENTAL METHODS

Materials. 3-Undecylthiophene was purchased from TCI America (Portland, OR). 3-Thiophenecarboxaldehyde, ferric chloride, and acetic acid were obtained from Aldrich Chemicals (Milwaukee, WI). Chloroform, dichloromethane, and methanol were also obtained from Aldrich Chemicals but distilled under nitrogen prior to use. 6-Biotinamido-hexano-hydrazide (or biotin-LC-hydrazide) was purchased from Pierce (Rockford, IL), and all reagents were used without further purification. Streptavidin-conjugated alkaline phosphatase, supplied as an aqueous preparation, was purchased as a part of the Southern-Light Chemiluminescent Detection System from Tropix, Inc. (Bedford, MA). Diethylamine (DEA), blocking buffer (0.2% casein in phosphate-buffered saline), and CSPD were among other components of the system. CSPD was received as a 25 mM aqueous solution. Sapphire, a luminescence-amplifying material (referred to as enhancer), was also obtained from Tropix, Inc. Magnesium chloride was purchased from Fisher Scientific (Fair Lawn, NJ). Paraoxon and methyl parathion were supplied by Sigma Chemicals Co. (St. Louis, MO). All other solvents were supplied by Aldrich Chemicals (Milwaukee, WI). Deionized and distilled water was used in all preparations.

Instrumentation. Characterization of the polymer was facilitated via IR and UV-vis spectroscopy. The IR spectra were recorded on a Perkin-Elmer 1760X FTIR spectrophotometer. UV-vis spectra were recorded on a Perkin-Elmer Lambda 9 spectrophotometer. Number and weight average molecular weights (M_n and M_w) of the polymers were determined with a Waters 410 differential refractometer using chloroform as an eluent. The gel permeation chromatography (GPC) calibration was based on polystyrene standards. The column set consisted of Waters styragel 100, 500, 1000, and 10 000 Å.

Silanization. Glass capillaries (accupette, 100 mL, Dade Diagnostics, Miami, FL) were cleaned with Chem-solv detergent (10% v/v in water) in an ultrasonic bath and rinsed thoroughly in water. The cleaned capillaries were dried under nitrogen and subsequently immersed in a 2% (w/v) solution of chlorodimethyloctadecylsilane (CDOS) in petroleum ether for 30 min. After silanization, the capillaries were washed with petroleum ether to remove any unbound silane compound, air dried, and stored in a dust-free environment at room temperature.

Polymer Synthesis. Synthesis of B-PUHT was carried out as follows. 3-Undecylthiophene and 3-thiophenecarboxaldehyde were copolymerized in a molar ratio of 1:1 by the chemical dehydrogenation method using anhydrous FeCl_3 (14). A 10 mmol solution of the mixture in chloroform was polymerized using 40 mmol of ferric chloride at room temperature under nitrogen. After being stirred for 5 h, this copolymer, PUAT, was precipitated in methanol, filtered, and washed with an acetone-methanol mixture. This was followed by a Soxhlet extraction with methanol for 3 days to remove oligomeric and iron impurities. The copolymer was finally washed with aqueous ammonia solution to remove any trace

amounts of iron impurities and dried under vacuum. The yield was 93%, the $M_n = 17\,000$, and the $M_w = 27\,800$ by GPC analyses. 6-Biotinamido-hexano-hydrazide (0.024 g, 0.064 mmol) and PUAT (50 mg, 0.125 mmol equiv of CHO) in dichloromethane (50 mL) was stirred at room temperature until the Schiff's base was formed. The reaction mixture was then washed with water (3×10 mL) and dried (MgSO_4). The B-PUHT solution was concentrated and reprecipitated in methanol. The precipitated polymer was repeatedly washed with acetone and dried overnight under vacuum, at room temperature. $M_n = 20\,000$ and $M_w = 32\,700$ for the B-PUHT.

Polymer Attachment. The silanized capillaries were immersed in a 10 mg/mL solution of B-PUHT in chloroform for 16 h at room temperature. The adsorption of the polymer on the silanized glass surface was attributed to van der Waals interactions between the extended octadecyl alkyl group of CDOS and the alkyl side chain of the biotinylated polymer. The control involved immersion of the silanized capillaries in a 10 mg/mL solution of PUAT (the nonbiotinylated polymer) for 16 h at room temperature. These capillaries were treated in a manner identical to that of those coated with B-PUHT in further experimental procedures.

Immobilization and Detection of Bound Streptavidin-Conjugated Alkaline Phosphatase. The capillaries were removed from the B-PUHT-containing solution, and the polymer solution was drained. These capillaries were immersed in blocking buffer for 30 min to minimize nonspecific interactions. Streptavidin-conjugated alkaline phosphatase solution (25 nM in blocking buffer) was drawn into a capillary. The tube was sealed and placed in a water bath at 45 °C for 90 min. The optimal incubation temperature was derived from a temperature dependence study.

Following the 90 min incubation, excess conjugate solution was blotted out. The enzyme-bound capillaries were immersed in blocking buffer for 10 min before being washed for 30 min (3×10 minutes) in wash buffer (1X phosphate-buffered saline solution, pH 7.2). The capillaries were equilibrated in a freshly prepared assay buffer (0.1 M DEA, 1 mM MgCl_2 , pH 10) for 10 min prior to the addition of the substrate. Typically, 100 mL of 0.4 mM CSPD (in assay buffer, 10% enhancer) was drawn into the capillary to initiate the reaction. The capillary was wrapped in aluminum foil and placed on end, in front of the photomultiplier tube (PMT) window for chemiluminescence signal detection.

Kinetic Measurements. Different substrate concentrations were used to initiate the chemiluminescent reaction by aliquoting or diluting appropriate amounts of CSPD from the stock solution. The substrate solution was always made up with assay buffer containing 10% enhancer. The substrate concentration was varied from 0.05 to 0.6 mM. Higher CSPD concentrations were not achievable due to solubility constraints. The corresponding changes in reaction rates as a function of substrate concentration were computed from the changes in the rates of chemiluminescence signal generation.

Pesticide Detection. Stock solutions of paraoxon (2400 ppm) and methyl parathion (50 ppm) were prepared by dissolving them separately in assay buffer (15). Appropriate fractions from the stock solution were aliquoted into a microfuge tube containing 90 mL of 0.4 mM CSPD. The final volume was always adjusted to 100 mL. This reaction mixture was drawn into the capillary to initiate the chemiluminescence signal generation. This procedure was repeated for each successive lower paraoxon and methyl parathion concentration until the detection limit was reached.

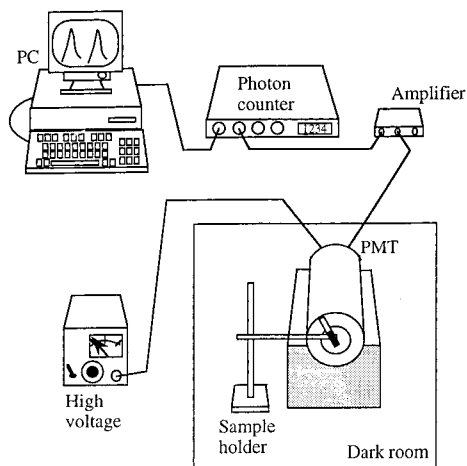


Figure 1. Schematic of the experimental setup for the detection of the chemiluminescence signal.

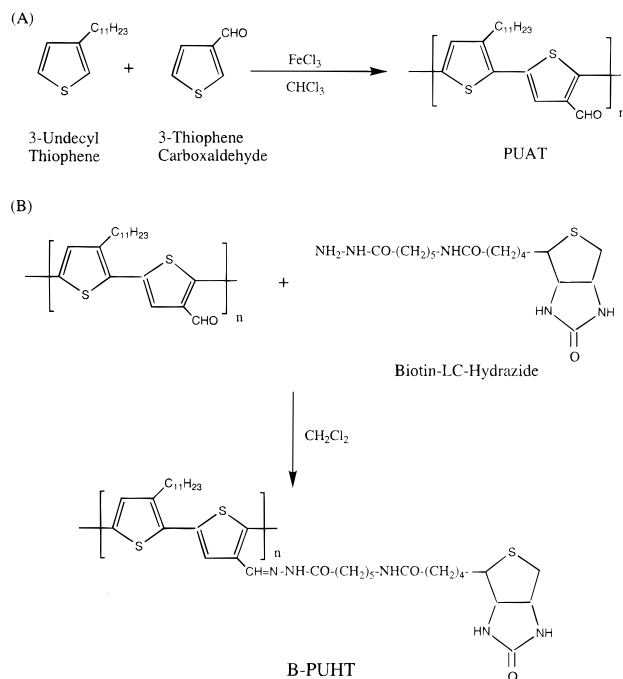


Figure 2. Schematic of the synthesis of B-PUHT. (A) Synthesis of the copolymer of 3-undecylthiophene and 3-thiophenecarboxaldehyde (PUAT) and (B) attachment of 6-biotinamido-hexanohydrazide through Schiff's base formation.

Signal Detection and Data Calibration. A photomultiplier tube (PMT, Hamamatsu R 943-02), a photon counter (Stanford Research Systems Inc., SR 400 2 channel-gated photon counter), and a microcomputer were used to collect and process the data. Figure 1 is a schematic of this simple experimental setup. The capillary was placed in front of the PMT glass window in a dark room. A photon counter connected to the PMT via an amplifier was placed in an anteroom. A personal computer hooked up to the photon counter was used to acquire and process the data. A typical experiment involved obtaining a real time curve of total photon counts per second versus time, as the photon counter was set up to count photons detected during 1 s intervals. Thus, enzymatic activity was the rate of chemiluminescence signal generation, which was the slope of the initial linear portion of the photon counts versus time plot. The final counts were corrected for background noise level. For kinetic studies, the raw data (real time curve) had to be integrated with time to generate a counts versus time plot. Initial rates of the reaction were then com-

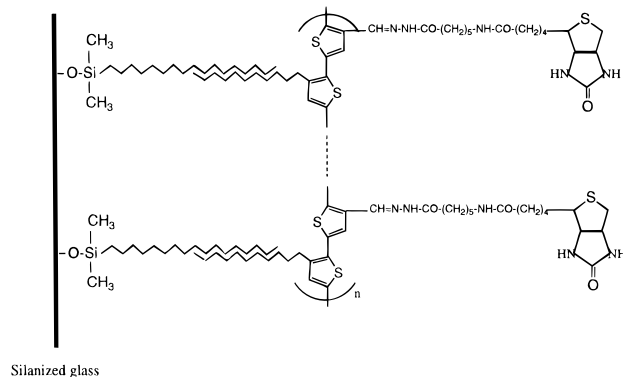


Figure 3. Hypothetical representation of biotinylated copolymer immobilization by hydrophobic interactions between the protruding octadecyl alkyl chain from a silanized glass surface and the extended undecyl alkyl side chain of the biotinylated thiophene polymer, B-PUHT.

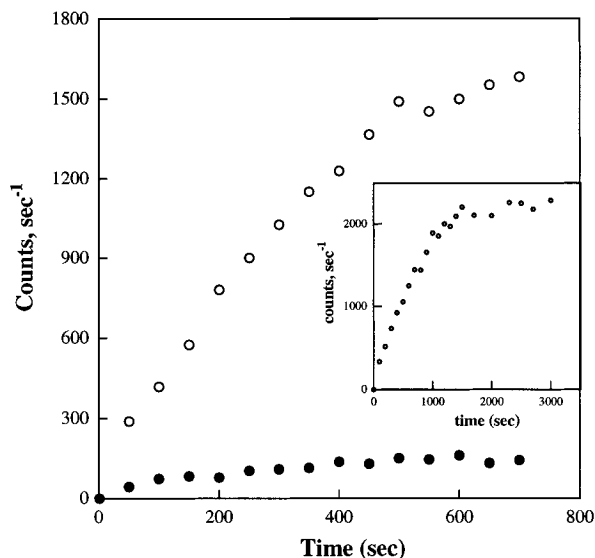


Figure 4. Typical chemiluminescence spectra of streptavidin-conjugated alkaline phosphatase immobilized on the inside walls of a glass capillary (0.4 mM CSPD, 10% enhancer): (○) enzyme conjugate interfaced with B-PUHT and (●) control—enzyme conjugate reacted with nonbiotinylated polymer.

puted from the slopes, calculated on the basis of initial data points collected. The signal profiles were highly reproducible. We observed minor batch variation effects with respect to the conjugate activity, and the data were normalized to account for these variations.

RESULTS AND DISCUSSION

The schematic for the synthesis of the conjugated polymer B-PUHT is shown in Figure 2 and involved two steps: (A) the synthesis of the copolymer of 3-undecylthiophene and 3-thiophenecarboxaldehyde (PUAT) and (B) subsequent attachment of 6-biotinamido-hexanohydrazide through Schiff base formation. The presence of the biotinamido-hexanohydrazide moiety was confirmed from FTIR results (data not shown). The Schiff's base formation was confirmed from the peak at 1627 cm^{-1} , the peak being characteristic of $\text{C}=\text{N}$ stretching vibrations.

Figure 3 is a schematic of interactive forces involved in the creation of the molecular assembly. B-PUHT was physisorbed onto the silanized glass capillary through hydrophobic interactions between the alkyl chain of the silane compound and the undecyl alkyl side chain of the polymer. Streptavidin-conjugated alkaline phosphatase was immobilized onto the polymer matrix via biotin-streptavidin interactions.

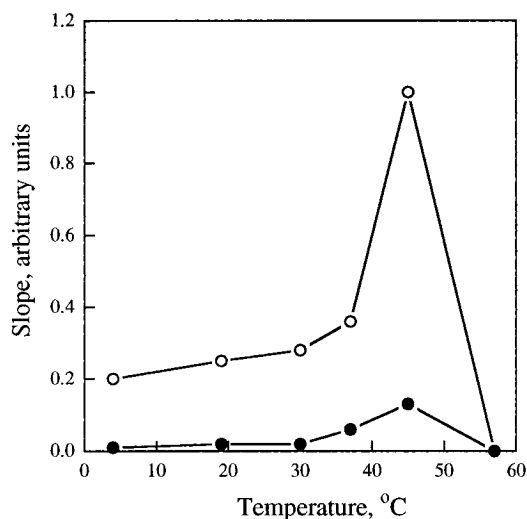


Figure 5. Effect of temperature on the conjugate binding step. The effect is also shown for the control—enzyme conjugate reacted with the nonbiotinylated polymer.

Table 1. Comparative Study of the Complete Molecular Assembly and Different Control Assemblies

controls	slope, arbitrary units
dark counts	0.0
unsilanized capillaries	0.01
silanized capillaries (no polymer)	0.06
enzyme complexed with free biotin	0.10
absence of the conjugated enzyme	0.06
absence of the substrate	0.05
nonbiotinylated polymer	0.15
experimental molecular assembly	4.0

Figure 4 illustrates a set of chemiluminescence data obtained with 100 μ L of 0.4 mM CSPD drawn into the glass capillary containing the immobilized enzyme. Typically, the signal increased steadily for 10 min and leveled off over a period of at least 50 min, as shown in the inset. The control consisted of silanized glass capillaries coated with PUAT (polythiophene copolymer lacking the biotin group of the side chain), and the resultant chemiluminescence signal observed was at background level as shown in the figure. Additional controls, shown in Table 1, were performed, including unsilanized capillaries, the absence of the enzyme conjugate, and the absence of CSPD. As is evident, the controls showed only background intensity levels. These results implied that the assembly successfully formed via specific biotin–streptavidin interactions and hydrophobic surface interactions with the polymer and retained enzymatic function.

The dependence of the conjugate binding step on temperature was investigated. Figure 5 represents the relative reaction rates computed from the generated chemiluminescence profiles of enzyme-immobilized capillaries, with the enzyme immobilization step being facilitated at the temperatures shown. The reaction rate at 45 °C was almost 3 times that observed at 25 °C. It is apparent from the figure that enzyme immobilization was maximized at 45 °C. This could be due to an increase in temperature, enhancing binding of the streptavidin conjugate by virtue of increased mobility of the biotinylated copolymer chain segments and the streptavidin binding site. This result led us to routinely process our conjugate binding step at 45 °C.

In an attempt to determine the amount of enzyme bound in the molecular assembly on the capillary surface, this activity was compared to known solution concentrations of the enzyme. First, the enzyme conjugate was dissolved in assay buffer at various concentrations. With

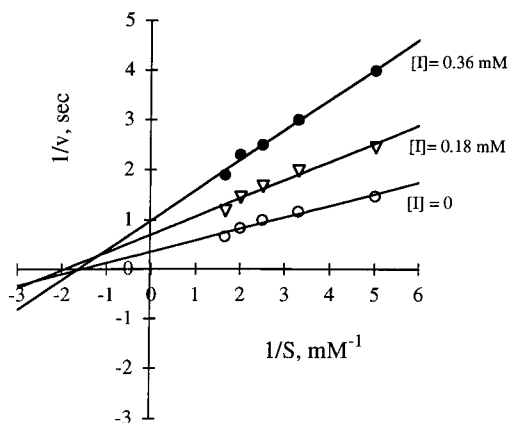


Figure 6. Lineweaver–Burk plot for paraoxon-mediated inhibition of alkaline phosphatase: (○) $[I] = 0$ mM, (Δ) $[I] = 0.18$ mM, and (●) $[I] = 0.36$ mM.

Table 2. Relative Activity of Immobilized Alkaline Phosphatase as a Function of Storage Time at 4 °C

days elapsed (after enzyme immobilization)	slope (normalized), arbitrary units
0	1.0
5	0.80
10	0.86
17	0.78
24	0.83
30	0.76

a 0.4 mM CSPD concentration, the reaction rates were computed for each enzyme concentration in the buffer. Next, 0.4 mM CSPD was drawn into the capillary containing the immobilized enzyme. The reaction rates were computed, and the enzyme concentration was determined from the solution calibration curve. This indicated the presence of 10^{-15} mol of the conjugate. Thus, the number of molecules immobilized was approximately 10^9 . Monolayer coverage involving the available surface area of the capillary and the size of the biomolecule [$110 \text{ \AA} \times 70 \text{ \AA}$ cross-section dimensions (16)] indicated approximately 1% surface coverage. We also estimated that 10% of the available enzyme was immobilized. The molecular assembly is mechanically robust and unaffected by vigorous washing. The immobilized enzyme retains up to 80% of its activity for a period of 30 days, when stored in 1X PBS at pH 7.2 at 4 °C as shown in Table 2.

As mentioned earlier, OP-based pesticides act as inhibitors during the process of alkaline phosphatase-catalyzed dephosphorylation of CSPD. Therefore, the kinetics of the immobilized enzyme in the molecular assembly were studied by a series of substrate dependent studies with the CSPD concentration varied from 0.05 to 0.6 mM, at a constant enzyme concentration. A plot of the reaction velocity, $[V]$, as a function of the substrate concentration, $[S]$, showed a classical $[S]$ versus $[V]$ dependence of an enzyme obeying Michaelis–Menten kinetics. A Lineweaver–Burk plot was generated for this set of data (Figure 6). The K_m for the immobilized enzyme was 0.58 mM^{-1} . This was close to the K_m measured for the free solution enzyme, 0.81 mM^{-1} (18). An investigation of paraoxon-mediated inhibition kinetics of capillary-immobilized alkaline phosphatase was carried out by varying the CSPD concentration while keeping the inhibitor (paraoxon) concentration at a constant value of either 0.36 or 0.18 mM. It can be observed in Figure 6 that the linear fits to these data do not intersect either on the x axis or on the y axis as would be the case for noncompetitive or competitive inhibition, respectively. This behavior, indicative of a mixed type of inhibition,

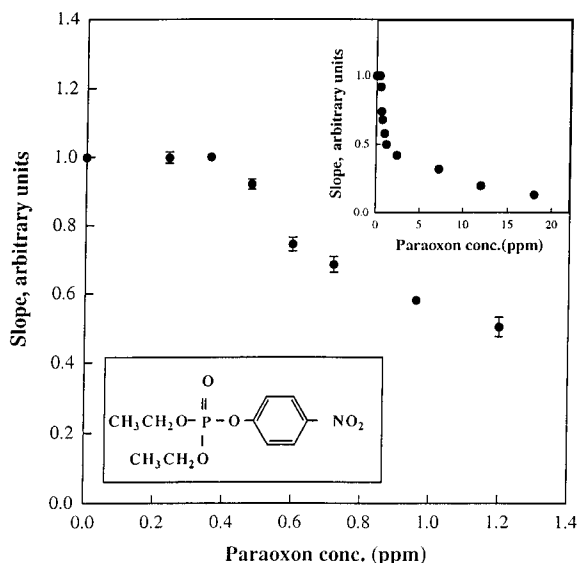


Figure 7. Calibration curve for paraoxon (0.4 mM CSPD, 10% enhancer). The inset shows the effect at higher paraoxon concentrations and the structure of the inhibitor.

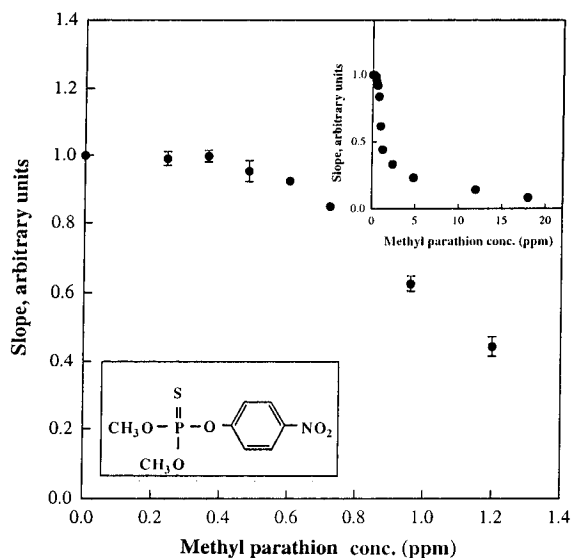


Figure 8. Calibration curve for methyl parathion (0.4 mM CSPD, 10% enhancer). The inset shows the profile for higher inhibitor concentrations and the structure of methyl parathion.

was similar to that observed and reported in solution-based studies of alkaline phosphatase (18).

Figure 7 demonstrated the extent of inhibition of the chemiluminescence signal in the presence of different paraoxon concentrations at fixed enzyme (10^{-15} mol) and substrate (0.4 mM CSPD) concentrations. The inset illustrated the chemiluminescence profile at higher paraoxon concentrations. The nonlinear behavior seen in the inset was commonly observed and may be attributed to high inhibitor concentrations altering the mechanism of inhibition. This system is capable of detecting 500 ppb paraoxon. Previously, we reported a 1.2 ppm detection limit utilizing a similar molecular assembly (17), but the conjugate binding step was performed at 25 °C. The improvement in the sensitivity of detection reported in this study is attributed to the higher levels of bound enzyme resulting from the change in the incubation temperature of the conjugate binding step to 45 °C. Figure 8 illustrates the extent of chemiluminescence signal inhibition for another organophosphorus pesticide, methyl parathion. The inset illustrates an increased chemiluminescence inhibition at higher methyl parathion

concentrations. This system is capable of detecting 700 ppb methyl parathion. Similar calibration curves for paraoxon and methyl parathion can be accounted for, presumably due to the structural similarity of these inhibitors.

We are currently investigating methods for enhancing the signal to noise ratio in the immobilized alkaline phosphatase assembly. Use of a conjugate with a higher specific activity and the utilization of a new enhancer (Sapphire-II), which affords a 3-fold signal amplification (19), are being pursued. Studies to investigate potential electrochemical signal transduction capabilities of the biotinylated thiophene copolymers and to create a more rugged molecular assembly are in progress. The biotinylated thiophene copolymer immobilization strategy holds promise for the development of a rapid and reasonably sensitive biosensor for OP-based pesticides.

CONCLUSIONS

A new methodology for the immobilization of biomolecules has been described. This generic approach was used to create a bioconjugate assembly for the detection of organophosphorus-based pesticides, paraoxon and methyl parathion. The enzyme alkaline phosphatase (conjugated with streptavidin in this case) was the sensing element in the detection of OP-based pesticides. The enzyme kinetics were studied and were in agreement with our previous demonstration of a mixed type of inhibition. In a simplified optical setup, 500 ppb paraoxon and 700 ppb methyl parathion were detectable and the molecular assembly was found to be reasonably stable and reusable. We have established the prototype of a conjugate alkaline phosphatase-based biosensor for organophosphorus compound detection in this study.

ACKNOWLEDGMENT

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LITERATURE CITED

- (1) Marx, K. A., Samuelson, L. A., Kamath, M., Sengupta, S., Kaplan, D. L., Kumar, J., and Tripathy, S. K. (1994) Intelligent Biomaterials Based on LB Monolayer Films. In *Molecular and Biomolecular Electronics* (R. R. Birge, Ed.) pp 395–412, Advances in Chemistry Series 240, American Chemical Society Books, Washington, DC.
- (2) Chaiet, L., and Wolf, F. J. (1964) The properties of Streptavidin, a biotin binding protein produced by Streptomycetes. *Arch. Biochem. Biophys.* 106, 1.
- (3) Green, N. M. (1975) *Adv. Protein Chem.* 29, 85.
- (4) Langer, P. R., Waldrop, A. A., and Ward, D. C. (1981) Enzymatic synthesis of biotin-labeled polynucleotides; Novel nucleic acid affinity probes. *Proc. Natl. Acad. Sci. U.S.A.* 78, 6633.
- (5) Wilchek, M., and Bayer, E. A. (1988) The Avidin-Biotin Complex in Bioanalytical Applications. *Anal. Biochem.* 171, 1.
- (6) Samuelson, L. A., Miller, P., Galotti, D. M., Marx, K. A., Kumar, J., Tripathy, S. K., and Kaplan, D. L. (1991) Oriented Fluorescent Streptavidin Conjugated Phycoerythrin Protein on Biotinylated Lipid L-B Monolayer Films. In *Proteins Structure, Dynamics and Design* (V. Renugopalakrishnan, P. R. Carey, I. C. P. Smith, S. G. Huang, and A. C. Storer, Eds.) pp 160–164, Escom Science Publishers BV, Leiden, The Netherlands.
- (7) Samuelson, L. A., Kaplan, D. L., Lim, J. O., Kamath, M. N., Marx, K. A., and Tripathy, S. K. (1994) Molecular

- Recognition Between a Biotinylated Polythiophene Copolymer and Phycoerythrin Utilizing the Biotin-Steptavidin Interaction. *Thin Solid Films* 242, 796.
- (8) Burroughs, J. H., Jones, C. A., and Friend, R. H. (1988) New Semiconductor device physics in polymer diodes and transistors. *Nature* 335, 137.
- (9) Naarman, H. (1990) The Development of Electrically Conducting Polymers. *Adv. Mater.* 2, 8, 345.
- (10) Elsenbaumer, R. L., Jen, K. Y., Miller, G. G., and Shacklette, L. W. (1987) Processable Environmentally stable, Highly Conductive forms of Polythiophene. *Synth. Met.* 18, 277.
- (11) Kamath, M., Lim, J. O., Chittibabu, K. G., Sarma, R., Samuelson, L. A., Akkara, J. A., Kaplan, D. L., Marx, K. A., Kumar, J., and Tripathy, S. K. (1993) Biotinylated Poly-(3-Hexylthiophene-co-3-Methanolphthiophene): A Langmuir Monolayer-Forming Copolymer. *J. M. S. Pure Appl. Chem. A* 30 (8), 493.
- (12) Green, N. M., Konieczny, L., Toms, E. J., and Valentine, R. C. (1971) The use of biotinyl compounds to determine the arrangement of subunits in avidin. *Biochem. J.* 125, 781.
- (13) Martin, C., Bresnick, L., Juo, R. R., Voyta, J. C., and Bronstein, I. (1991) Improved Chemiluminescent DNA Sequencing. *BioTechniques* 11, 1, 110.
- (14) Sugimoto, R., Takeda, S., Gu, H. B., and Yoshino, K. (1986) Preparation of soluble polythiophene derivatives transition metal halides as catalysts and their property. *Chem. Express* 1, 635.
- (15) *The Merck Index* (1989) p 1112, Merck & Co. Ltd., Rahway, NJ.
- (16) Coleman, J. E. (1992) Structure and Mechanism of Alkaline Phosphatase. *Annu. Rev. Biophys. Biomol. Struct.*, 441.
- (17) Ayyagari, M. S., Pande, R., Kamtekar, S., Marx, K. A., Kumar, J., Tripathy, S. K., Akkara, J. A., and Kaplan, D. L. (1995) Molecular Assembly of Proteins and Conjugated Polymers: Toward Development of Biosensors. *Biotechnol. Bioeng.* 45, 116.
- (18) Ayyagari, M. S., Kamtekar, S., Pande, R., Marx, K. A., Kumar, J., Tripathy, S. K., Akkara, J. A., and Kaplan, D. L. (1995) *Biotechnol. Prog* 11, 699.
- (19) *Tropix catalog* (1994) p 8, Tropix Inc., Bedford MA (Sapphire, Sapphire-II, Emerald, Emerald-II, Nitro BlocLuminescence Enhancers.
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REVIEWS

Vehicles, Chelators, and Radionuclides: Choosing the "Building Blocks" of an Effective Therapeutic Radioimmunoconjugate

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1. INTRODUCTION

The selective destruction of malignant cells using an immunoconjugate is attractive because of the apparent simplicity of the concept. A carrier molecule possessing desired affinity for the target tumor should serve as a selective delivery system for a cytotoxic agent, thereby achieving a localized cell killing while sparing normal body tissues. The field of immunotargeting as a whole consists of numerous diverse strategies, based around the delivery of cytotoxic proteins (Wawrzynczak, 1991), conventional chemotherapeutics (Smyth et al., 1986), and high-linear energy transfer (LET) radionuclides (Langmuir, 1992). Each strategy has its own set of related problems which serve to illustrate just how deceptive the conceptual simplicity of the "magic bullet" is. The latter approach, termed radioimmunotherapy (RIT), has suffered in that the targeting capacity of the carrier molecules used to date has barely been adequate for delivery of therapeutic doses to tumor, whereas their relatively long half-lives *in vivo* together with the radiation characteristics of the commonly used radionuclide ¹³¹I have resulted in high whole body radiation burdens and correspondingly poor therapeutic indices. In clinical RIT studies performed to date, a large variation in efficacy has been observed. This variation depends on many contributory factors, including radiosensitivity of the target tumor, its accessibility, and the distribution characteristics of the antibody selected for use. As a result, many studies have been performed on hematological cancers which have good systemic accessibility (Scheinberg et al., 1991; DeNardo et al., 1990) or employ-

ing a regional therapeutic approach such as that used against ovarian tumors in which the radioimmunoconjugate can be administered directly into the peritoneum (Epenetos, 1987). The generally disappointing clinical results so far obtained are not wholly surprising. RIT, as an experimental therapy, has often been applied to patients of poor prognosis. Despite this, in a recent clinical study using ¹³¹I-labeled monoclonal antibodies (mAbs) against the CD20 and CD37 antigens expressed on B-cell lymphoma, Press was able to achieve complete remission in 16 of the 19 relapsed patients with poor prognosis and who had been selected on the basis of favorable mAb distribution as determined in diagnostic studies. A further two patients showed partial remission, with one showing only a minor response (Press et al., 1993). These encouraging results were obtained using large doses of ¹³¹I of between 8.6 and 28.7 GBq, and all patients were given autologous bone marrow infusion to protect against myelosuppression. Similarly, in a contemporary study employing a ¹³¹I-labeled anti-CD20 antibody against B-cell lymphoma, Kaminski achieved objective responses in 9 of 10 patients for whom chemotherapy had failed (Kaminski et al., 1993). In 4 cases, complete remission was observed, lasting from 8–11 months. In this study, much lower levels of radioactivity were used, ranging from 1.26 to 2.44 GBq, using various administration protocols, and only mild to no myelosuppression was observed.

The future application of RIT as an adjuvant component in "first choice" therapies offered to newly diagnosed patients enjoying a better prognosis should allow a more realistic assessment of the value of this approach to be made. For the present, clinical results such as those referred to above encourage us to believe that, as the design of radioimmunoconjugates (RICs) is improved, the number of clinical situations in which a role for RIT can

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Table 1. Doses to the Least Irradiated Cell Nuclei

nuclide distribution	β^-					α , ^{211}At (≥ 5900 keV)
	Auger ^{67}Ga (< 10 keV)	^{131}I (180 keV)	^{111}Ag (350 keV)	^{90}Y (930 keV)		
homogeneous	1	1	1	1		1
cell nucleus	~ 20	~ 1	~ 1	~ 1		~ 1
cytoplasm	< 0.2	~ 1	~ 1	~ 1		~ 1
cell surface ($20\ \mu\text{m}$)	0	~ 1	~ 1	~ 1		~ 0.9
capillary ($50\ \mu\text{m}$)	0	~ 0.9	~ 1	~ 1		~ 0.8
capillary ($200\ \mu\text{m}$)	0	~ 0.8	~ 0.9	~ 1		0
capillary ($1000\ \mu\text{m}$)	0	~ 0.3	~ 0.7	~ 0.9		0

be identified will increase. In this review, we consider the broadening choice of therapeutic and carrier moieties available for use in RIC construction and the means whereby the two are coupled.

2. CHOICE OF THERAPEUTIC RADIONUCLIDE

2.1. Optimal Properties of Therapeutic Radionuclides. In RIT, potentially therapeutic radionuclides are transported to the desired site of action by target-selective vehicles. As a consequence, any consideration of the optimal properties of such nuclides must take into account the nature and *in vivo* behavior of the respective vehicle. Despite this obvious limitation, some generalizations can be made about the required physical characteristics of therapy nuclides, and these have formed the basis of numerous publications [e.g. Wessels and Rogus (1984) and Schubiger and Andres (1987)]. Of primary importance when selecting a nuclide for application with a given vehicle are the nature of the associated radiation, the ratio of penetrating to nonpenetrating radiation, the physical half-life ($t_{1/2}$) of the nuclide, the stability of any daughter nuclides formed, and, of practical significance, the route of production or availability.

Tumor cell killing is most effectively achieved by radionuclides emitting relatively high LET radiations which deposit the majority of their ionizing energy within the targeted tumor. Due to the diversity of vehicles and their sites of action, the optimal range in tissue of the particulate high-LET emissions varies accordingly. For illustration, some possible tissue distribution patterns are shown (Figure 1), and for each pattern, radiation doses to the cell nucleus have been calculated (Wernli, 1986), assuming the targeted nuclide to be either Auger electron-emitting, β -emitting (both high and low energies), or α -emitting. In Table 1, the resulting doses to the least irradiated cell nucleus, normalized to the doses delivered by homogeneously distributed nuclide, are given. The Auger electron emitters are shown to be quite potent but only if they can penetrate the cell membrane and come into close proximity with the nucleus. The α -emitters are also very potent cell-killing agents but are restricted in range to around $50\ \mu\text{m}$ in tissue, so that little "crossfire" can be expected. The clear implication here is that in both cases the nuclide distribution must be highly homogeneous with respect to the tumor volume in order to uniformly irradiate all tumor cells. As pointed out by Wheldon, even very modest levels of heterogeneity involving small numbers of untargeted cells are catastrophic in terms of tumor cure (Wheldon, 1993).

β -particle emitters yield a homogeneous dose distribution even when they are heterogeneously distributed within the target tissue. Depending on the β -particle energies, the tumor size for optimal curability will vary, for example from 3 mm for ^{131}I to 3 cm for ^{90}Y (Wheldon, 1993). Smaller metastases will receive a reduced dose due to deposition of energy outside the desired volume. A very detailed discussion of radiation dosimetry in RIT has been given by Humm (1986).

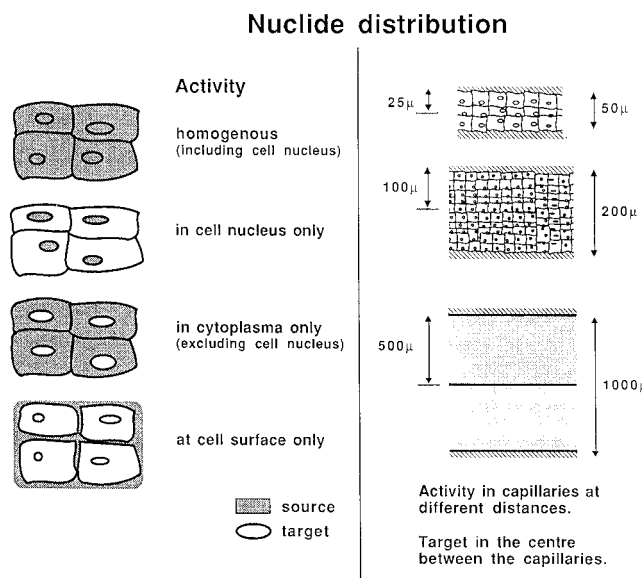


Figure 1. Effect of the microdistribution of α , β , and Auger electron-emitting radionuclides on the dose to target cell nuclei. Assuming seven different patterns of distribution—homogeneous, in the cell nucleus only, in the cytoplasm only, at the cell surface only, and then contained within capillaries 25, 100, or 500 μm from the target—doses to the least irradiated cell nucleus are calculated and normalized to the dose calculated assuming homogeneous distribution (Table 1).

The highly penetrating γ -radiation associated with many radionuclides contributes significantly to the whole body radiation burden borne by patients undergoing a program of RIT but does not contribute significantly to the tumor dose. Dosimetric calculations for RIT based on patient data with ^{131}I mAb have been published (Langmuir, 1992), suggesting that penetrating γ -radiation contributes up to 50% of the dose-limiting radiation toxicity to the bone marrow. The other 50% comes from nonpenetrating radiation (i.e. particulate, high-LET radiation). Similar calculations based on animal data and extrapolated to man resulted in a value of 30% as the contribution of penetrating radiation to the bone marrow dose (Buehler et al., 1991). Additionally, the high γ -component associated with ^{131}I is problematic for nuclear medicine personnel who either are repeatedly exposed to this danger or are forced to adopt impractical working methods. These factors taken together make a strong case for the replacement of the most commonly used nuclide, ^{131}I (main $\gamma = 360$ keV, 82% abundance), with nuclides displaying a lower accompanying γ -component. Ideally, however, nuclides possessing a low abundance (5–20%) of γ -radiation in the range of 100–200 keV may be optimal, as this allows easy measurement during experimental workup, biodistribution studies, scintigraphy, and dosimetric calculations.

In the case of high-energy β -emitters such as ^{90}Y , the bone marrow is subjected to a more uniform irradiation due to the higher penetrance of the emitted particles. This suggests it may be beneficial to use β -emitters of

lower energy, such as ^{67}Cu , so as to obtain a marrow-sparing effect (Langmuir, 1992). Elaborate accounts of tumor dosimetry following application of therapeutic nuclides have been made (Saenger et al., 1979; Humm, 1986; Jönsson and Strand, 1986; Langmuir, 1992), and dosimetric calculations relating to the use of Auger electrons have also been published (Booz, 1984; Humm and Cobb, 1990; Johnson et al., 1992; Sgouros, 1992, 1993).

In terms of the physical $t_{1/2}$ of any given radionuclide, this should approach the biological $t_{1/2}$ of the radioimmunoconjugate at the tumor. Clearly, attaining a sufficiently high integrated tumor dose to achieve sterilization (>50 Gy, Rösler et al., 1986) will prove problematic if a nuclide possesses a physical half-life which is inappropriately short for use with a chosen vehicle. On the other hand, a certain minimal dose rate (>0.4 Gy/h, Andres et al., 1986) must be achieved over a long enough period so as to ensure that all cells are irradiated during the most radiation sensitive M and G₂ phases of the cell cycle. For these reasons, a physical $t_{1/2}$ between 10 h and 10 d seems to be appropriate. Spencer has argued for the application of nuclides possessing short half-lives, on the order of a few hours, especially in the context of irradiating synovial membranes where leakage into the lymphatic channels, nodes, and distant parts of the body can be a problem (Spencer, 1987). Also, he has suggested the use of such nuclides in fractionated therapy. He noted, however, the shortcomings of this recommendation when considering applications where a significant delivery time is required (from the site of administration to the point of uptake by the lesion) and also the problem of the removal of necrotic tissue following rapid destruction.

Following the initial disintegration of a radioactive nuclide which has been chemically linked to a carrier molecule, the residual atom may be liberated and then distribute itself *in vivo* according to a pattern very different from that observed for the original therapeutic agent. If the resulting daughter nuclide is unstable, this could lead to unwanted and uncontrollable irradiation of nontarget tissues. For example, with the α -emitter ^{211}At , one has to take into account the biological fate of the daughter nuclides ^{211}Po and ^{207}Bi (Franke et al., 1986). As a consequence, only nuclides with stable or very long-lived daughter nuclides should be considered for application in RIT.

Biological vehicles take advantage of naturally occurring structures presented by the target cell. For any given vehicle, such sites of recognition are limited in number, resulting in a corresponding limitation in the number of molecules which can interact with the target cell (Spencer, 1987). In order to deliver an adequate dose to the tumor, it is therefore important that therapeutic nuclides can be produced in either a carrier-free form, or at least with high specific activity. As a consequence, production may require high-flux reactors to obtain sufficiently high neutron fluence or high-energy/high-beam cyclotrons as a source of protons. Finally, the separation of desired therapy nuclide from precursor nuclide can require sophisticated methods, such as that for the separation of ^{111}Ag from the target material Pd (Alberto et al., 1992).

2.2. Potential Therapeutic Nuclides. Having defined the optimal properties desirable in a therapeutic nuclide, a search can be made through the table of nuclides to find potential candidates. Depending on the precise definition and preferred properties, many lists have emerged in the literature [e.g. Schubiger and Andres (1987), Adelstein and Kassis (1987), Hoefnagel

Table 2. Physical Characteristics of Potential Therapeutic Radionuclides

nuclides	$T_{1/2}$	radiation type (MeV)	particle maximum range	authors
^{80}mBr	4.42 h	Auger, γ (0.037)	<10 nm	<i>d</i>
^{67}Ga	3.26 d	Auger, γ (0.09)	10 nm	<i>b</i>
^{125}I	60.0 d	Auger, γ (0.027)	10 nm	<i>d</i>
^{211}At	7.2 h	α (6.8)	65 μm	<i>b,d,e</i>
^{212}Bi	1.0 h	α (7.8), γ (0.72)	70 μm	<i>d,e</i>
^{169}Er	9.5 d	β (0.34)	1.0 mm	<i>c,d</i>
^{177}Lu	6.7 d	β (0.497), γ (0.208)	1.5 mm	<i>b</i>
^{161}Tb	6.91 d	β (0.51), γ (0.025)	1.7 mm	<i>b</i>
^{67}Cu	2.58 d	β (0.54), γ (0.185)	1.8 mm	<i>c,d</i>
^{105}Rh	1.48 d	β (0.57), γ (0.320)	1.9 mm	<i>b</i>
^{131}I	8.04 d	β (0.6), γ (0.364)	2.0 mm	<i>c-f</i>
^{77}As	1.62 d	β (0.68), γ (0.239)	2.5 mm	<i>b</i>
^{127}Te	9.4 h	β (0.7)	2.6 mm	<i>c</i>
^{153}Sm	1.95 d	β (0.8), γ (0.103)	3.0 mm	<i>b,d,f</i>
^{198}Au	2.7 d	β (0.97), γ (0.411)	4.4 mm	<i>c,d</i>
^{111}Ag	7.47 d	β (1.05), γ (0.34)	4.8 mm	<i>a,b</i>
^{149}Pm	2.21 d	β (1.07), γ (0.289)	5.0 mm	<i>b</i>
^{186}Re	3.77 d	β (1.08), γ (0.131)	5.0 mm	<i>c,d,e,f</i>
^{89}Sr	50.5 d	β (1.49)	8.0 mm	<i>d,f</i>
^{32}P	14.3 d	β (1.71)	8.7 mm	<i>b-d,f</i>
^{188}Re	16.95 h	β (2.13), γ (0.155)	11.0 mm	<i>b,c,e</i>
^{142}Pr	19.1 h	β (2.16), γ (1.6)	11.3 mm	<i>c</i>
^{90}Y	2.67 d	β (2.28)	12.0 mm	<i>b-f</i>

^a Hahn and Sheppard (1949). ^b Schubiger and Andres (1987). ^c Adelstein (1987). ^d Hoefnagel (1991). ^e Eary (1991). ^f Lewington (1993).

(1991), Eary (1991), and Lewington (1993)]. Table 2 shows the physical characteristics of those nuclides proposed in the above-mentioned reviews.

Some of these proposed nuclides do not fulfill the conditions relating to length of half-life (^{80}mBr , 4.42 h; ^{212}Bi , 1.0 h; ^{89}Sr , 50.5 d). As a result, these nuclides cannot be considered as realistic candidates for use in RIT when the vehicles presently available are employed. Others have a high abundance of γ -radiation (e.g. ^{131}I with a total of $\sim 90\%$ and ^{67}Cu with $\sim 60\%$). However, as ^{131}I has been used most extensively in RIT up to this point, it must also come into consideration and serves as a benchmark for comparison. ^{67}Cu is presently available on a limited basis and can also be used as a model system for studies on the behavior of metal nuclides *in vivo*.

For further discussion, some promising nuclides of differing types have been selected. As a typical Auger electron emitter, ^{67}Ga is chosen, from the β -emitters ^{131}I , ^{186}Re , ^{111}Ag , ^{67}Cu , and ^{90}Y and for the α -emitters ^{211}At .

The β -emitting nuclide ^{131}I , by far the most commonly used nuclide in RIT, has serious disadvantages. The high abundance of γ -radiation ($\sim 90\%$) contributes insignificantly to the dose to the tumor but does add heavily to whole body dose and to the dose-limiting toxicity to the bone marrow. As detailed in the preceding section, the penetrating radiation from ^{131}I may account for 30–50% of the radiation burden to the bone marrow. In this respect, ^{67}Cu (γ -radiation abundance $\sim 60\%$) would lead to a reduction of the radiation burden to the bone marrow, an effect even more significant in the cases of ^{111}Ag (γ -abundance $\sim 7\%$) and ^{186}Re (γ -abundance $\sim 16\%$). ^{90}Y has no γ -radiation, but its relatively high-energy β -particles together with its predilection for accumulation in the bone may add to the dose-limiting toxicity to the bone marrow (Langmuir, 1992).

The ranges of the β -particles emitted by ^{186}Re , ^{111}Ag , and ^{67}Cu permit the irradiation of several layers of tumor cells, a prerequisite for successful RIT. Of course, it must be borne in mind that there is no single ideal therapy nuclide, rather a number of candidates which are suited

to a greater or lesser extent to the employed vehicle and the clinical target. The development of vehicles capable of targeting tumor cell DNA may allow Auger-emitting nuclides such as ^{67}Ga to come into consideration. In the case of the α -emitting nuclides, their relatively short path length means that their effective application requires that almost every tumor cell be targeted by the radio-immunoconjugate, as may be achievable with certain blood-borne malignancies. Additionally, their application together with β -emitting nuclides as part of a nuclide "cocktail" approach may be feasible.

Another more practical consideration when selecting radionuclides for application in RIT is availability and quality. Methods for the production of therapeutic nuclides are not dealt with here, and the interested reader is referred to the review article of Volkert et al. (1991). It must be emphasized that one of the most important aspects of a production method is the achievable specific activity of the desired radionuclide. Obviously, therapeutic nuclides produced from an inactive isotope (e.g. ^{186}Re from ^{185}Re by n,γ -reaction) can hardly be carrier-free. However, strategies are occasionally available which allow for the enhancement of specific activities. For example, in the case of ^{186}Re , specific activity can be increased significantly by applying the long-known Szilard–Chalmers reaction, in which neutron capture by an inactive nucleus leads to emission of a γ -quantum, the recoil energy of which strips the active nucleus from its chemical environment. It is then usually stabilized in another chemical form, and if the two are sufficiently different, they can be separated by conventional chemical means. We have recently shown that the application of the Szilard–Chalmers reaction with the organometallic rhenium compound $[(\text{C}_5(\text{CH}_3)_5\text{ReO}_3)]$ yielded specific activities up to 30 times higher with only 1% of the total irradiation time necessary for conventional saturation (Cahn, 1995). For any nuclide of interest for therapy or diagnostic application, the resultant specification has to be adequate and reproducible within a narrow range of reliability as trace amounts of impurities may affect labeling yields.

At present, our own interests lie in the β -emitting nuclides ^{186}Re , ^{111}Ag , and ^{67}Cu , which we feel to be among the most promising for application in RIT.

2.3. Nuclide Chelators and Chemistry of Linkage. In some cases, a direct approach to radiolabeling of antibodies with therapeutic radionuclides can be adopted by reducing internal disulfide bonds within the antibody and allowing, for example, Re to react directly with the resultant sulfhydryl groups (Griffiths et al., 1991). This is directly analogous to the known direct-labeling methods for the diagnostic radionuclide $^{99\text{m}}\text{Tc}$. However, labeling strategies usually rely on the utilization of a multidentate ligand (or chelator) capable of selective chelation of the desired radionuclide. If the therapeutic nuclide is introduced into its chelator after the chelator has been attached to the carrier antibody, this is referred to as a postlabeling. However, one could also consider a prelabeling procedure, forming the radionuclide–chelator complex prior to activation and substitution into the antibody. The decision as to which labeling strategy is to be adopted will be influenced by a variety of considerations. For example, if the radionuclide is not abundantly available, the efficiency of each given step in the labeling procedure needs to be considered and may lead to the conclusion that a direct one-step postlabeling is most appropriate. Similarly, where complex formation between nuclide and chelator can only be achieved under nonaqueous or otherwise harsh condi-

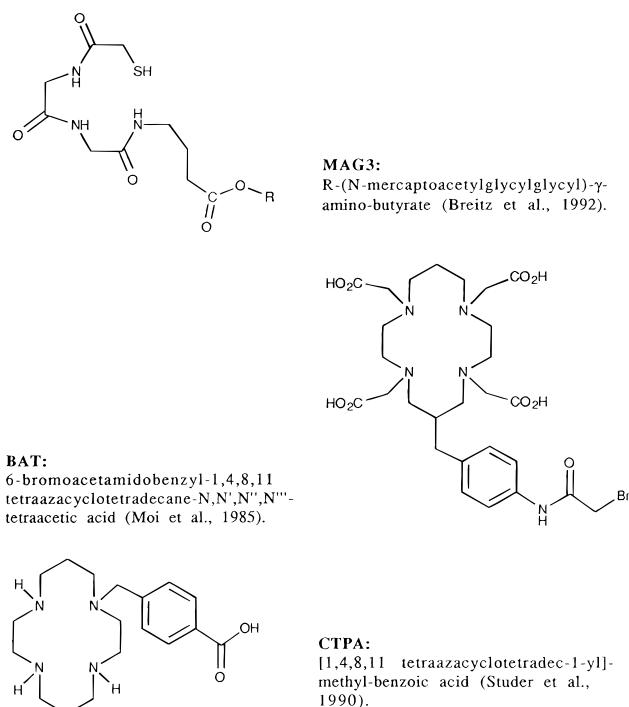


Figure 2. Representative chelators for use with the therapeutic radionuclides rhenium and copper: the open-chained MAG3 which has found application with ^{186}Re and the 14-membered macrocycles BAT and CTPA which have been used extensively with ^{67}Cu .

tions (i.e. in organic solvents or with heating), then clearly a prelabeling is indicated.

The choice of chelator for any given nuclide, if there is choice, may be influenced by the metabolic fate of the free nuclide and its chelated form. Ideally, the chelated nuclide would be rapidly excreted via the urinary tract without intracellular retention, with intact conjugate accumulating and being retained selectively at the tumor site. This is however clearly not always the case, and in several instances now, persistent and unwanted retention of metallic nuclides, presumably still in their chelated forms, has been reported. For example, Anderson et al. compared the biodistribution of the anti-colon carcinoma mAb 1A3 labeled with ^{64}Cu using BAT and CTPA (see Figure 2). Their conclusion was that there were no significant differences between the two ^{64}Cu -labeled intact mAb preparations in terms of the overall *in vivo* biodistribution but the accumulation of ^{64}Cu -CTPA-F(ab')₂ in the kidneys was 2.5 times greater than that of ^{64}Cu -TETA-F(ab')₂ (Anderson et al., 1993). As the *in vivo* behavior of a given nuclide–chelator complex cannot be predicted easily, the choice of potential multidentate ligands is guided by the field of conventional chelation chemistry. Once a radionuclide (and therefore an element) has been selected for use, the logical subsequent step is the development of a bifunctional form of the matched multidentate ligand, consisting of a coordination site for metal complexation and a linking site for covalent coupling to an available functional group within the protein (often an ϵ -amino group of lysine). Ligands which are capable of coordinating a given nuclide strongly (from a thermodynamic point of view) in a specific valency are known for all of the radionuclides of interest for an application in RIT. However, the demand for bifunctionality limits the application of these numerous ligands as it is often difficult from a synthetic point of view to introduce a reasonable linking group without changing the coordinative properties of the given ligand. Nevertheless, a relatively wide variety of such bifunctional

systems for different radionuclides of interest has been prepared and a number tested under *in vivo* conditions. The range of such ligands mirrors very well the chemical properties of the corresponding nuclides for which they were synthesized. For "simple" aquo-ions, such as Y(III), it was logical to apply polyamino-polycarboxylic ligands such as diethylenetriaminepentaacetic anhydride (DTPA), as they provide a linkage and a coordination site without the necessity for derivatization. This system has been applied in numerous studies (Kosmas et al., 1995; Stewart et al., 1990; Vriesendorp et al., 1991; Quadri et al., 1993a).

From a thermodynamic point of view, the philosophy of ligand choice is also clear in the case of Cu(II), where macrocyclic tetradentate amines of variable ring size have been chosen due to their very high stability. Within the group of ligands described for use with ^{67}Cu or ^{64}Cu , the coordination sphere of four nitrogen atoms can be found in most studies but the number of bridging methylene groups or the linking moiety differs for individual reasons in each study. Typical examples are TETA (Mausner et al., 1994) with a 14-membered macrocycle and DOTA with a 12-membered macrocycle. In the case of Sm(III), it was obvious to choose polyamino-polycarboxylates (Izard et al., 1992) and, more recently, polyphosphonate ligands, particularly EDTMP [ethylenediaminetetra(methylene phosphonate)], and derivatives thereof (Goeckler et al., 1987; Kasi et al., 1991).

The choice of the ligand is less obvious in the case of those elements with several possible oxidation states, such as the potential therapeutic nuclides ^{186}Re or ^{188}Re , or in the case of elements forming only weak or labile compounds with their ligands such as Au(I) ($^{199}\text{Au}/^{198}\text{Au}$) or Ag(I) (^{111}Ag). In the case of rhenium, the most promising results have been achieved with ligands similar or identical to those used for $^{99\text{m}}\text{Tc}$, for example ligands based generally on tetradentate N,S systems, the most prominent of which being of the MAG3 type (Figure 2). Ligands based on these systems but with different numbers of methylene groups between two coordinating atoms, and together with additional groups providing higher rigidity, have been investigated extensively (Goldschmidt et al., 1990; Beaumier et al., 1991; Breit et al., 1992; Visser et al., 1993). Although the labeling procedure is more complicated, the compound $[\text{ReO}(\text{MAG3})]$ seems to be of sufficiently high *in vivo* stability for clinical application, and phase I trials have already been published. Besides the MAG3 ligands, a different approach has been described using a hexadentate N_2S_4 ligand. One of the ligand thiol groups was used for covalent linkage to thiol groups in the protein which had been generated by reduction of disulfide bridges. Although the nature of the formed complex is not described, it seems reasonable to assume a similar coordination to a $\text{Re}=\text{O}$ moiety as occurs with the MAG3 ligands (Najafi et al., 1992). A new approach in this respect was the application of organometallic Re(I) compounds of the type $[(\text{C}_5\text{H}_5\text{R})\text{Re}(\text{CO})_3]$ where R stands for a linking group such as propionic acid (Salmain et al., 1993). Although this study was performed with the inactive complex only, the authors could prove with classical analytical methods the high *in vitro* stability of this complex when bound to antibody. This study could not be performed with radioactive material as the starting compound $[\text{Re}_2(\text{CO})_{10}]$ has to be prepared by a complicated high-pressure synthesis. Our own approach is similar in that we also intend to apply organometallic Re(I) complexes. We have recently described a convenient access to the fragment $[\text{Re}(\text{OH}_2)_3(\text{CO})_3]^+$ on the macroscopic and microscopic level with ^{188}Re (Alberto et al., 1995a). Although this

compound is organometallic in nature, it proved to be very stable in aerobic conditions in water even at elevated temperatures. We could show that $[\text{Re}(\text{OH}_2)_3(\text{CO})_3]^+$ reacts very readily with tridentate thiomacrocycles, such as derivatized 1,4,7-trithiacyclononane (9-aneS3) systems in buffer, to form extremely stable compounds of the general formula $[(9\text{-aneS3-R})\text{Re}(\text{CO})_3]^+$ where R stands for a linking group (Figure 3a). The inertness of the complex under widely varying chemical conditions leads to the expectation of a reasonable *in vivo* stability, although the radioimmunoconjugate so produced has not yet been fully tested. In addition, the complex $[\text{Re}(\text{OH}_2)_3(\text{CO})_3]^+$ can be considered as an aquo-ion and convenient postlabeling with, for example, macrocyclic thioethers should be possible in contrast to the prelabeling used with the conventional MAG3 ligands. These two examples in the case of rhenium serve to demonstrate the variety of possibilities to be faced when choosing a radionuclide with multiple potential oxidation states. The problems are different in the case of the second group of radionuclides such as Au(I) or Ag(I) where few thermodynamic stability constants have been quantified. One interesting study on the labeling of antibody with ^{199}Au or ^{198}Au did not use multidentate ligands for the stabilization of Au(I) but simple monodentate phosphane ligands which are known to form cluster-like compounds with Au(I). Such clusters were bound to antibody and were found to be stable, at least under *in vitro* testing, and also to retain their immunoreactivity. This study showed that multidenticity of ligands is not an absolute requirement for successful labeling, although the nature of the interaction between nuclide and protein was not confirmed in detail (Hainfeld et al., 1990; Kolsky et al., 1993).

We are working in our own group with the promising therapeutic radionuclide ^{111}Ag . From coordination chemistry, that Ag(I) is a very labile metal center which forms complexes of only moderate stability with most of the ligands investigated in the literature has to be appreciated. Stability constants rarely exceed 10^{12} , and the highest constant described for an 18-membered N_2S_4 system was 10^{14} (Craig et al., 1990). It has to be considered in the latter case that this is an absolute and not a conditional constant. This means that the amines will be protonated in physiological solution, with the conditional constants being therefore much lower. In our own group, we have decided to prepare hexadentate bifunctional thiocrown ethers, as we expect them to have relative high thermodynamic stability constants and, in addition, the ability to encapsulate the labile Ag(I) centers and thus avoid rapid transmetalation. An 18-membered hexadentate thiocrown ether has been published recently and its structure elucidated. The structure reveals an octahedral coordination geometry so that the macrocycle envelopes the Ag(I) center. This ligand, [18-aneS6], was not bifunctionalized, and we have extended the ring size by one hydroxyl-bearing methylene group to the 19-membered ring [19-aneS6-OH]. The structure of its Ag(I) complex $[\text{Ag}(19\text{-aneS6-OH})]^+$ could be resolved. It exhibits, in contrast to $[\text{Ag}(18\text{-aneS6})]^+$, only tetrahedral coordination geometry but with all Ag-S bond lengths being much shorter (Figure 3b). The probable reasons for this behavior are discussed elsewhere (Alberto et al., 1995b). The ^{111}Ag complex with [19-aneS6-OH] can be prepared quantitatively within minutes on the millimolar scale without applying a large excess of ligand. *In vitro* investigations proved that the complex is stable at least over a 10 d period without significant decomposition. Stability constants have been determined for the 18-, 19-, and 20-membered rings and

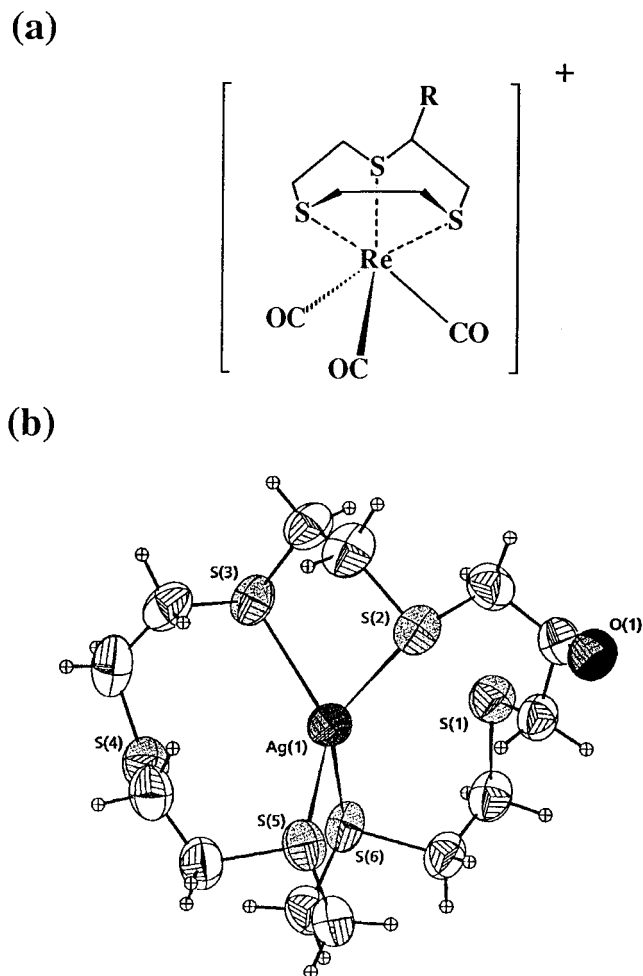


Figure 3. $[\text{Re}(\text{OH}_2)_3(\text{CO})_3]^+$ was found to react very readily with tridentate thiomacrocycles, such as derivatized [9-aneS3] systems, in buffer to form extremely stable compounds of the general formula $[(9\text{-aneS3-R})\text{Re}(\text{CO})_3]^+$ (a) where R stands for a linking group and an ORTEP representation (b) of the complex cation $[\text{Ag}(19\text{-aneS6-OH})]^+$.

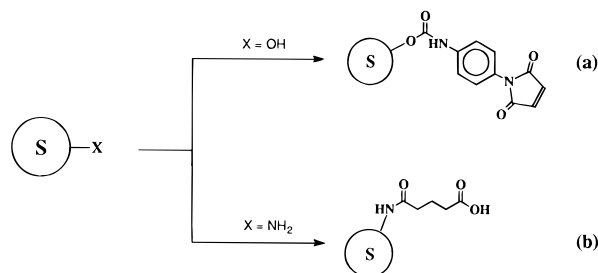


Figure 4. Functionalization of the hydroxyl of a multidentate ligand. Strategies are available to convert such a group into, for example, a maleimide (a) or more conveniently a carboxyl group (b).

found to be about 10^{13} for the former two and about 10^{12} for the latter complex. Compared to those of the Cu(II) complexes mentioned earlier, these constants are not particularly high, but in contrast, they are not dependent on the pH value of the solution and are thus both conditional and absolute stabilities. Despite the tetrahedral coordination in $[\text{Ag}(19\text{-aneS6-OH})]^+$ the Ag(I) center is rather well-shielded as could be shown by molecular-modeling studies based on the X-ray structure. The hydroxyl group can be functionalized in two different ways which are shown in Figure 4. In a recent paper, hydroxyl groups within various protein hormones were derivatized with a maleimide derivative (Annunziato et al., 1993), and we have used the same compound to

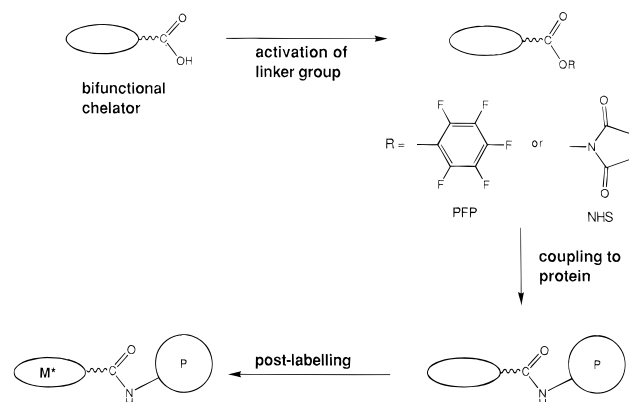


Figure 5. Activation, coupling to antibody, and subsequent radiolabeling of bifunctional ligands. In this example, NHS or PFP is used to convert a carboxyl group of the chelator into an active ester which can then react with primary amino groups in protein. The radiolabeling step is then performed in an appropriate buffer system.

introduce a maleimide function into the macrocycle as presented in path a. A more convenient approach is the exchange of the hydroxyl group for an amine which is subsequently reacted with glutaric anhydride to yield the bifunctional macrocycle presented in path b. This approach seems to be advantageous as carboxylic groups can be linked directly to the protein, whereas in the case of maleimide, additional thiol groups have to be generated in the protein.

Although the ligand has been coupled successfully to protein, postlabeling failed, probably due to nonspecific binding of ^{111}Ag to the protein. It can be concluded from these results that the prelabeling procedure might be the approach of choice with this radionuclide. It has to be emphasized in this respect that any approach employing direct labeling failed. Direct labeling of unmodified protein or a protein with additional thiol groups introduced by different standard protocols resulted either in aggregation, even with almost carrier-free ^{111}Ag , and/or in complete loss of immunoreactivity. We found additionally that such labeled protein exchanged the ^{111}Ag very quickly with potential competing ligand systems or other proteins.

From the above examples resulting from our own work and from the literature, it is clear that thermodynamic stability is not the only characteristic used to decide if a ligand or metal complex allows application under *in vivo* conditions. Kinetic stability is often more important, but as such data are rare, it is difficult to decide if a ligand of low stability, but possibly higher inertness, is worthy of investigation.

When one possesses an appropriate bifunctional ligand, the next step in the procedure is often the activation of the functional group so as to allow its reaction with the carrier moiety. A common strategy for ligand activation is given in Figure 5, where the functional group is a COOH group and potential leaving groups, R, are pentafluorophenol (PFP) or *N*-hydroxysuccinimide (NHS). Esterification with these groups is aided by the participation of variously substituted carbodiimides such as 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride (EDC), which are able to withdraw water resulting from the esterification process by forming urea compounds. Finally, the bifunctional ligand is reacted with an accessible primary amino group (predominantly on lysines) in the antibody. Obviously, this last step has to be performed in aqueous solution, and hydrolysis is a strong competitor to aminolysis. Usually, a very high excess of activated ligand has to be applied (up to a 100-

fold excess) to achieve a reasonable coupling yield. A study of the literature reveals that the linking function (the second function) is indeed very often a carboxylic acid; however, for almost any other reactive functional group, activation reagents and protocols have been described (Bodansky and Bodansky, 1984). One special case of the conventional coupling induced by NHS or other ester groups is the so-called anhydride method. This procedure for linkage is confined to particular ligands such as polyamino-polycarboxylic acids and is only possible when the system contains two carboxylic acids which are able to form 6-membered cyclic anhydrides. Amide formation again takes place at the primary amine group of lysine. One carboxylic acid group of the anhydride is subsequently again available for coordination to the nuclide. Most of the labeling experiments dealing with typical hard metal centers such as Y(III), In(III), and the lanthanides have been performed with this approach and been treated extensively in the literature.

A further general and very prominent example of labeling strategy is the intraconversion in organic solvents of primary amines by thiophosgene into isothiocyanato groups. The subsequent labeling protocol is easy because the isothiocyanato group reacts in water selectively with primary amine residues from lysine to form stable thiourea bridges. Loss of water solubility often causes practical problems when amine groups, which are positively charged at physiological pH, are converted into a neutral isothiocyanato function.

Should a bifunctional ligand bear thiol groups for linkage, this function can be used directly as a reducing agent for the disulfide bridges present within the carrier antibody. These disulfide bridges are cleaved under concomitant formation of a disulfide bridge to the ligand. Reduction has to be performed very carefully since reduction of too many disulfide bridges is often accompanied by loss of biological activity. A very detailed investigation in this respect was published by Najafi et al. (1992). This latter example demonstrates that linking protocols are not confined only to the most prominent ϵ -amino group in lysine but can take advantage of almost any other reactive group in the protein. An extension of the above-described strategy for binding ligands covalently to proteins consists of a previous derivatization of the latter by various chemical methods. One of these methods has already been mentioned and consists of the reduction of disulfide bridges for generation of reactive thiol groups in the protein. A more selective route for the introduction of thiol groups is by reaction of the ϵ -amino groups on lysines with various organic molecules such as 2-iminothiolane or *N*-succinimidyl 3-(2-pyridyldithio)propionate (SPDP). The introduced thiol groups react selectively with a number of functional groups on the bifunctional ligand such as a maleimide, which has already been mentioned in this text, or with a bromoacetamide function. One study which discussed this procedure in detail took advantage of 2-iminothiolane derivatization to bind the ligand 1,4,8,11-tetraazacyclotetradecane-*N'*,*N''*,*N'''*,*N''''*-tetraacetic acid (BAT, Figure 2) over a 6-*p*-(bromoacetamido)benzyl bridge to proteins (McCall et al., 1990). The advantage of this strategy over the activation of carboxylic acid by esterification is that hydrolysis does not significantly compete with the linking process; thus, yields are often higher and allow application of stoichiometric amounts of ligand. However, proteins with free thiol groups tend to cross-link by disulfide bridge formation in the presence of air,

and carefully controlled anaerobic conditions are the basis of a successful linking or labeling for these protocols.

An example of a labeling strategy designed to avoid interference with the mAb binding site is the selective oxidation of carbohydrates using periodate, which cleaves those C–C bonds with an adjacent OH group, to yield dialdehydes. These can then be reacted with a number of reagents, among them amines which result in a stable Schiff's base, which can be further stabilized by reduction with pyridine borane. Another interesting and truly "site-specific" approach has also been applied by Rose et al. (1991) in using the protease *Achromobacter lyticus* to effect reverse proteolysis and thereby attach carbonyldrazide to the C-terminus of des-Ala^{B30}. This allowed the attachment of an aldehyde-containing form of the chelator ferrioxamine by formation of a hydrazone bond. This approach was subsequently employed to form an F(ab')₂-like fragment from the mAb B72.3 (Fisch et al., 1992). Such labeling strategies, which seek to attach chelators at sites removed from the N-terminal region of the carrier protein and which thus avoid impairment of the binding site, are expected to be beneficial with respect to the retention of immunoreactivity, that is, the proportion of antibody molecules capable of binding to antigen following the labeling procedure.

Extensive and thorough discussions of many of the general techniques available and of interest for practical application in the field of protein conjugation have been published (Bodansky and Bodansky, 1984; Wong, 1993).

The *in vivo* application of mAbs labeled with metallic radionuclides, by methods such as those described above, has revealed unwanted accumulation in nontarget tissues. An important consideration in this context is the nature of the linker, or chemical bridge, between the carrier protein and the chelator, as this can significantly alter patterns of biodistribution (Meares et al., 1988; Deshpande et al., 1989). The latter study examined the effect of using disulfide, thioether, thiourea, and peptide linkers between the Lym-1 mAb and a benzyl ethylenediaminetetraacetic acid chelator. The latter three conjugates showed similar whole body and blood clearance curves, but the disulfide-linked conjugate was found to be rapidly cleaved in the blood and excreted. Despite their similar rates of clearance, the thioether-, thiourea-, and peptide-linked conjugates showed altered liver uptake and clearance, with liver:blood ratios significantly worse in the case of the peptide linkage. Similarly, the introduction of a diester linkage between a DTPA chelator and an anti-melanoma mAb resulted in more rapid clearance from the blood, and liver, spleen, and kidney accumulation were reduced when compared to that of the analogous peptide-linked radioimmunoconjugate (Paik et al., 1989).

In those instances where radiometal-labeled mAb fragments have been used, the kidney has proven to be the site of unwanted radionuclide accumulation (*vide infra*). In a recent study, four mAbs were labeled with ^{99m}Tc using the RP-1 complex as described by Weber et al. (1990) (Tibben et al., 1994). This complex consists of an N₃S chelator together with a linker containing an ester group. When the complex was tested in nude mice bearing ovarian tumor xenografts, the use of an ester linkage was again shown to significantly reduce nonspecific radionuclide uptake, whereas no significant differences in tumor accumulation were observed when comparing the RP-1 and directly labeled Fab' preparations.

3. CHOICE OF CARRIER MOIETY

3.1. Monoclonal Antibodies and Their Antigen-Binding Fragments. The development of selective anti-cancer therapeutics based on the principle of antibody targeting received new impetus in 1975 following the description of, at that time, innovative methodology allowing the generation of immortal murine cell lines secreting monoclonal antibodies (mAbs) of predetermined specificity (Kohler and Milstein, 1975). This technique centers around the fusion of antibody-secreting "B" lymphocytes, initially murine although a variety of other sources have more recently been used, with immortal myeloma cell lines. This technique brought with it the possibility of generation of a constant supply of a desired mAb directed against a defined tumor specific antigen (TSA). In initial *in vivo* studies performed in model systems consisting of mice bearing human tumor xenografts, selective uptake was modest with values around 1% of the injected dose per gram of tumor (%ID/g). The continual search for better antibodies of higher affinity has improved these values so that xenograft accumulation of 10–35%ID/g is commonly observed (Buchegger et al., 1990; Smith et al., 1994), with even higher values in particularly well-vascularized xenograft systems, although such manipulation of model test systems clearly has no relevance to clinical performance of a given RIC. Radioimmunoconjugates consisting of such murine mAbs and radiolabeled with ^{131}I have been used successfully to cure mice bearing a variety of xenografted human tumors such as neuroblastoma (Cheung et al., 1986), colon carcinoma (Buchegger et al., 1990), and small cell lung cancer (Smith et al., 1990). Despite intense efforts, no mAbs have yet been generated which fulfill original expectations of being truly tumor specific. They all display reactivity to antigens which are to some extent expressed on normal tissue and thus define tumor-associated antigens (TAA) rather than TSAs, the latter appearing to be a less realistic objective than was initially thought.

The pharmacokinetic characteristics of mAbs can be modified in that they can be subjected to selective proteolytic degradation to generate either a divalent immunoreactive fragment of 100 kDa, the F(ab')_2 fragment, or a monovalent immunoreactive Fab fragment of 50 kDa. The most commonly used proteolytic enzymes for F(ab')_2 and Fab generation are pepsin and papain, respectively, although a number of alternative enzymes are now available (Milenic et al., 1989). Apart from alteration of the pharmacokinetic behavior of the mAb, fragmentation may offer improvement by virtue of reduced immunogenicity.

Fab and F(ab')_2 fragments have been shown to possess pharmacokinetic characteristics which are distinctly different from those observed for the 150 kDa parent molecule and which may bring advantages in terms of therapeutic ratios. Both fragments display a more rapid partitioning and clearance *in vivo* and because of their reduced size show a more rapid and uniform penetration of tumor (Buchegger et al., 1983). Particularly in the case of the Fab, which is small enough to be cleared over the kidney by glomerular filtration, the lower levels of these fragments observed circulating in the blood and normal tissues have been shown to result in improved tumor to nontumor localization ratios and to allow improved radioimmunoscintigraphy for diagnostic purposes. Rapid clearance of Fab fragments together with their monovalency has, however, a second consequence in that absolute tumor uptake is much lower than with intact mAb. When considering the difficulty presently experienced in trying to achieve a therapeutic dose to the

tumor, requiring many gigabecquerel to be injected, we come to the conclusion that intact antibodies are currently to be favored over mAb fragments for therapeutic application. In addition, recent studies undertaken with radiometal-labeled F(ab')_2 fragments (Brown et al., 1987a; Anderson et al., 1992; Smith et al., 1993, 1994) have revealed altered patterns of *in vivo* distribution when compared with that of conventional radioiodine-labeled F(ab')_2 and, in particular, a high level of kidney uptake which appears to translate to the patient situation and may negate the use of such fragments with metal nuclides (Halpern et al., 1988). The reason for the altered patterns of distribution observed when comparing radioiodine and radiometals seems to center around the ability of radioiodide or radioiodotyrosine to exit cells following uptake and degradation of the immunoconjugate, whereas many metal nuclide-chelator complexes cannot exit the cell so readily (Press et al., 1990).

The transfer of murine mAbs from predominantly mouse model systems into the clinical situation is not without consequence, so that only limited conclusions can be drawn from preclinical work. For example, metal nuclide-labeled murine mAbs show little evidence of liver accumulation in mouse model systems but can show very high levels in patient studies using, for example, ^{67}Ga (Pochon et al., 1989; Ryser et al., 1992) and ^{67}Cu (Smith et al., 1993; Bischof-Delaloye et al., 1996). In contrast, the high accumulation of metal nuclides in the kidney, following administration of radiolabeled mAb fragments, is an effect which is observed both in animal model studies (Brown et al., 1987a; Anderson et al., 1992) and in patient studies (Halpern et al., 1988).

Human mAbs would be expected to offer a number of advantages over murine mAbs. For example, immunization of mice with human tumor cells tends to elicit responses against very obvious antigenic determinants, such as the HLA system. Patients clearly will not respond to such antigens which determine "self" and are therefore more likely to respond to more subtle and obscure TSAs (Sikora et al., 1983). Also, as a protein of human origin, any patient immune response to RICs constructed using such mAbs should be restricted to anti-idiotypic responses directed against the complementarity-determining regions (CDR) of the antibody or against the chelator. This would be expected to help in circumvention of the human anti-mouse antibody (HAMA) response associated with clinical studies involving murine mAbs and which is considered to be a hindrance to fractionated application of RIT. The most frequently used approach to the generation of human mAbs has been the fusion of B cells from the draining lymph nodes or from the peripheral blood of cancer patients with established murine myeloma cell lines. Initial studies on human mAbs, in which human antibody-secreting cells were fused with murine myeloma cells, were unfortunately plagued with problems in that a preferential deletion of human chromosomes by the resultant hybridomas was observed together with instability in culture (Glassy et al., 1987). Substitution of human myeloma cells so that human/human hybridomas were produced resolved the problem of instability but yielded only low amounts of mAb. Another approach to the immortalization of human cells which secrete antibody has been direct transformation using the Epstein-Barr virus, although this has also been found to lead to low-secreting cell lines requiring frequent recloning to ensure stability and often producing mAbs of the IgM class (Steinitz et al., 1977; Kozbor and Roder, 1981). These approaches seem now to have been largely surpassed by the techniques of genetic molecular engineering.

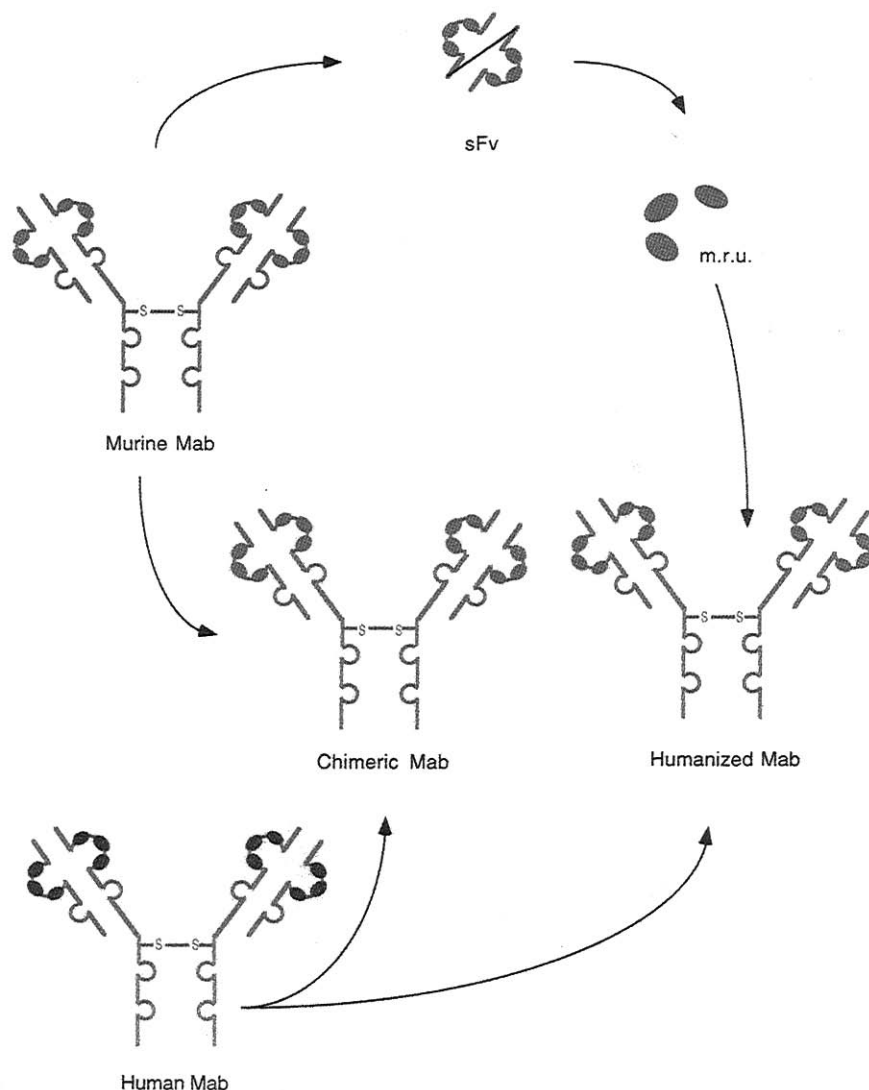


Figure 6. Genetic engineering of vehicles for use in RIT. Using these techniques, a range of possible constructs can be generated such as chimeric antibodies consisting of murine variable and human constant regions, humanized antibodies in which only the murine CDRs are grafted into a human immunoglobulin variable framework region, and single chain Fvs.

3.2. Genetic Engineering of Antibody Constructs.

The introduction of genetic engineering to the field of immunotargeting may well prove to be an even more significant event than the introduction of the original monoclonal antibody technology which motivated the field in the late 1970s. Perhaps the most obvious advantage to be gained from this methodology is the capacity to "humanize" mAbs so as to make them appear less foreign, and therefore less immunogenic, when used in clinical studies and, in some instances, to enhance recruitment of human effector mechanisms.

The first application of this approach was to construct chimeric mAbs in which the entire murine constant region was replaced with its human counterpart. For example, murine variable region genes from the B6.2 anti-cancer mAb and human constant region exons were fused to yield chimeric immunoglobulin genes which were cotransfected into murine myeloma cells which then secreted the intact chimeric antibody, cB6.2 (Brown et al., 1987b). However, this approach did not completely resolve the problem of immunogenicity of the murine variable region, although HAMA responses were markedly reduced and occasionally absent. It appears that each chimeric mAb is unique in its immunogenicity (Khazaeli et al., 1991).

Recognizing the degree of conformational similarity between the framework regions of the majority of anti-

bodies, Jones (Jones et al., 1986) suggested that the specificity of antibody binding may be independent of the framework regions, thereby opening up the possibility of further minimizing the murine nature of the antibody by grafting the rodent hypervariable CDRs into a human variable region framework. The resultant three-dimensional configuration of such constructs, and accordingly the affinity, has proven sometimes to be detrimentally effected, although the selection of human framework regions bearing strong similarity to the original rodent regions was found to reduce this risk (Queen et al., 1989). Additionally, molecular modeling can be used to identify, and subsequently preserve, critical amino acids outside the CDRs which may influence conformation.

Apart from the potential advantages to be gained by humanizing a given mAb, genetic engineering brings the possibility of altering pharmacokinetic behavior by a variety of means. For example, the same logic which was applied to conventional mAbs has also been applied to genetically engineered carriers. On the assumption that smaller molecules should partition more rapidly throughout the body, a series of genetically engineered mAb fragments have been generated (Figure 6). Antigen-binding Fv fragments are noncovalently bound heterodimers consisting of a variable light chain (V_L) and variable heavy chain (V_H). These have proven to be prone to dissociation, although the extent to which this occurs

varies from construct to construct. This tendency to dissociate can be overcome through insertion of a peptide sequence linking the two chains to give a single chain Fv, or sFv. The first *in vivo* studies involving an sFv were performed by Colcher. V_L and V_H regions taken from the murine B6.2 anti-cancer mAb were linked via a synthetic peptide bridge and the biological properties of the resulting sFv compared with the original B6.2 Fab' fragment (Colcher et al., 1990). *In vitro* cell binding studies revealed that the affinity constant of the sFv was not significantly different from that of the B6.2 Fab' fragment. Pharmacokinetic differences were however observed *in vivo*, in a mouse model system, where the reduced molecular mass of the sFv at 25 kDa led to more rapid clearance than was seen with the 50 kDa Fab'. Despite this, tumor xenograft accumulation of the two materials was found to be comparable. These initial encouraging results were found, unfortunately, to be the exception rather than the rule, and subsequent studies have revealed very low absolute accumulation of sFv fragments in tumor xenograft model systems (Schott et al., 1992).

Still smaller than the sFvs, but still possessing antigen binding activity, are single V_H domains (dAb) and even single CDRs, known as minimal recognition units (mru). In relation to such fragments, it must be borne in mind that there is a clear conflict between the need for rapid clearance from the body to reduce whole body toxicity and the need for higher tumor accumulation. It would seem that, at least at the moment, the two are mutually exclusive.

3.3. Chemical Engineering of Antibody Constructs. The powerful techniques of genetic engineering are not so widely available that they can be employed at will. A more direct and accessible approach to the generation of antigen binding constructs, possessing binding and pharmacokinetic characteristics other than those displayed by intact mAbs and their conventionally derived fragments, is the use of chemical linkage methodology to build novel constructs from naturally occurring mAb subunits. Using this approach, a number of fundamental questions relating to the modification of the *in vivo* behavior of carrier molecules can be addressed. For example, what is the effect on tumor localization of an increase in the valency of a mAb from divalent to trivalent? Or is a chemically stable form of an $F(ab)_2$ fragment less likely to find its way into the kidney and thereby reduce the problem of metallic nuclide uptake in this organ?

Schott has produced synthetically cross-linked multivalent constructs generated from antitumor antibodies by chemical engineering (Schott et al., 1993). Starting with a mAb directed against the TAG-72 TAA, Fab fragments were first made by enzymatic digestion and then reassembled using the cross-linking agents tris- and tetrakis-maleimide to give tri- and tetravalent constructs, respectively. The biodistribution patterns of these constructs, following labeling with the radionuclide ^{105}Rh , were evaluated in a nude mouse tumor xenograft model system. The tetravalent construct, $F(ab)_4$, showed blood clearance similar to that of normal $F(ab)_2$ fragment, but accumulations in the liver, spleen, and whole body were markedly higher. Only kidney accumulation was reduced, in line with the higher molecular mass and stability of the $F(ab)_4$ construct. The high liver accumulation observed in these studies prevented further *in vivo* evaluation. The trivalent construct, $F(ab)_3$, revealed a very different pattern of distribution. The residence time in the circulation of this construct was found to be longer than that of the $F(ab)_2$, and a

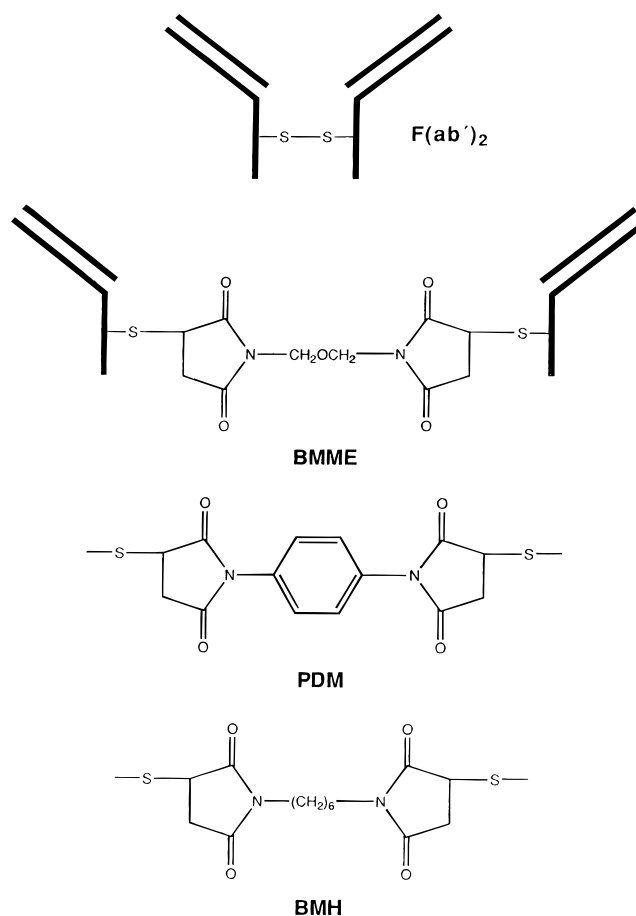


Figure 7. Chemical stabilization of murine $F(ab')_2$ fragments as described by Quadri et al. Following reduction of the native fragment, cross-linking over the available SH groups was performed using the commercially available reagents BMME, PDM, and BMH.

decreased level of kidney accumulation was seen. As a consequence of the longer circulating half-life, higher accumulation was seen in the tumor with the $F(ab')_3$ fragment than with the $F(ab')_2$. However, significantly higher levels were also found in the liver, spleen, and whole body, with values between those of the $F(ab')_2$ and the intact mAb.

In an analogous study, Quadri (Quadri et al., 1993b) used commercially available cross-linking agents based on bis-maleimide to covalently cross-link the heavy chains of a mAb $F(ab')_2$ fragment (Figure 7). The agents used were bis(maleimido)methyl ether (BMME), *N,N'*-p-phenylenedimaleimide (PDM), and *N,N'*-bis(maleimido)hexane (BMH). The stabilized $F(ab')_2$ fragments generated using these linkers were identical to the original $F(ab')_2$ in terms of immunoreactivity and serum stability, suggesting that differences in *in vivo* distribution were due to differences in linkage chemistry. Following labeling with the metallic nuclide ^{111}In , all three constructs behaved similarly *in vivo* in a nude mouse xenograft model, showing very altered pharmacokinetics and distribution when compared with the conventional $F(ab')_2$ fragment. Tumor uptake in this system at 24 h postinjection was 2–2.5 times higher for the constructs than for normal $F(ab')_2$ and similar to that observed with the intact mAb. This improved accumulation was presumably the consequence of the extended circulating half-lives of the constructs. In addition, liver uptake for the three constructs was only around 40% of that observed with the intact mAb, offering hope of circumvention of the excessive liver uptake seen in the clinical situation

by ourselves and others when employing metallic radionuclides in conjunction with intact mAbs (Ryser et al., 1992; Bischof-Delaloye et al., 1996). This approach also seems to offer grounds for optimism in relation to a second significant problem, that of the nuclide accumulation normally seen in the kidneys when using $F(ab')_2$ fragments labeled with metallic nuclides. Much reduced levels of radiolabel were observed in the kidney in the case of the three constructs. This implies that, at least in some instances, instability of $F(ab')_2$ fragments results in reduction to the Fab form which, with a molecular mass of only 50 kDa, is subject to glomerular filtration and subsequent renal absorption. The application of such stabilized $F(ab')_2$ fragments in a clinical setting will hopefully yield interesting results.

More recently, King et al. (1994) have used chemically cross-linked recombinant Fab and scFv antibody fragments derived from the anti-TAG72 antibody B72.3. To make these constructs, bis- and tris-maleimide linkers containing an appropriate multidentate ligand to allow the direct chelation of ^{90}Y were synthesized. In the case of the Fab, expression was performed with a cysteine in the hinge region to facilitate coupling, and this same hinge region was attached to the terminal carboxyl group of the scFv, thus permitting use of the same linkers. Following labeling with ^{125}I , tumor:blood ratios in a nude mouse xenograft system were found to be improved, particularly at later time points, for the di- and trivalent constructs made by linking Fab fragments. In the case of the trivalent material, absolute accumulation was not far short of that achieved using the original intact mAb. Patterns of *in vivo* distribution following labeling with ^{90}Y were markedly different. The divalent material showed unusually high kidney accumulation, as did the parent immunoglobulin. Only in the case of the trivalent material, which displayed very rapid blood clearance, were positive tumor:normal tissue ratios seen against all tissues. Tumor accumulation was poor for the scFv, di-scFv, and tri-scFv materials, and substantial uptake was seen in liver, kidney, and spleen.

In our own collaborative studies with the Department of Medical Biochemistry at the University Medical Center, Geneva, a trivalent construct has been generated from the anti-CEA MAb35 by use of a novel polyoxime chemistry according to the strategy first applied by Rose (1994). Our testing of the ^{125}I -labeled form of the construct in nude mice bearing human colon carcinoma xenografts revealed much improved pharmacokinetic behavior with higher tumor:normal tissue ratios when compared with the parent murine monoclonal (Figure 8a) and with higher accumulation and longer residence times in the tumor than were observed with the $F(ab')_2$ fragment of the mAb (Werlen et al., 1996). The circulating half-life of the construct was found to be much reduced when compared with that of the intact antibody, thereby resulting in encouragingly high tumor:blood ratios, reaching over 12 for the construct at day 5 postinjection compared with around 2 for the intact MAb35 (Figure 8b).

4. CONCLUSION

Following initial overoptimism as to the potential of RIT, which accompanied the widespread introduction of monoclonal antibody technology, there has followed a period of consolidation in which several key problems restricting the broad application of RIT have been identified. The failure to identify any truly tumor-specific antigens is undoubtedly one of the major disappointments, and the tumor-associated antigens targeted today are often the very same antigens which were initially

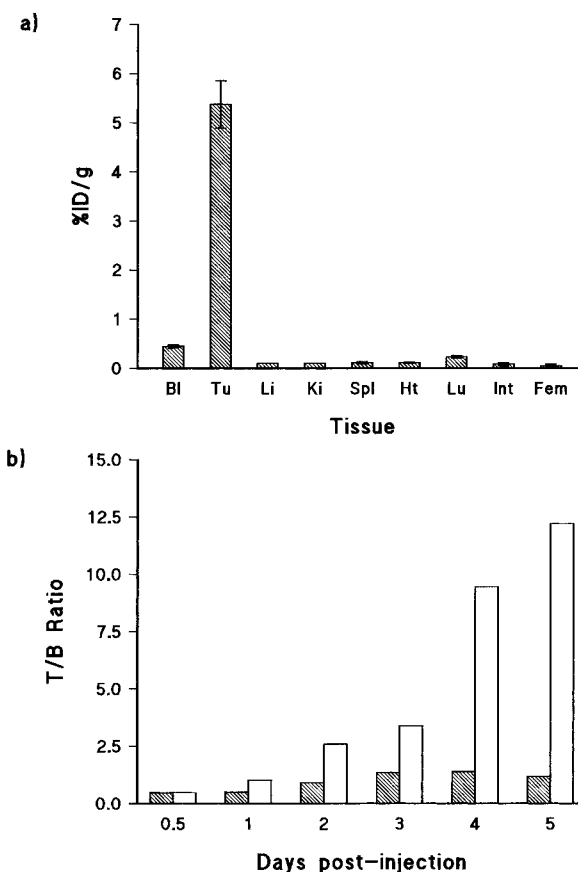


Figure 8. *In vivo* distribution of a ^{125}I -labeled trivalent antibody construct. Made by the chemical linkage of three Fab fragments from the anti-colon carcinoma mAb MAb35, the construct was tested in a nude mouse model system. Clearance from all normal tissues examined was greatly accelerated, leading to improved tumor:normal tissue ratios as suggested by the %ID/g values in a. In comparison with the parent MAb35, which has approximately the same molecular weight, the rapid clearance of the construct from the blood gave rise to encouraging increases in tumor:blood ratios, reaching over 12 at 5 d postinjection compared to around 1 for the parent material.

selected, such as the CEA antigen, TAG-72, or NCAM. To some extent then, the almost universally disappointing results obtained using systemic RIT against solid tumors can be said to be the consequence of the nature of the target malignancy.

However, the various strategies which have been used to try to circumvent the dose-limiting toxicity to the bone marrow have brought with them a new set of considerations. The use of alternative radionuclides to replace ^{131}I certainly offers advantages in terms of better radiation characteristics and more appropriate half-lives. However, as shown in the case of ^{67}Cu , ^{111}In , ^{67}Ga , and ^{177}Lu , there is often a clear problem in relation to intracellular retention of metallic radionuclides. This reveals itself as high liver accumulation following administration of intact mAbs and as high kidney levels observed following administration of mAb fragments. The extent to which these effects occur seems also to be related to the nature of the chelator employed, with the degree of hydrophobicity playing a role. Although such accumulation in nontarget organs is problematic, and efforts are now being aimed at its reduction, there is a positive aspect in that nuclide accumulation within target tumor cells tends also to be raised as a result of this effect should the carrier moiety be an internalizing antibody (Novak-Hofer et al., 1995). A strategy achieving a differential "wash-out" of nuclide from nontarget tissue while preserving levels within tumor is currently difficult

to envisage, although the application of mAbs which target noninternalizing tumor antigens may offer advantages.

If a successful RIT is currently to be achieved, it is clear that a real effort must be made to choose only clinical targets which lend themselves to this approach. This implies of course the selection of easily accessible and highly radiosensitive small cellular deposits. On these grounds, the blood-borne malignancies have long been considered suitable targets for RIT. Additionally, disease states in which an intracavitary administration of RIC can be attempted are seen as particularly attractive. In such cases there exists the possibility of introduction of the RIC into direct contact with the target tissue without significant systemic leakage, thereby avoiding adverse toxicity. A good example of such an approach would perhaps be the treatment of superficial carcinoma of the urinary bladder, and toward this end, studies have already been undertaken using an ^{111}In -labeled NCRC-48 mAb which was found to give a mean tumor:normal tissue ratio of 12:1 at 2 h postinstillation. Encouragingly, no radioactivity could be detected in blood taken 24 h postadministration, and no HAMA response could be identified in any of the 12 patients (Kunkler et al., 1994).

Crucial to the future of systemic RIT is an improvement in the absolute amount of radioactivity which can be delivered to the target tumor without corresponding increases in dose-limiting toxicities. With this as our goal, the alteration of the pharmacokinetics of a given radioimmunoconjugate by modification of the carrier moiety appears now to hold the key to successful RIT. Whether this will best be achieved by genetic engineering or by chemical modification remains open. However, the encouraging results described involving chemically engineered mAb fragments suggest this direction may act as a useful evaluation step prior to production by genetic engineering. Whatever the nature of the constructs chosen for future application in RIT, the availability of a panel of appropriate radionuclides, matched in terms of half-life, radiation characteristics, and *in vivo* behavior, will hopefully serve to enhance efficacy and improve therapeutic indices.

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LITERATURE CITED

- Adelstein, S. J., and Kassis, A. I. (1987) Radiobiologic implications of the microscopic distribution of energy from radionuclides. *Nucl. Med. Biol.* 14 (3), 165–169.
- Alberto, R., Blauenstein, P., Novak-Hofer, I., Smith, A., and Schubiger, P. A. (1992) An improved method for the separation of ^{111}Ag from irradiated natural palladium. *Appl. Radiat. Isot.* 43, 869–872.
- Alberto, R., Schibli, R., Egli, A., Schubiger, P. A., Herrmann, W. A., Artus, G., Abram, U., and Kaden, T. A. (1995a) Low pressure carbonylation of $[\text{MOC}_4\text{L}_4]^-$ and $[\text{MO}_4]^-$: The technetium(I) and rhenium(I) complexes $[\text{NET}_4]_2[\text{MCl}_3(\text{CO})_3]$. *J. Organomet. Chem.* 493, 119–127.
- Alberto, R., Nef, W., Smith, A., Schubiger, P. A., Kaden, T. A., Neuburger, M., Zehnder, M., Frey, U., and Abram, U. (1995b) Silver(I) Complexes of the Derivatized Crown Thioether Ligands 3,6,9,12,15,18-Hexathianonadecanol (19-aneS6-OH) and 3,6,9,13,16,19-Hexathiaicosanol (20-aneS6-OH). Determination of Stability Constants and the Crystal Structures of $[\text{Ag}(19\text{-aneS6-OH})][\text{CF}_3\text{SO}_3]$ and $[\text{Ag}(20\text{-aneS6-OH})][\text{BF}_4]$. *J. Inorg. Chem.* (submitted for publication).
- Anderson, C. J., Connett, J. M., Schwarz, S. W., Rocque, P. A., Guo, L. W., Philpott, G. W., Zinn, K. R., Meares, C. F., and Welch, M. J. (1992) Copper-64-labeled antibodies for PET imaging. *J. Nucl. Med.* 33, 1685–1691.
- Anderson, C. J., Rogers, B. E., Connett, J. M., Guo, L. W., Schwarz, S. W., Zinn, K. R., and Welch, M. J. (1993) Comparison of two bifunctional chelates for labeling ^{64}Cu to MAb 1A3 and 1A3-F(ab')₂: Chemistry and animal biodistribution. Tenth International Symposium on Radiopharmacy Chemistry Abstracts, p 313.
- Andres, R. Y., Blattmann, H., Pfeiffer, G., Schubiger, P. A., Vogt, M., Weinreich, R., and Wernli, C. (1986) Radionuclides for therapy: a review. In *Radionuclides for therapy* (P. A. Schubiger and P. H. Hasler, Eds.) pp 11–20, Hoffman-LaRoche, Basel.
- Annunziato, M. E., Patel, U. S., Ranade, M., and Palumbo, P. S. (1993) *p*-Maleimidophenyl Isocyanate: A novel Heterobifunctional Linker for Hydroxyl to Thiol Coupling. *Bioconjugate Chem.* 4, 212–218.
- Beaumier, P. L., Venkatesan, P., Vanderheyden, J.-L., Burgua, W. D., Kunz, L. L., Fritzberg, A. R., Abrams, P. G., and Morgan, A. C. (1991) ^{186}Re Radioimmunotherapy of small cell lung carcinoma xenografts in Nude mice. *Cancer Res.* 51, 676–681.
- Bischof-Delaloye, A., Buchegger, F., Delaloye, B., Smith, A., Poncioni, M., Mach, J.-P., and Schubiger, P. A. (1996) Clinical evaluation of a Cu-67 labeled anti-CEA MAb (manuscript in preparation).
- Bodansky, M., and Bodansky, A. (1984) Practice of peptide synthesis. ISBN 3-540-13471-9, Springer-Verlag, Berlin.
- Booz, J. (1984) Microdosimetric basis of individualized cancer radiotherapy by radionuclides including Auger effect and the photon activation approach. *Strahlentherapie* 160, 745–754.
- Breitz, H. B., Weiden, P. L., Vanderheyden, J. L., Appelbaum, J. W., Bjorn, M. J., Fer, M. F., Wolf, S. B., Ratliff, B. A., Seiler, C. A., Foisie, D. C., Fisher, D. R., Schroff, R. W., Fritzberg, A. R., and Abrams, P. G. (1992) Clinical experience with rhenium-186-labeled monoclonal antibodies for radioimmunotherapy: results of Phase 1 trials. *J. Nucl. Med.* 33, 1099–1112.
- Brown, B. A., Comeau, R. D., Jones, P. L., Liberatore, F. A., Neacy, W. P., Sands, H., and Gallagher, B. M. (1987a) Pharmacokinetics of monoclonal antibody B72.3 and its fragments labeled with either ^{125}I or ^{111}In . *Cancer Res.* 47, 1149–1154.
- Brown, B. A., Davis, G. L., Saltzgaber-Muller, J., Simon, P., Ho, M. K., Shaw, P. S., Stone, B. A., Sands, H., and Moore, G. P. (1987b) Tumor-specific genetically engineered murine/human chimeric monoclonal antibody. *Cancer Res.* 47, 3577–3583.
- Buchegger, F., Haskell, C. M., Schreyer, M., Scazziga, B. R., Randin, S., Carrel, S., and Mach, J.-P. (1983) Radiolabeled fragments of monoclonal antibodies against carcinoembryonic antigen for localization of colon carcinoma grafted into nude mice. *J. Exp. Med.* 158, 413–427.
- Buchegger, F., Pelegrin, A., Delaloye, B., Bischof-Delaloye, A., and Mach, J. P. (1990) Iodine-131-labeled MAb F(ab')₂ fragments are more efficient and less toxic than intact anti-CEA antibodies in radioimmunotherapy of large human colon carcinoma grafted in Nude mice. *J. Nucl. Med.* 31, 1035–1044.
- Buchegger, F., Chalandon, Y., Pelegrin, A., Hardman, N., and Mach, J. P. (1991) Bone marrow dosimetry in rats using direct tissue counting after injection of radioiodinated intact monoclonal antibodies or F(ab')₂ fragments. *J. Nucl. Med.* 32, 1414–1421.
- Cahn, R. (1995) High specific activity ^{186}Re by the Szilard-Chalmers reaction. Ph.D. Thesis, Technical University of Munich, Munich, Germany.
- Cheung, N.-K. V., Landmeier, B., Neeley, J., Nelson, A. D., Abramowsky, C., Ellery, S., Adams, R. B., and Miraldi, F. (1986) Complete tumor ablation with iodine 131-radiolabeled disialoganglioside GD2-specific monoclonal antibody against human neuroblastoma xenografted in Nude mice. *J. Natl. Cancer Inst.* 77, 739–745.

- Colcher, D., Bird, R., Roselli, M., Hardman, K. D., Johnson, S., Pope, S., Dodd, S. W., Pantoliano, M. W., Milenic, D. E., and Schlom, J. (1990) *In vivo* tumor targeting of a recombinant single-chain antigen-binding protein. *J. Natl. Cancer Inst.* 28, 1191–1197.
- Craig, A. S., Katakay, R., Matthews, R. C., Parker, D., Ferguson, G., Lough, A., Adams, H., Bailey, N., and Schneider, H. (1990) Synthesis of 1,10-Dithia-4,7,13,16-tetra-azacyclo-octadecane, 1-aza-4,7-dithiacyclononane and *N,N'*-1,2-Bis(1-aza-4,7-dithiacyclononyl)ethane. Structural studies of their silver complexes. *J. Chem. Soc., Perkin Trans. 2*, 1523–1531.
- DeNardo, G. L., DeNardo, S. J., O'Grady, L. F., Mills, S. L., Lewis, J. P., and Macey, D. J. (1990) Radiation treatment of B cell malignancies with immunoconjugates. In *Frontiers of radiation therapy and oncology: The present and future role of monoclonal antibodies in the management of cancer* (J. M. Vaeth, Ed.) Vol. 24, pp 194–201, Karger, Basel.
- Deshpande, S. V., DeNardo, S. J., Meares, C. F., McCall, M. J., Adams, G. P., and DeNardo, G. L. (1989) Effect of different linkages between chelates and monoclonal antibodies on levels of radioactivity in the liver. *Nucl. Med. Biol.* 16 (6), 587–597.
- Eary, J. (1991) Fundamentals of radioimmunotherapy. *Nucl. Med. Biol.* 18 (1), 105–108.
- Epenetos, A. A., Munro, A. J., Stewart, S., Rampling, R., Lambert, H. E., McKenzie, C. G., Soutter, P., Rahemtulla, A., Hooker, G., Sivolapenko, G. B., Snook, D., Courtney-Luck, N., Dhokia, B., Krausz, T., Taylor-Papadimitriou, Durbin, H., and Bodmer, W. F. (1987) Antibody-guided irradiation of advanced ovarian cancer with intraperitoneally administered radiolabeled monoclonal antibodies. *J. Clin. Oncol.* 5, 1890–1899.
- Fisch, I., Künzi, G., Rose, K., and Offord, R. E. (1992) Site-Specific Modification of a Fragment of a Chimeric Monoclonal Antibody Using Reverse Proteolysis. *Bioconjugate Chem.* 3, 147–153.
- Franke, W. G., Wunderlich, G., Dreyer, R., and Fischer, S. (1986) Astatine 211: a powerful α -emitter for potential application in metabolic and contact therapy. In *Radionuclides for therapy* (P. A. Schubiger and P. H. Hasler, Eds.) pp 111–124, Hoffman-LaRoche, Basel.
- Glassy, M. C., Handley, H. H., Surh, C. S., and Royston, I. (1987) Genetically stable human hybridomas secreting tumor reactive human monoclonal IgM. *Cancer Invest.* 5, 447–455.
- Goeckler, W. F., Edwards, B., Volkert, W. A., Holmes, R. A., Simon, J., and Wilson, D. (1987) Skeletal localization of Sm-153 chelates: Potential therapeutic bone agents. *J. Nucl. Med.* 28, 495–504.
- Goldrosen, M. H., Biddle, W. C., Pancook, J., Bakshi, S., Vanderheyden, J.-L., Fritzberg, A. R., Morgan, A. C., and Foon, K. A. (1990) Biodistribution, pharmacokinetic and imaging studies with ^{186}Re -labeled NR-LU-10 whole antibody in LS174T colonic tumor bearing mice. *Cancer Res.* 50, 7973–7978.
- Griffiths, G. L., Goldenberg, D. M., Knapp, F. F., Callahan, A. P., Chang, C. H., and Hansen, H. J. (1991) Direct radio-labeling of monoclonal antibodies with generator-produced rhenium-188 for radioimmunotherapy: labeling and animal biodistribution studies. *Cancer Res.* 51, 4594–4602.
- Hahn, P. F., and Sheppard, C. W. (1947) The therapeutic use of radioactive elements in malignancy. *Ann. Intern. Med.* 28, 98–606.
- Hainfeld, J. F., Foley, C. J., Srivastava, S. C., Mausner, L. F., Feng, N. I., Meinken, G. E., and Steplewski, Z. (1990) Radioactive gold cluster immunoconjugates: Potential agents for cancer therapy. *Nucl. Med. Biol.* 17 (3), 287–294.
- Halpern, S., Haindl, W., Beauregard, R. N., Hagan, P., Clutter, M., Amox, D., Merchant, B., Unger, M., Mongovi, C., Bartholomew, R., Jue, R., Carlo, D., and Dillman, R. (1988) Scintigraphy with In-111-labeled monoclonal antibodies: kinetics, biodistribution and tumor detection. *Radiology* 168, 529–536.
- Hoefnagel, C. A. (1991) Radionuclide therapy revisited. *Eur. J. Nucl. Med.* 18, 408–431.
- Humm, J. L. (1986) Dosimetric aspects of radiolabeled antibodies for tumor therapy. *J. Nucl. Med.* 27, 1490–1497.
- Humm, J. L., and Cobb, L. M. (1990) Nonuniformity of tumor dose in radioimmunotherapy. *J. Nucl. Med.* 31, 75–83.
- Izard, M. E., Boniface, G. R., Hardman, K. L., Brechbiel, M. W., Gansow, O. A., and Walkers, K. Z. (1992) An Improved Method for Labeling Monoclonal Antibodies with Samarium-153: Use of the Bifunctional Chelate 2-(*p*-Isothiocyanatobenzyl)-6-methyldiethylenetriaminepentaacetic Acid. *Bioconjugate Chem.* 3, 346–350.
- Johnson, J. C., Langhorst, S. M., Loyalka, S. K., Volkert, W. A., and Ketring, A. R. (1992) Calculation of radiation dose at a bone-to-marrow interface using Monte-Carlo modeling techniques (EGS4). *J. Nucl. Med.* 33, 623–628.
- Jones, P. T., Dear, P. H., Foote, J., Neuberger, M. S., and Winter, G. (1986) Replacing the complementarity determining regions in a human antibody with those from a mouse. *Nature* 321, 522–525.
- Jönsson, B. A., and Strand, S. E. (1986) Auger electron emitters as therapeutic agents or sources of hazard in diagnosis - a review. In *Radionuclides for therapy* (P. A. Schubiger and P. H. Hasler, Eds.) pp 91–99, Hoffman-LaRoche, Basel.
- Kaminski, M. S., Zasadny, K. R., Francis, I. R., Milik, A. W., Ross, C. W., Moon, S. D., Crawford, S. M., Burgess, J. M., Petry, N. A., Butchko, G. M., et al. (1993) Radioimmunotherapy of B-cell lymphoma with (131I)anti-B1 (anti-CD20) antibody. *N. Engl. J. Med.* 329, 459–465.
- Kasi, L. P., Fossella, F., Holoye, P., Podoloff, D., Kim, E., Crane, J., and Gordon, E. E. (1991) Evaluation of multidose Sm-153-EDTMP for bone pain palliation in cancer patients. *Proceedings of the 7th International Symposium of Radiopharmacology*, Boston, p 11.
- Khazaali, M. B., Saleh, M. N., Liu, T. P., Merideth, R. F., Wheeler, R. H., Baker, T. S., King, D., Secher, D., Allen, L., Rogers, K., Colcher, D., Schlom, J., Shochat, D., and LoBuglio, A. F. (1991) Pharmacokinetics and immune response of ^{131}I -chimaeric mouse/human B72-3 (human $\gamma 4$) monoclonal antibody in humans. *Cancer Res.* 51, 5461–5466.
- King, D. J., Turner, A., Farnsworth, A. P. H., Adair, J. R., Owens, R. J., Pedley, R. B., Baldock, D., Proudfoot, K. A., Lawson, A. D. G., Beeley, N. R. A., Millar, K., Millican, T. A., Boyce, B. A., Antoniow, P., Mountain, A., Begent, R. H. J., Shochat, D., and Yarranton, G. T. (1994) Improved tumor targeting with chemically cross-linked recombinant antibody fragments. *Cancer Res.* 54, 6176–6185.
- Kohler, G., and Milstein, C. (1975) Continuous cultures of fused cells secreting antibodies of predefined specificity. *Nature* 256, 495–497.
- Kolsky, K. L., and Mausner, L. F. (1993) Production of no-carrier added ^{199}Au for gold cluster labelled antibodies. *Appl. Radiat. Isot.* 44 (3), 553–560.
- Kosmas, C., Maraveyas, A., Gooden, C. S., Snook, D., and Epenetos, A. A. (1995) Anti-chelate antibodies after intraperitoneal yttrium-90 labeled monoclonal antibody immunoconjugates for ovarian cancer therapy. *J. Nucl. Med.* 36 (5), 746–753.
- Kozbor, D., and Roder, J. C. (1981) Requirements for the establishment of high-titered human monoclonal antibodies against tetanus toxoid using the Epstein-Barr virus technique. *J. Immunol.* 127, 1275–1280.
- Kunkler, R. B., Bishop, M. C., Green, D. J., Pimm, M. V., and Price, M. R. (1994) Biodistribution and imaging of 111-Indium labeled monoclonal antibody (MAb) NCRC-48 following intravesical administration for bladder cancer. *Br. J. Cancer* 70 (Suppl. XXII), 24.
- Langmuir, V. K. (1992) Radioimmunotherapy: clinical results and dosimetric considerations. *Nucl. Med. Biol.* 19 (2), 213–225.
- Lewington, V. J. (1993) Targeted radionuclide therapy for bone metastases. *Eur. J. Nucl. Med.* 20, 66–74.
- Mausner, L. F., Srivastava, S. C., Kolsky, K. L., Mease, R. C., Joshi, V., Meinken, G. E., Kurczak, S., Chatal, J. F., and Steplewski, Z. (1994) Development and evaluation of copper-67 labeled DOTA and TETA immunoconjugates for radioimmunotherapy. *J. Labelled Compd. Radiopharm.* 35, 374–376.
- McCall, M. J., Diril, H., and Meares, C. F. (1990) Simplified Method for Conjugating Macrocytic Bifunctional Chelating Agents to Antibodies via 2-Iminothiolane. *Bioconjugate Chem.* 1, 222–226.

- Meares, C. F., McCall, M. J., Deshpande, S. V., DeNardo, S. J., and Goodwin, D. A. (1988) Chelate radiochemistry: cleavable linkers lead to altered levels of radioactivity in the liver. *Int. J. Cancer* 2 (Suppl.), 99–102.
- Milenic, D. E., Esteban, J. M., and Colcher, D. (1989) Comparison of methods for the generation of immunoreactive fragments of a monoclonal antibody (B72.3) reactive with human carcinomas. *J. Immunol. Methods* 120, 71–83.
- Moi, M. K., Meares, C. F., McCall, M. J., Cole, W. C., and DeNardo, S. J. (1985) Copper chelates as probes of biological systems: stable copper complexes with a macrocyclic bifunctional chelating agent. *Anal. Biochem.* 148, 249–253.
- Najafi, A., Alauddin, M. M., Sosa, A., Ma, G. Q., Chen, D. C. P., Epstein, A. L., and Siegel, M. E. (1992) The evaluation of ^{186}Re -labeled antibodies using N_2S_4 chelate *in vitro* and *in vivo* using tumor-bearing nude mice. *Nucl. Med. Biol.* 19 (2), 205–212.
- Novak-Hofer, I., Amstutz, H. P., Maecke, H. R., Schwarzbach, R., Zimmerman, K., Morgenthaler, J. J., and Schubiger, P. A. (1995) Cellular processing of copper-67-labeled monoclonal antibody chCE7 by human neuroblastoma cells. *Cancer Res.* 55, 46–50.
- Paik, C. H., Yokoyama, K., Reynolds, J. C., Quadri, S. M., Min, C. Y., Shin, S. Y., Maloney, P. J., Larson, S. M., and Reba, R. C. (1989) Reduction of background activity by introduction of a diester linkage between antibody and chelate in radioimmunodetection of tumor. *J. Nucl. Med.* 30, 1693–1701.
- Pochon, S., Buchegger, F., Pelegrin, A., Mach, J.-P., Offord, R. E., Ryser, J. E., and Rose, K. (1989) A novel derivative of the chelon desferrioxamine for site-specific conjugation to antibodies. *Int. J. Cancer* 43, 1188–1194.
- Press, O. W., DeSantes, K., Anderson, S. K., and Geissler, F. (1990) Inhibition of catabolism of radiolabeled antibodies by tumor cells using lysosomotropic amines and carboxylic ionophores. *Cancer Res.* 50, 1243–1250.
- Press, O. W., Eary, J. F., Appelbaum, F. R., Martin, P. J., Badger, C. C., Nelp, W. B., Glenn, S., Butchko, G., Fisher, D., Porter, B., Matthews, D. C., Fisher, L. D., and Bernstein, I. D. (1993) Radiolabeled-antibody therapy of B-cell lymphoma with autologous bone marrow support. *N. Engl. J. Med.* 329, 1219–1224.
- Quadri, S. M., Vriesendorp, M., Lechner, P. K., and Williams, J. R. (1993a) Evaluation of indium-111 and yttrium-90 labeled linker immunoconjugates in Nude mice and dogs. *J. Nucl. Med.* 34 (6), 938–945.
- Quadri, S. M., Lai, J., Mohammadpour, H., Vriesendorp, H. M., and Williams, J. R. (1993b) Assessment of radiolabeled stabilized F(ab')_2 fragments of monoclonal antiferritin in Nude mouse model. *J. Nucl. Med.* 34, 2152–2159.
- Queen, C., Schneider, W. P., Selick, H. E., Payne, P. W., Landli, N. F., Duncan, J. F., Avdalovic, N. M., Levitt, M., Junghans, R. P., and Waldman, T. A. (1989) A humanized antibody that binds to the interleukin 2 receptor. *Proc. Natl. Acad. Sci. U.S.A.* 86, 10029–10033.
- Rose, K. (1994) Facile Synthesis of Homogeneous Artificial Proteins. *J. Am. Chem. Soc.* 116, 30–33.
- Rose, K., Vilaseca, L. A., Werlen, R., Meunier, A., Fisch, I., Jones, R. M. L., and Offord, R. E. (1991) Preparation of Well-Defined Protein Conjugates Using Enzyme-Assisted Reverse Proteolysis. *Bioconjugate Chem.* 2, 154–159.
- Rösler, H., Noelp, U., Bläuenstein, P., Triller, K. J., and Steffen, R. (1986) Endoarterial radionuclide embolizing tumor therapy. In *Radionuclides for therapy* (P. A. Schubiger and P. H. Hasler, Eds.) pp 227–244, Hoffman-La Roche, Basel.
- Ryser, J. E., Jones, R. M. L., Egeli, R., Pelegrin, A., Rose, K., Kurt, A. M., Perin, M., Broquet, P. E., Ambrosetti, P., Fisch, I., Pochon, S., Donath, A., Mach, J.-P., and Offord, R. E. (1992) Colon carcinoma immunoscintigraphy by monoclonal anti-CEA antibody labeled with gallium-67-aminooxyacetyldeferoxamine. *J. Nucl. Med.* 33, 1766–1773.
- Saenger, E. L., Kereiakes, J. G., Sodd, V. J., and David, R. (1979) Radiotherapeutic agents: properties, dosimetry and radiobiologic considerations. *Sem. Nucl. Med.* 9 (2), 72–84.
- Salmain, M., Gunn, M., Gorfti, A., Top, S., and Jaouen, G. (1993) Labeling of Proteins by Organometallic Complexes of Rhenium(I): Synthesis and Biological Activities of the Conjugates. *Bioconjugate Chem.* 4, 425–433.
- Scheinberg, D. A., Lovett, D., Divgi, C. R., Graham, M. C., Berman, E., Pentlow, K., Feirt, N., Finn, R. D., Clarkson, B. D., Gee, T. S., Larson, S. M., Oettingen, H. F., and Old, L. J. (1991) A Phase 1 trial of monoclonal antibody M195 in acute myelogenous leukemia: specific bone marrow targeting and internalization of radionuclide. *J. Clin. Oncol.* 9, 478–490.
- Schott, M. E., Milenic, D. E., Yokota, T., Whitlow, M., Wood, J. F., Fordyce, W. A., Cheng, R. C., and Schlom, J. (1992) Differential metabolic patterns of iodinated versus radiometal chelated anticarcinoma single-chain Fv molecules. *Cancer Res.* 52, 6413–6417.
- Schott, M. E., Frazier, K. A., Pollock, D. K., and Verbanac, K. M. (1993) Preparation, Characterization, and *In Vivo* Biodistribution Properties of Synthetically Cross-Linked Multivalent Antitumor Antibody Fragments. *Bioconjugate Chem.* 4, 153–165.
- Schubiger, P. A., and Andres, R. Y. (1987) Radionuclides for therapy: a review. In *Klinische anforderungen an die Nuklearmedizin*, pp 15–19, Schattauer Verlag, Stuttgart.
- Sgouros, G. (1992) Plasmaphoresis in radioimmunotherapy of micrometastases: a mathematical modeling and dosimetric analysis. *J. Nucl. Med.* 33, 2167–2179.
- Sgouros, G. (1993) Bone marrow dosimetry for radioimmunotherapy: theoretical considerations. *J. Nucl. Med.* 34, 689–694.
- Sikora, K., Alderson, T., Ellis, J., Phillips, J., and Watson, J. (1983) Human hybridomas from patients with malignant disease. *Br. J. Cancer* 47, 135–145.
- Smith, A., Waibel, R., and Stahel, R. A. (1990) Selective immunotherapy of small cell cancer xenografts using ^{131}I -labeled SWA11 antibody. *Br. J. Cancer* 64, 263–266.
- Smith, A., Alberto, R., Bläuenstein, P., Novak-Hofer, I., Maecke, H., and Schubiger, P. A. (1993) Preclinical evaluation of ^{67}Cu -labeled intact and fragmented anti-colon carcinoma monoclonal antibody MAb35. *Cancer Res.* 53, 5727–5733.
- Smith, A., Zangemeister-Wittke, U., Waibel, R., Schenker, T., Schubiger, P. A., and Stahel, R. A. (1994) A comparison of ^{67}Cu and ^{131}I -labeled forms of monoclonal antibodies SEN7 and SWA20 directed against small cell lung cancer. *Int. J. Cancer* 8, 43–48.
- Smyth, M. J., Pietersz, G. A., Glasson, M. J., and McKenzie, I. F. C. (1986) The specific targeting of chlorambucil to tumors. *J. Natl. Cancer Inst.* 76, 503–510.
- Spencer, R. P. (1987) Short lived radionuclides in therapy. *Nucl. Med. Biol.* 14 (6), 537–538.
- Steinitz, M., Klein, G., Koskimies, S., and Makela, O. (1977) EB virus-induced B lymphocyte cell lines producing specific antibody. *Nature* 269, 420–422.
- Stewart, J. S. W., Hird, V., and Snook, D. (1990) Intraperitoneal yttrium-90 labeled monoclonal antibody in ovarian cancer. *J. Clin. Oncol.* 8, 1941–1950.
- Studer, M., Kaden, T. A., and Maecke, H. R. (1990) Reactivity studies of the pendant carboxylic group in a macrocyclic Cu^{2+} complex towards amide formation and its use as a protein-labeling agent. *Helv. Chim. Acta* 73, 149–153.
- Visser, G. W. M., Gerretson, M., Herscheid, J. D. M., Snow, G. B., and van Dongen, G. (1993) Labeling of monoclonal antibodies with rhenium-186 using the MAG3 chelate for radioimmunotherapy of cancer: a technical protocol. *J. Nucl. Med.* 34 (11), 1953–1963.
- Volkert, W. A., Goeckeler, W. F., Ehrhardt, G. J., and Ketring, A. R. (1991) Therapeutic radionuclides: production and decay property considerations. *J. Nucl. Med.* 32, 174–185.
- Vriesendorp, H. M., Herpst, J. M., Germack, M. A., Klein, J. L., Lechner, P. K., Loudenslager, D. M., and Order, S. E. (1991) Phase I–II studies of yttrium labeled antiferritin treatment for end-stage Hodgkin's disease. *J. Clin. Oncol.* 9, 918–928.
- Wawrzynczak, E. J. (1991) Systemic immunotoxin therapy of cancer: advances and prospects. *Br. J. Cancer* 64, 624–630.
- Weber, R. W., Boutin, R. H., Nedelman, M. A., Lister-Jones, J., and Dean, R. T. (1990) Enhanced Kidney Clearance with an Ester-Linked $^{99\text{m}}\text{Tc}$ -Radiolabeled Antibody Fab'–Chelator Conjugate. *Bioconjugate Chem.* 1, 431–437.
- Werlen, R., Lankinen, M., Offord, R. E., Schubiger, P. A., Smith, A., and Rose, K. (1996) Preparation of a trivalent antigen-binding construct using polyoxime chemistry: improved

- biodistribution and potential for therapeutic application. *Cancer Res.* (in press).
- Wernli, C. (1986) Effective range of different radiation types: a comparative study. In *Radionuclides for therapy* (P. A. Schubiger and P. H. Hasler, Eds.) pp 105–110, Hoffman-LaRoche, Basel.
- Wessels, B. W., and Rogus, R. D. (1984) Radionuclide selection and model absorbed dose calculations for radiolabeled tumor associated antibodies. *Med. Phys.* 11, 638–645.
- Wheldon, T. E. (1993) Radionuclide therapy of cancer: particle range and therapeutic effectiveness. *Nucl. Med. Commun.* 14, 408–410.
- Wong, S. S. (1993) Chemistry of protein conjugation and cross-linking. ISBN 0–8493–5886–8, CRC Press Inc., Boca Raton, Florida.

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ARTICLES

Synthesis of Short Polyoxyethylene-Based Heterobifunctional Cross-Linking Reagents. Application to the Coupling of Peptides to Liposomes

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We describe the synthesis of [2-[2-[2-[(2-bromoacetyl)amino]ethoxy]ethoxy]ethoxy]acetic acid (**7**), [2-[2-(2,5-dioxo-2,5-dihydropyrrol-1-yl)ethoxy]ethoxy]acetic acid (**11**), and [2-[2-(pyridin-2-yl)disulfanyl]ethoxy]ethoxy]acetic acid (**16**), three new thiol-reactive heterobifunctional reagents, and the preparation of their corresponding dipalmitoylphosphatidylethanolamine derivatives (**8**, **12**, and **17**). Such phospholipid amide derivatives were aimed to be incorporated into the bilayers of liposomal constructs used for immunization with e.g. synthetic peptides. The spacer arms introduced by **8**, **12**, and **17** are hydrophilic polyoxyethylene chains of variable lengths that were expected to provide a good accessibility to their conjugates and have a lesser intrinsic immunogenicity than the spacer introduced by *N*-[4-(*p*-maleimidophenyl)butyryl]phosphatidylethanolamine (MPB-PE), a classical reagent used for conjugation of ligands to the surface of liposomes. Such an immunogenicity might be prejudicial (e.g. carrier-induced epitopic suppression) to the development of synthetic vaccination formulations. Moreover, the derivatives **8**, **12**, and **17** allowed the coupling of peptides, bearing a thiol function, to their liposomal carrier via two types of linkages, i.e. stable thio ether (**8** and **12**) and bioreducible disulfide (**17**) bonds; this might be of importance in the mechanism of antigen presentation by competent cells. Using CG-IRGERA as a model peptide, the rate of coupling to **8**, **12**, and **17** was assessed as a function of pH.

INTRODUCTION

Synthetic peptide representatives of epitopes from e.g. microorganisms of transformed cells are useful for the development of therapeutic vaccines in infectious diseases or cancer (Arnon and Horwitz, 1992). However, most peptides of relatively small size are hampered by poor immunogenicity, and to trigger an immune response, they must be coupled to macromolecular carriers such as proteins or synthetic polymers (Defoort et al., 1992) or associated to particulate systems such as liposomes (Gregoriadis, 1994a,b) or immunostimulating complexes (e.g. ISCOMs, Löfvén and Larsson, 1994). In most cases, the presence of an adjuvant is also required. In this respect, to preserve the structural integrity of the peptide, it is important to design bioconjugation procedures that couple peptides in a chemically well-defined fashion to the carriers (Muller, 1988). Our group has elaborated an efficient strategy that confers a particularly important immunogenicity to small synthetic peptides, such as IRGERA which corresponds to the C-terminal of histone H3 (Frisch et al., 1991; Friede et al., 1993). It is based on the covalent coupling of the peptide to the surface of preformed small unilamellar vesicles (SUV) that carry an adjuvant such as monophosphoryl lipid A (MPLA) in their bilayer (Figure 1). When applied to a cyclic nine-

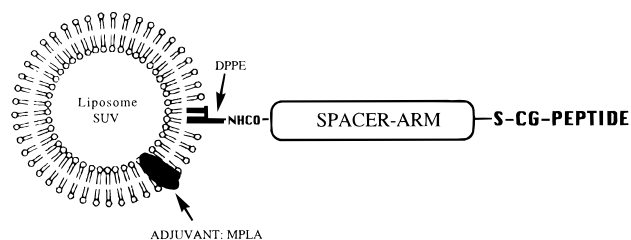


Figure 1. Liposome-based construct for immunization with synthetic peptides. The peptide carrying a cysteine residue, e.g. at its N-terminus, is conjugated to the surface of preformed SUV containing a thiol-reactive phosphatidylethanolamine derivative and MPL as adjuvant.

residue peptide mimicking the A site of influenza virus hemagglutinin, over 70% protection was achieved in a murine vaccination model (Friede et al., 1994).

The coupling of the peptides to liposomes was performed according to the strategy originally developed by Martin and Papahadjopoulos (1982) for the preparation of immunoliposomes. Thus the peptide, which was extended by a Cys-Gly spacer arm at its N-terminal, in the presence of preformed liposomes that contain *N*-[4-(*p*-maleimidophenyl)butyryl]phosphatidylethanolamine (MPB-PE), readily reacts with the maleimide moiety to give a stable thio ether linker linkage. This thiol-reactive phospholipid derivative is obtained by reaction between phosphatidylethanolamine and succinimidyl 4-(*p*-maleimidophenyl)butyrate (SMPB), a commercially available heterobifunctional coupling reagent. In the development of synthetic vaccines, this particular reagent, which also introduces a spacer arm between the peptide immunogen and the vesicle surface, has drawbacks. First, it is intrinsically immunogenic and therefore could engender

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secondary immune responses that are prejudicial to a vaccination formulation (e.g. carrier-induced epitopic suppression). Such an immunogenicity is classical for structures that carry aromatic groups (Bernatowicz and Matsueda, 1986; Jones et al., 1989; Peeters et al., 1989). Second, the spacer introduced is fairly hydrophobic, and the possibility exists that it will partition into the bilayer of the liposome, thus limiting an optimal presentation of the antigen to the cells involved in the immune response. Since many commercial heterobifunctional reagents that are useful for such a purpose carry hydrophobic groups, we have decided to synthesize new reagents with polyoxyethylene spacers. The main advantage of this approach is that these more hydrophobic and flexible arms should be, *a priori*, less immunogenic (Zalipsky, 1995); moreover, their length can be easily modulated, thus allowing a good accessibility of the linked peptide.

In the present article, we describe the synthesis of three new such heterobifunctional reagents **7**, **11**, and **16** (Schemes 1–3). One end of these reagents has been functionalized to react with a thiol group carried by a peptide to give a stable thio ether bond (maleimide or bromoacetyl moieties) or a disulfide linkage (dithiopyridine moiety) that can easily regenerate the free peptide by reduction or by thiol exchange reaction. Such a difference in the mode of attachment to the carrier is of importance in defining the mechanisms involved in the presentation of the peptide to the immune system (Friede et al., 1995). The other end bears a free carboxylic function destined to be coupled to the amine of PE. These thiol-reactive phospholipid derivatives have also been studied for their reactivity with the thiol function of a model peptide and for their immunogenicity (Boeckler et al., submitted for publication).

EXPERIMENTAL SECTION

General. Reagent-grade solvents were used without further purification. Dicyclohexylcarbodiimide (DCC), bromoacetic acid, *N*-hydroxysuccinimide, 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), methyl chloroformate, bromoacetic chloride, [2-(2-chloroethoxy)ethoxy]ethanol, and 2,2'-dipyridyl disulfide were purchased from Aldrich Chemical Co. (L'Isle d'Abeau Chesnes, France). Methanesulfonyl chloride and ninhydrin were obtained from Lancaster Synthesis (Bischeim, France). Tetra(ethylene glycol), triethylamine, and triphenylphosphine were from Janssen Chemica (Geel, Belgium). Sodium azide and aluminum oxide 90 active, basic were purchased from Merck (Darmstadt, Germany) and maleimide and *N*-methylmorpholine from Fluka Chemical Co. (Buchs, Switzerland). Phosphomolybdenum blue and dithiothreitol were obtained from Sigma Chemical Co. Bio-Sil, HA was purchased from Bio-Rad (Ivry/Seine, France). (Benzotriazol-1-yl-oxy)tris(dimethylamino)phosphonium hexafluorophosphate (BOP) was from Neosystem (Strasbourg, France), and thiourea was obtained from Prolabo (Paris, France). 1,2-Dipalmitoyl-*sn*-glycero-3-phosphoethanolamine (DPPE) was a gift from D3F (Doullens, France). The peptide, CGIRGERA, was a kind gift from Dr. J. P. Briand (CNRS-IBMC, Strasbourg, France). Thin layer chromatography was performed on silica gel 60 F 254 plates (Merck, Darmstadt, Germany). Flash chromatography was carried out using silica gel 60, 40–63 μ m (230–400 mesh) (Merck). ¹H-NMR spectra were recorded at 200 MHz on a Bruker WP-200 SY spectrometer. Mass spectra were recorded on a VG ZAB-HF mass spectrometer.

Methanesulfonic Acid 2-[2-[2-(2-Hydroxyethoxy)ethoxy]ethoxy]ethyl Ester (2). Methanesulfonyl chlo-

ride (10 mL, 0.129 mol) was added dropwise to a solution of 25 g (0.129 mol) of tetra(ethylene glycol) (**1**) and 17.91 mL (0.129 mol) of triethylamine in 200 mL of tetrahydrofuran (THF) at 0 °C. The reaction mixture was stirred for 2 h at 0 °C and left overnight at room temperature (RT). After elimination of the precipitate by filtration, the solution was evaporated under reduced pressure. The residue was purified by chromatography on a silica gel column eluted with EtOAc/hexane (80:20 to 100:0), and the title product was obtained (8.79 g, 25% yield) as a colorless oil: *R*_f 0.2 (EtOAc); ¹H-NMR (CDCl₃) δ 4.40–4.35 (m, 2H, CH₂OS), 3.78–3.57 (m, 14H, CH₂OCH₂, HOCH₂), 3.07 (s, 3H, SCH₃).

2-[2-[2-(2-Azidoethoxy)ethoxy]ethoxy]ethanol (3). A solution of **2** (8 g, 29.4 mmol) and sodium azide (2.8 g, 43 mmol) in 50 mL of acetonitrile was heated under reflux for 36 h. After return to room temperature, 50 mL of water was added and the mixture was extracted with CH₂Cl₂. The organic phase was then dried on MgSO₄ and concentrated. The residue was chromatographed on a silica gel column eluted with EtOAc. Compound **3** was obtained as a colorless oil (5.78 g, 90% yield): *R*_f 0.5 (EtOAc); ¹H-NMR (CDCl₃) δ 3.73–3.57 (m, 14H, CH₂OCH₂, HOCH₂), 3.37 (t, *J* = 5.0 Hz, 2H, CH₂N₃).

2-[2-[2-(2-Aminoethoxy)ethoxy]ethoxy]ethanol (4). Compound **3** (4.88 g, 22.28 mmol), triphenylphosphine (6.43 g, 24.51 mmol), and water (600 mg, 33.42 mmol) were mixed with 25 mL of THF. After the solution was stirred for 4 h at room temperature, the solvent was eliminated under reduced pressure and the residual product was purified on a silica gel column that was eluted with CHCl₃/CH₃OH/Et₃N (3:3:1). The title compound was obtained as colorless oil (4.07 g, 95% yield): *R*_f 0.4 (CHCl₃/CH₃OH/Et₃N, 3:3:1); ¹H-NMR (CDCl₃) δ 3.72–3.44 (m, 14H, CH₂OCH₂, HOCH₂), 2.79 (t, *J* = 4.9 Hz, 2H, CH₂N).

2-Bromo-*N*-[2-[2-[2-(2-hydroxyethoxy)ethoxy]ethoxy]ethyl]acetamide (6). Compounds **4** (193 mg, 1 mmol) and **5** (236 mg, 1 mmol) were dissolved in 10 mL of CHCl₃ (0 °C), and the mixture was left at room temperature overnight. The solvent was then removed under reduced pressure, and the product was purified on a silica gel column that was eluted with CH₂Cl₂/CH₃OH (95:5). A colorless oil was obtained (220 mg, 70% yield): *R*_f 0.2 (CHCl₃:CH₃OH, 95:5); ¹H-NMR (CDCl₃) δ 7.66 (s, 1H, NH), 3.87 (s, 2H, CH₂Br), 3.82–3.42 (m, 16H, HOCH₂, CH₂OCH₂, CH₂N), 2.16 (s, 1H, OH).

[2-[2-[2-(2-Bromoacetyl)amino]ethoxy]ethoxy]ethoxy acetic Acid (7). Compound **6** (270 mg, 0.8 mmol) was dissolved in 10 mL of acetone. Under stirring, 0.5 mL of 2.67 M Jones reagent (26.72 g of chromic trioxide in 23 mL of concentrated sulfuric acid brought to a final volume of 100 mL of water) was added dropwise over 15 min. After an additional 30 min, a few drops of 2-propanol were added, followed by 10 mL of water to dissolve the precipitated chromium salts. The organic solvent was then removed under reduced pressure and the aqueous phase extracted two times with 20 mL of CH₂Cl₂. The combined CH₂Cl₂ extracts were dried on MgSO₄ and concentrated, and the residue was chromatographed on silica gel column eluted with CH₂Cl₂/CH₃OH (80:20). The product was obtained as a colorless oil (180 mg, 64% yield): *R*_f 0.26 (CH₂Cl₂/MeOH, 80:20); ¹H-NMR (CDCl₃) δ 7.18 (s, 1H, NH), 4.17 (s, 2H, O₂CCH₂O), 3.88 (s, 2H, CH₂Br), 3.74–3.49 (m, 12H, CH₂CH₂O); HRMS (FAB⁺) *m/z* calcd for C₁₀H₁₉O₆NBr (M + H⁺) 328.0396, found 328.039.

Hexadecanoic Acid 3-[[[2-[2-[2-[2-[2-(2-Bromoacetyl)amino]ethoxy]ethoxy]ethoxy]acetyl]amino]ethoxy]hydroxyphosphoryl]oxy]-2-(hexadecanoyloxy)propyl Ester (8). DPPE (100 mg, 155 μ mol) was added

to a solution of 1 mL of anhydrous CH_3OH and 10 mL of anhydrous CHCl_3 containing **7** (80 mg, 244 μmol), DCC (50 mg, 243 μmol), and *N*-hydroxysuccinimide (5 mg, 43 μmol). The reaction was carried out overnight under argon at room temperature. Thin layer chromatography revealed a complete conversion of DPPE to a less polar product (R_f 0.6; $\text{CH}_2\text{Cl}_2/\text{CH}_3\text{OH}/\text{H}_2\text{O}$, 65:25:4). Solvents were then removed under reduced pressure and the reaction products redissolved in CHCl_3 . The organic phase was extracted twice with 1% NaCl to remove water soluble byproducts. The title compound was purified by chromatography on a 10 mL silica gel (Bio-Sil, HA) column that had been activated (150 °C, overnight) and prewashed with 100 mL of CHCl_3 . The products were eluted with an additional 20 mL of CHCl_3 followed by 20 mL of $\text{CHCl}_3/\text{CH}_3\text{OH}$ (40:1, 30:1, 25:1, 20:1, and 15:1) and 100 mL of $\text{CHCl}_3/\text{CH}_3\text{OH}$ (10:1) and finally with 100 mL of $\text{CHCl}_3/\text{CH}_3\text{OH}$ (10:2). The phosphate-containing fractions eluting with 10:2 $\text{CHCl}_3/\text{CH}_3\text{OH}$ were concentrated under reduced pressure, affording a colorless oil (60 mg, 42% yield). Analysis by TLC (R_f 0.6; $\text{CH}_2\text{Cl}_2/\text{CH}_3\text{OH}/\text{H}_2\text{O}$, 65:25:4) indicated a single phosphate-positive and ninhydrin-negative spot: $^1\text{H-NMR}$ ($\text{CDCl}_3/\text{CD}_3\text{OD}$, 1:1) δ 5.30–5.20 (m, 1H, CHCH_2OP), 4.44–4.12 (m, 2H, $\text{CH}_2\text{CHCH}_2\text{OP}$), 4.10–3.90 (m, 6H, CHCH_2OP , $\text{POCH}_2\text{CH}_2\text{N}$, COCH_2O), 3.85 (s, 2H, NCOCH_2Br), 3.72–3.38 (m, 14H, $\text{POCH}_2\text{CH}_2\text{N}$, OCH_2CH_2), 2.34–2.29 (m, 4H, CH_2CO_2), 1.70–1.50 (m, 4H, $\text{CH}_2\text{CH}_2\text{CO}_2$), 1.28 (m, 48H, $(\text{CH}_2)_{24}$), 0.89 (t, J = 6.6 Hz, 6H, CH_3); MS (FAB^+) m/z 1001.1 ($\text{M} + \text{H}^+$), 1023 ($\text{M} + \text{Na}^+$), 1039 ($\text{M} + \text{K}^+$).

[2-[2-(2,5-Dioxo-2,5-dihydropyrrol-1-yl)ethoxy]ethoxy]acetic Acid (11). [2-(2-Aminoethoxy)ethoxy]acetic acid (**10**) (760 mg, 4.55 mmol) in solution in 20 mL of a saturated solution of NaHCO_3 was treated, at 0 °C under vigorous stirring, with finely ground **9** (723 mg, 4.66 mmol). After 5 min, the mixture was diluted with 75 mL of water and stirred at 20 °C for 35 min, after which the pH was brought to 6 with concentrated sulfuric acid. The reaction mixture was reduced to 20 mL by evaporation, acidified to pH 1–2 with 1 M sulfuric acid, and extracted 8-fold with 100 mL of EtOAc/HOAc (95:5). The organic phase was dried on MgSO_4 and evaporated to dryness under reduced pressure. The residue was purified on a silica gel column eluted with $\text{CHCl}_3/\text{ethanol}/\text{HOAc}$ (60:28:2). It afforded a yellowish solid (790 mg, 70% yield): R_f 0.44 ($\text{CHCl}_3/\text{CH}_3\text{OH}/\text{HOAc}$, 70:28:2); mp 65–67 °C; $^1\text{H-NMR}$ (CDCl_3) δ 6.73 (s, 2H, CHCH), 4.11 (s, 2H, CH_2CO_2), 3.79–3.66 (m, 8H, $\text{CH}_2\text{CH}_2\text{O}$); HRMS (FAB^+) m/z calcd for $\text{C}_{10}\text{H}_{14}\text{N}_1\text{O}_6$ ($\text{M} + \text{H}^+$) 244.0821, found 244.0820.

Hexadecanoic Acid 3-[[[2-[2-[2-(2,5-Dioxo-2,5-dihydropyrrol-1-yl)ethoxy]ethoxy]acetyl amino]ethoxy]hydroxyphosphoryl]oxy]-2-(hexadecanoyloxy)-propyl Ester (12). To a solution of DPPE (20 mg, 28 μmol) in 4 mL of anhydrous CHCl_3 (40 °C) were added **11** (5 mg, 20 μmol), DCC (4.5 mg, 22 μmol), and *N*-ethyl-diisopropylamine (5 μL , 29 μmol). The reaction mixture was left under argon at room temperature for 15 h. *N,N'*-dicyclohexylurea was removed by filtration, and the organic phase was extracted twice with 1% NaCl. The title compound was purified by chromatography on a 10 mL silica gel (Bio-Sil, HA) column that had been activated (150 °C, overnight) and prewashed with 100 mL of CHCl_3 . The column was washed with an additional volume of 20 mL of CHCl_3 followed by 20 mL of $\text{CHCl}_3/\text{CH}_3\text{OH}$ mixtures (40:1, 30:1, 25:1, 20:1, and 15:1) and finally with 60 mL of 10:1 $\text{CHCl}_3/\text{CH}_3\text{OH}$. Analysis by TLC ($\text{CHCl}_3/\text{CH}_3\text{OH}/\text{HOAc}$, 60:20:3) indicated a single phosphate-positive and ninhydrin-negative spot for the fractions eluting with 15:1 and 10:1 $\text{CHCl}_3/\text{CH}_3\text{OH}$.

These fractions were pooled and concentrated under reduced pressure to afford a yellowish oil (10 mg, 54% yield): R_f 0.67 ($\text{CHCl}_3/\text{CH}_3\text{OH}/\text{HOAc}$, 60:20:3); $^1\text{H-NMR}$ ($\text{CDCl}_3/\text{CD}_3\text{OD}$, 1:1) δ 6.78 (s, 2H, COCHCHCO), 5.30–5.20 (m, 1H, CH_2CHCH_2), 4.59–4.15 (m, 2H, $\text{CH}_2\text{CHCH}_2\text{OP}$), 3.99 (s, 2H, NCOCH_2O), 3.74–3.66 (m, 8H, $\text{CH}_2\text{CH}_2\text{O}$), 2.36–2.28 (m, 4H, CH_2CO_2), 1.65–1.57 (m, 4H, $\text{CH}_2\text{CH}_2\text{CO}_2$), 1.25 (m, 48H, $(\text{CH}_2)_{12}$), 0.89 (t, J = 6.0 Hz, 6H, CH_3); MS (FAB^+) m/z 936.6 ($\text{M} + \text{Na}^+$).

[2-(2-Chloroethoxy)ethoxy]acetic Acid (14). To a solution of [2-(2-chloroethoxy)ethoxy]ethanol (**13**) (15 g, 89 mmol) in 500 mL of acetone was added dropwise over 15 min, and under stirring, 45 mL of 2.67 M Jones reagent (see above). The solution was allowed to stir for an additional 30 min period, after which a few drops of 2-propanol were added followed by 300 mL of water. The aqueous phase obtained after evaporation of the organic layer was added to 150 mL of a saturated NaCl solution and extracted seven times with 200 mL of CHCl_3 . The combined extracts were dried on MgSO_4 and evaporated to dryness. The residue was chromatographed on a silica gel column eluted with $\text{CHCl}_3/\text{ethanol}/\text{HOAc}$ (30:5:3), and a colorless oil was obtained (10.5 g, 64% yield): R_f 0.46 ($\text{CH}_2\text{Cl}_2/\text{CH}_3\text{OH}/\text{HOAc}$, 25:1:1); $^1\text{H-NMR}$ (CDCl_3) δ 10.11 (s, 1H, OH), 4.18 (s, 2H, OCH_2CO_2), 3.82–3.58 (m, 8H, $\text{CH}_2\text{CH}_2\text{O}$).

[2-(2-Mercaptoethoxy)ethoxy]acetic Acid (15). A solution of **14** (1.56 g, 8.6 mmol) and thiourea (2.7 g, 35 mmol) in 23 mL of water was heated under reflux for 4 h. After the addition of 30 mL of 2.5 M NaOH, the heating was resumed for an additional period of 4 h. After the mixture was cooled at room temperature, the pH was adjusted to 1 with concentrated HCl. The resulting solution was extracted seven times with 20 mL of CHCl_3 . The combined extracts were dried on MgSO_4 and evaporated to dryness, leaving an oil which was chromatographed on an aluminum oxide column. The products were eluted with $\text{CH}_2\text{Cl}_2/\text{ethanol}/\text{HOAc}$ (60:40:5). The title compound was obtained as a colorless oil (1.17 g, 75% yield): R_f 0.23 ($\text{CH}_2\text{Cl}_2/\text{ethanol}$, 3:2); $^1\text{H-NMR}$ (CD_3OD) δ 4.19 (s, 2H, OCH_2CO_2), 3.77–3.61 (m, 6H, CH_2O , OCH_2), 2.70 (t, J = 6.5 Hz, 2H, SCH_2).

[2-[2-(Pyridin-2-yl)disulfanyl]ethoxy]ethoxy]acetic Acid (16). 2,2'-Dipyridyl disulfide (2.7 g, 12.4 mmol) was dissolved in 15 mL of absolute ethanol containing 0.5 mL of HOAc, and **15** (1.12 g, 6.2 mmol) was added dropwise over 10 min. The mixture was stirred at room temperature under nitrogen for 24 h, and the solvent was evaporated under reduced pressure. The residue was chromatographed on a column of active aluminum oxide (activated at 150 °C overnight). The byproducts were eliminated with $\text{CH}_2\text{Cl}_2/\text{ethanol}$ (60:40), and the desired reaction product was eluted with $\text{CH}_2\text{Cl}_2/\text{ethanol}/\text{HOAc}$ (60:40:4). The presence of the dithiopyridine bond was assessed by a spectrophotometrical analysis. Aliquots (0.1 mL) of the chromatographic fractions were mixed with 3 mL of 0.1 M NaHCO_3 containing a small amount of dithiothreitol, and the formed 2-thiopyridone was detected by its absorbance at 343 nm. The 2-thiopyridone-generating fractions were pooled and concentrated under reduced pressure, affording a colorless oil (740 mg, 50% yield): R_f 0.3 ($\text{CH}_2\text{Cl}_2/\text{ethanol}$, 3:2); $^1\text{H-NMR}$ (CDCl_3) δ 8.94 (s, 1H, CO_2H), 8.47 (d, J = 4.3 Hz, 1H, CHN), 7.79–7.63 (m, 2H, CHCHCHN), 7.11 (t, J = 5.8 Hz, 1H, NCCH), 4.15 (s, 2H, OCH_2CO_2), 3.76–3.61 (m, 6H, $\text{CH}_2\text{OCH}_2\text{CH}_2\text{O}$), 2.99 (t, J = 6.2 Hz, 2H, CH_2SS); HRMS (FAB^+) m/z calcd for $\text{C}_{11}\text{H}_{16}\text{O}_4\text{NS}_2$ ($\text{M} + \text{H}^+$) 290.0520, found 290.0517.

Hexadecanoic Acid 2-(Hexadecanoyloxy)-3-[[hydroxy[2-[2-[2-(pyridin-2-yl)disulfanyl]ethoxy]ethoxy]

oxyl]acetylaminol[ethoxy]hydroxyphosphoryl]oxyl-propyl Ester (17). A solution of **16** (40 mg, 138.4 μ mol) and BOP (73 mg, 165 μ mol) in 5 mL of anhydrous CHCl_3 was prepared at room temperature. After 30 min, 100 mg (144.5 μ mol) of DPPE and 50 mL (276 μ mol) of diisopropylethylamine, previously dissolved at 40 $^\circ\text{C}$ in 5 mL of anhydrous CHCl_3 , was added. The reaction mixture was stirred at room temperature for 24 h. The volume was then adjusted to 30 mL with CHCl_3 and the organic phase washed successively four times with 20 mL of 1% citric acid and three times with 20 mL of water. Finally, the organic extract was dried on MgSO_4 and evaporated to dryness. The residue was purified on a silica gel column eluted with $\text{CHCl}_3/\text{CH}_3\text{OH}/\text{HOAc}$ (30:5:1). The title product was dissolved in *tert*-butanol/water (95:5) and was recovered after lyophilization as a white solid (84.7 mg, 64% yield): R_f 0.67 ($\text{CHCl}_3/\text{CH}_3\text{OH}/\text{HOAc}$, 60:20:3); $^1\text{H-NMR}$ ($\text{CDCl}_3/\text{CD}_3\text{OD}$, 1:1) δ 8.42 (d, $J = 4.8$ Hz, 1H, CHNC), 7.85–7.74 (m, 2H, CHCHCHNC), 7.23 (t, $J = 5.9$ Hz, 1H, NCCH), 5.30–5.20 (m, 1H, CHCH₂OP), 4.44–4.12 (m, 2H, CH₂CHCH₂OP), 4.04 (s, 2H, NCOCH₂O), 4.03–3.96 (m, 4H, CH₂OPOCH₂), 3.80–3.65 (m, 6H, CH₂CH₂OCH₂), 3.54 (t, $J = 6.0$ Hz, 2H, CH₂-NHCO), 3.05 (t, $J = 6.0$ Hz, 2H, CH₂SS), 2.36–2.29 (m, 4H, CH₂CH₂CO₂), 1.65–1.56 (m, 4H, CH₂CH₂CO₂), 1.28 (m, 48H, (CH₂)₂₄), 0.89 (t, $J = 6.6$ Hz, 6H, CH₃); MS (FAB⁺) m/z 961.4 ($\text{M} - \text{H}^+$).

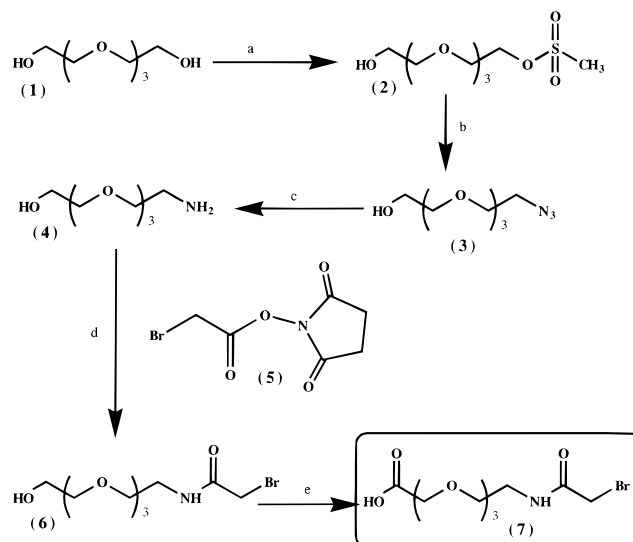
Rate of Reaction of Thiol-Reactive Functions in 8, 12, and 17 with the Peptide CGIRGERA. Solutions of **8**, **12**, and **17** (100 nmol) in CHCl_3 were evaporated under vacuum. The resulting lipidic film was resuspended, at room temperature, by sonication (2 min in a sonicator bath) in 800 μL of 50 mM sodium phosphate/citric acid (pH 5.0, 6.0, and 7.0) or sodium borate (pH 8.0 and 9.0) buffers containing 5% (v:v) methanol. The suspensions were then put in a bath thermostated at 25 $^\circ\text{C}$, and at time zero, CGIRGERA (200 nmol in 8.6 μL of water) was added. For **8** and **12**, reaction progress was measured at given times by determination of residual thiols (Riddles et al., 1979) on 100 μL aliquots with 1 mL of Ellman's reagent [2 mM DTNB and 1 mM ethylenediaminetetraacetic acid (EDTA) in 0.2 M sodium phosphate buffer (pH 7.27)]. The absorbance was read at 412 nm ($\epsilon = 14\,150\text{ M}^{-1}\text{ cm}^{-1}$). For **17**, reaction progress was measured spectrophotometrically at 343 nm by quantification of the release of 2-thiopyridone ($\epsilon = 8080\text{ M}^{-1}\text{ cm}^{-1}$) (Carlsson et al., 1978).

RESULTS AND DISCUSSION

The thiol-reactive heterobifunctional reagents **7**, **11**, and **16** (Schemes 1–3) were synthesized according to methodologies outlined in this paper. They contain flexible di- or tri(ethylene glycol) spacer arms and bear maleimide, bromoacetyl, or dithiopyridine moieties whose reactivities with the terminal cysteine of a model octapeptide were determined to evaluate the best coupling conditions. To investigate the immunogenicity of their spacer arms, these reagents were also conjugated, via their carboxylic function, to DPPE to yield respectively compounds **8**, **12**, and **17** (Figure 2) that could be incorporated into liposomes for immunological studies (Boeckler et al., submitted for publication).

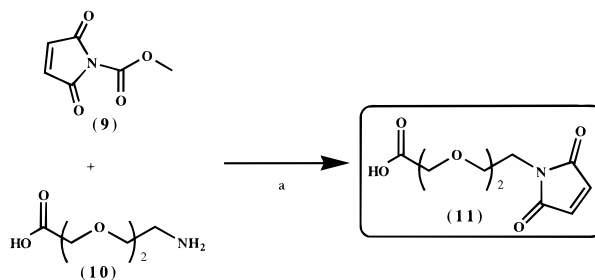
Synthesis of Compounds 7 and 8. Commercial tetra(ethylene glycol) was converted into monomesylate derivative **2** (Scheme 1) which in turn was converted into the azide **3** by reaction with sodium azide in acetonitrile under reflux. Compound **3** was conveniently reduced into the primary amine **4** with triphenylphosphine in the presence of water (Gololobov et al., 1981). A first attempt to transform **4** into **7** by oxidation of the alcohol function

Scheme 1. Synthesis of Compound 7^a



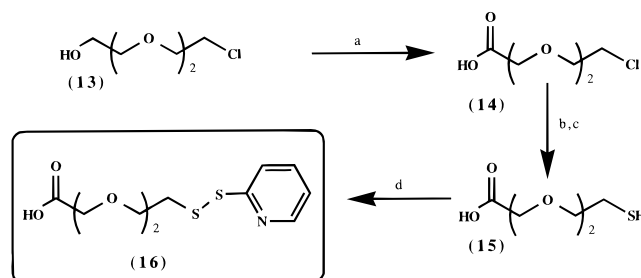
^a Key: (a) triethylamine, methanesulfonyl chloride, THF, overnight, RT; (b) NaN_3 , CH_3CN , reflux 36 h; (c) triphenylphosphine, H_2O , THF, 10 h, RT; (d) CHCl_3 , overnight, RT; (e) Jones reagent, acetone, 1 h, RT.

Scheme 2. Synthesis of Compound 11^a



^a Key: (a) NaHCO_3 , H_2O , 5 min 0 $^\circ\text{C}$; H_2O , 35 min, 20 $^\circ\text{C}$.

Scheme 3. Synthesis of Compound 16^a



^a Key: (a) Jones reagent, acetone, 30 min, RT; (b) thiourea, H_2O , reflux 4 h; (c) NaOH (2.5 M), reflux 4 h; (d) 2,2'-dipyridyl disulfide, ethanol, EtOAc, 24 h, RT.

into a carboxylic group followed by introduction of the bromoacetamide moiety proved unsuccessful. This was due to the very poor solubility of the intermediary amino acid in organic solvents and to side reactions with the carboxylic group in the last reaction. Therefore, **4** was first transformed, in good yields, into **6** by reaction with the *N*-hydroxysuccinimidyl ester of bromoacetate (**5**; Bernatowicz and Matsueda, 1986). Finally, the heterobifunctional reagent **7** was obtained by oxidation of the alcohol function with Jones reagent. In a final step, we wanted to activate the carboxylic function of **7** by formation of *N*-hydroxysuccinimidyl or *p*-nitrophenyl esters. Such esters proved to be very unstable and therefore difficult to purify. This is attributable to the polarity of the spacer arm which requires fairly polar solvents as eluents, e.g. methanol on silica gel and water on reverse

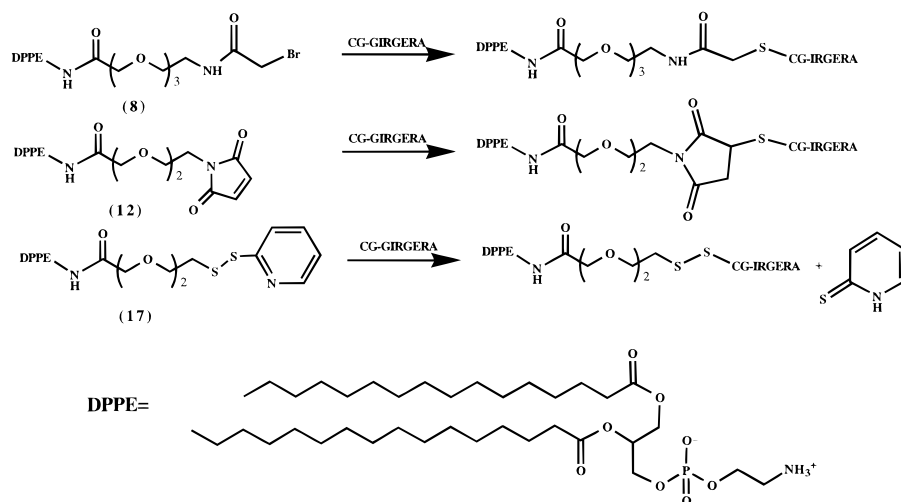


Figure 2. Structure of the thiol-reactive DPPE derivatives **8**, **12**, and **17** and their coupling to the model peptide CG-IRGERA.

phase media, which are detrimental to these esters. Moreover, the intrinsic reactivity of polyoxyethylene succinimidyl esters has been documented, and the carboxymethyl-NHS derivatives were found to be the least stable (Shearwater, 1995). Compound **8** was readily obtained by reaction of the carboxylic function of **7** with the free amine of DPPE in the presence of DCC and *N*-hydroxysuccinimide. The latter reagent assists in the formation of the amide bond (Weygand et al., 1966; Mattson et al., 1993). Compound **8** was purified by chromatography on BioSil,HA; it is stable for several months when stored dry at -20°C under argon.

Synthesis of Compounds 11 and 12. The synthesis of compound **11** is outlined in Scheme 2. The known amino acid **10** was prepared according to Slama and Rando (1980), and to introduce the maleimide moiety, a variety of methods were tested without success. For example, the classical reaction of the free amine with maleic anhydride (Coleman et al., 1958) which should produce the maleimide after the elimination of a water molecule, e.g. in the presence of toluene in a Dean–Stark apparatus (Rich et al., 1975) or with anhydrous sodium acetate in the presence of acetic anhydride (Witter and Tuppy, 1960), gave very poor yields. Similar results were obtained with other compounds that also contained a carboxylic function (Keller and Rudinger, 1975). The best method was that of Keller and Rudinger (1975) which makes use of 2,5-dioxo-2,5-dihydropyrrole-1-carboxylic acid methyl ester (**9**). This reaction of **10** with this maleimide donor must be carefully controlled. The conversion of the reaction intermediate into **11** is very dependent on pH, temperature, and time. At pH 8.5, in the presence of bicarbonate ions which catalyze the cyclization reaction, the formation of the maleimide is in competition with its own hydrolysis. The optimal experimental conditions were found by monitoring spectrophotometrically the formation of the maleimide at 297 nm (this peak disappears in the presence of an excess of thiol). They involve two temperature stages (5 min at 0°C followed by 35 min at 20°C) and a final acidification of the reaction medium (pH 6) to stabilize the maleimide moiety. Under these conditions, compound **11** was obtained with an average yield of 70%. For the same reasons as above, the preparation of the *N*-hydroxysuccinimidyl ester of **11** failed and **12** was prepared directly by reaction with DPPE in the presence of DCC and *N*-hydroxysuccinimide.

Preparation of Compounds 16 and 17. Commercial compound **13** (Scheme 3) was oxidized with Jones

reagent to give **14** in good yields. The introduction of the thiol group (compound **15**) was not as straightforward as anticipated. Thus, reaction of **14** with NaSH gave poor yields. We have therefore adapted a reaction which is classically used for the preparation of e.g. *S*-glycosides (Chipowsky and Lee, 1973). The chloro bond in **14** was reacted with thiourea to give an intermediary isothiurea which was directly hydrolyzed, under basic conditions, into a free thiol. Compound **15** was purified by chromatography through basic aluminum oxide. The heterobifunctional reagent **16** was finally obtained by derivatization with 2,2'-dipyridyl disulfide, according to Carlsson et al. (1978); this reaction was followed spectrophotometrically by measuring the formation of 2-thiopyridone. The formation of the derivative **17**, by reaction of DPPE with **16** in the presence of DCC, gave many side products that were difficult to eliminate during the purification step. We therefore used BOP, a reagent frequently used in peptide synthesis (Castro et al., 1975), as the coupling agent. The advantage of this approach was the relatively facile elimination of excess reagent and secondary products by extraction.

Reactivity of the Thiol-Reactive Moieties in 8, 12, and 17 with the Cysteine Residue of a Model Peptide CGIRGERA. The aim of our study is to replace MPB-PE in our liposomal construct (see Introduction) with these newly synthesized thiol-reactive DPPE derivatives. To conjugate peptides to the surface of such preformed vesicles, it was important to define the experimental conditions (time and pH) which give quantitative coupling yields. We have therefore studied the rate of reaction of **8**, **12**, and **17** with CG-IRGERA, the model peptide we have been using for optimizing our immunization system (see Introduction) and which carries at the N-terminus a cysteine residue (Figure 2). The reactions were carried out at 25°C and at fixed pH values, with a molar ratio of 2 in favor of the peptide. Reaction progress for **8** and **12** (i.e. formation of the thio ether derivatives) was determined by measuring residual free thiols with Ellman's reagent, whereas for **17**, the thiol exchange reaction was assessed by following spectrophotometrically the formation of 2-thiopyridone. Controls, under these different reaction conditions, allowed us to determine the spontaneous oxidation of CG-IRGERA (e.g. dimerization) and to correct the experimental data. The reaction progress curves are given in Figure 3A–C. As expected, the maleimide moiety of **12** (Figure 3B) reacted rapidly with the peptide, especially at basic pH due to great reactivity of the thiolate group (pK_a thiol

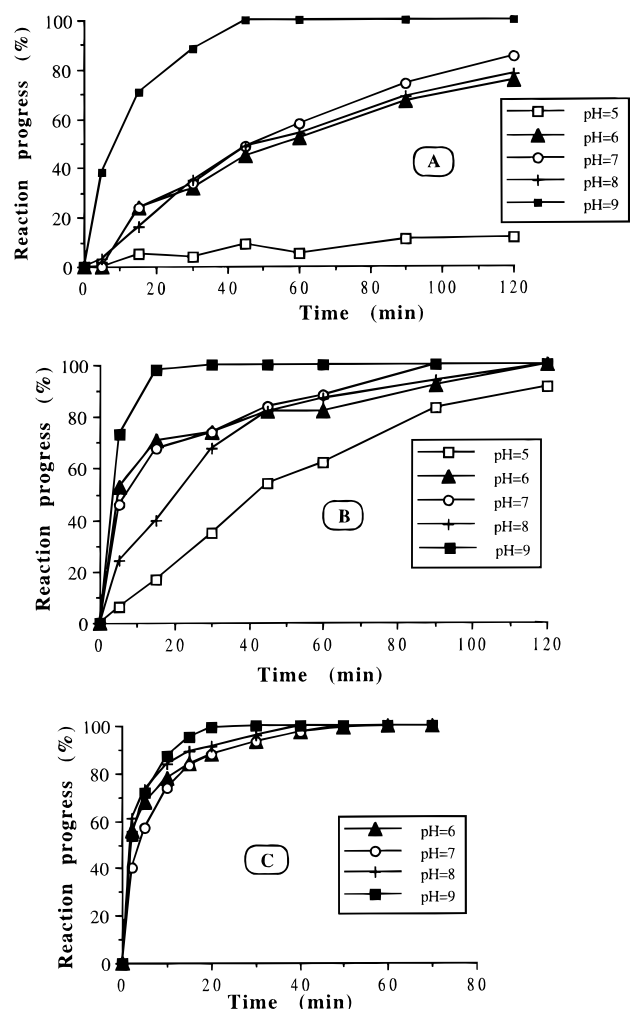


Figure 3. Rate of reaction between CG-IRGERA and the thiol-reactive DPPE derivatives **8** (A), **12** (B), and **17** (C), at different pH values. The coupling reactions were performed at 25 °C in the presence of a 2-fold molar excess of peptide (see text), and reaction progress was determined by measuring residual thiols with Ellman's reagent (A and B) or by following the formation of 2-thiopyridone (C).

of cysteine ≈ 8.5); however even at pH 6.0, which preserves the maleimide group, the coupling was essentially quantitative after 2 h. It should be noted that at pH 9.0 the reaction of the maleimide moiety with CGIRGERA is fast enough to keep its hydrolysis by hydroxyl ions marginal. It follows that, similar to MPB-PE (Martin and Papahadjopoulos, 1982), the coupling of the thiol-bearing peptide to **12** can be obtained in excellent yields under mild (neutral) pH and temperature conditions, making this PE derivative suitable for our synthetic vaccine program. Comparatively, the reactivity of the bromoacetyl moiety of **8** was less pronounced (Figure 3A). Whereas a quantitative conjugation was observed at pH 9.0 within 50 min of reaction, at lower pH values, the coupling was found more sluggish. The thiol exchange reaction between the peptide and compound **17** proceeded smoothly and was essentially quantitative within a pH range from 6 to 9 (Figure 3C). The driving force here is the release of 2-thiopyridone. This coupling reaction can therefore be performed using similar experimental conditions as given above for the coupling to a maleimido group.

Size of the Spacer in Peptide Derivatives of **8, **12**, and **17**.** Since the immune response against the peptides conjugated to the surface liposomes could, in principle, depend on the separation of the peptide from its carrier,

i.e. on the length and the flexibility of the spacer arms (Figure 1), we have measured the distances between the carbon atom of the amide group of the DPPE derivatives **8**, **12**, **17**, and MPB-PE and the sulfur atom of the conjugated peptide. In extended conformations, as calculated with the Personal CAChe program, the following distances, in angstroms, were found: **8**, 16.1; **12**, 13.1; **17**, 11.3; and MPB-PE, 12.5.

CONCLUSION

Three new thiol-reactive polyoxyethylene-based heterobifunctional reagents were synthesized with the aim of providing spacer arms of relatively low immunogenicity in liposomal constructs used for eliciting an immune response against synthetic peptides. After coupling to DPPE, they allowed the conjugation, under defined conditions, of synthetic peptides e.g. to the surface of preformed liposomes (Frisch et al., 1991) via spacers of variable lengths. Moreover, two types of linkages between the peptides, which carry a cysteine residue, and the vesicles were introduced: i.e. stable thio ether and bio-reducible disulfide bonds which might be of importance in the processing of such constructs by immunocompetent cells. As described elsewhere (Boeckler et al., submitted for publication), an important immune response was obtained against IRGERA, the model hexapeptide used as the immunogen (Figure 1), which did not depend on the length of the spacer or on the linkage type. The two spacer arms in **8** and **17** were also found to be less immunogenic than the one introduced with the classical MPB-PE derivative. These new heterobifunctional reagents should also find applications in other fields; for example, we have used this approach in the design of multiantennary glycoconjugates (Kichler and Schuber, 1995), and their corresponding phospholipid derivatives were found to strongly enhance gene delivery with neutral complexes of lipospermine (Kichler et al., 1995).

LITERATURE CITED

- Arnon, R., and Horwitz, R. J. (1992) Synthetic peptides as vaccines. *Curr. Opin. Immunol.* 4, 449–453.
- Bernatowicz, M. S., and Matsueda, G. R. (1986) Preparation of peptide-protein immunogens using N-succinimidyl bromoacetate as a heterobifunctional crosslinking reagent. *Anal. Biochem.* 155, 95–102.
- Carlsson, J., Drevin, H., and Axen, R. (1978) Protein thiolation and reversible protein-protein conjugation. N-succinimidyl 3-(2-pyridyldithio) propionate, a new heterobifunctional reagent. *Biochem. J.* 173, 723–734.
- Chipowsky, S., and Lee, Y. C. (1973) Synthesis of 1-thioaldosides having an amino group at the aglycon terminal. *Carbohydr. Res.* 31, 339–346.
- Coleman, L. E., Bork, J. F., and Dunn, H. (1959) Reaction of primary aliphatic amines with maleic anhydride. *J. Org. Chem.* 24, 135–136.
- Defoort, J. P., Nardelli, B., Huang, W., and Tam, J. P. (1992) A rational design of synthetic peptide vaccine with built-in adjuvant. *Int. J. Pept. Protein Res.* 40, 214–221.
- Friede, M. (1995) Liposomes as carriers of antigens. In *Liposomes as tools in basic research and industry* (J. R. Philippot and F. Schuber, Eds.) pp. 189–199, CRC Press Inc., Boca Raton, FL.
- Friede, M., Muller, S., Briand, J.-P., Van Regenmortel, M. H. V., and Schuber, F. (1993) Induction of immune response against a short synthetic peptide antigen coupled to small neutral liposomes containing Monophosphoryl Lipid A. *Mol. Immunol.* 30, 539–547.
- Friede, M., Muller, S., Briand, J.-P., Plaué, S., Fernandes, I., Frisch, B., Schuber, F., and Van Regenmortel, M. H. V. (1994) Selective induction of protection against influenza virus

- infection in mice by a lipid-peptide conjugate delivered in liposomes. *Vaccine* 12, 791–797.
- Frisch, B., Muller, S., Briand, J.-P., Van Regenmortel, M. H. V., and Schubert, F. (1991) Parameters affecting the immunogenicity of liposome-associated synthetic peptide antigen. *Eur. J. Immunol.* 21, 185–193.
- Gololobov, Y. U., Zhmurova, I. N., and Kasukhin, L. F. (1981) Sixty years of Staudinger reaction. *Tetrahedron* 37, 437–472.
- Gregoriadis, G. (1994a) The immunological adjuvant and vaccine carrier. Properties of liposomes. *J. Drug Targeting* 2, 351–356.
- Gregoriadis, G. (1994b) Liposomes as immunoadjuvants and vaccine carriers: antigen entrapment. *Immunomethods* 4, 210–216.
- Gregory, J. D. (1955) The stability of *N*-ethylmaleimide and its reaction with sulfhydryl groups. *J. Am. Chem. Soc.* 77, 3922–3923.
- Jones, G. L., Edmundson, H. M., Spencer, L., Gale, J., and Saul, A. (1989) The use of maleimidocaproyloxysuccinimide to prepare malarial peptide carrier immunogens. *J. Immunol. Methods* 123, 211–216.
- Keller, O., and Rudinger, J. (1975) Preparation and some properties of maleimido acids and maleoyl derivatives of peptides. *Helv. Chim. Acta* 58, 531–541.
- Kichler, A., and Schubert, F. (1995) Versatile synthesis of bi- and tri-antennary galactose ligands: interaction with the Gal/GalNAc receptor of human cells. *Glycoconjugate J.* 12, 275–281.
- Kichler, A., Remy, J.-S., Boussif, O., Frisch, B., Boeckler, C., Behr, J.-P., and Schubert, F. (1995) Efficient gene delivery with neutral complexes of lipospermine and thiol-reactive phospholipids. *Biochem. Biophys. Res. Commun.* 209, 444–450.
- Klausner, Y. S., and Bodansky, M. (1972) Coupling reagents in peptide synthesis. *Synthesis*, 453–463.
- Lövgren, K., and Larsson, M. (1994) Conjugation of synthetic peptides to carriers Iscoms: affecting the immunogenicity of the conjugate. *J. Immunol. Methods* 173, 237–243.
- Martin, F. J., and Papahadjopoulos, D. (1982) Irreversible coupling of immunoglobulin fragments to preformed vesicles. *J. Biol. Chem.* 257, 286–288.
- Martin, F. J., Heath, T. D., and New, R. R. C. (1989) Covalent attachment of proteins to liposomes. In *Liposomes a practical approach* (R. R. C. New, Ed.) Chapter 4, pp 163–182, IRL Press, Oxford University Press, Oxford, England.
- Mattson, G., Conklin, E., Desai, S., Nielander, G., Savage, M. D., and Morgensen, S. (1993) A practical approach to crosslinking. *Mol. Biol. Rep.* 17, 167–183.
- Muller, S. (1988) Peptide-carrier conjugation. In *Synthetic polypeptides as antigens* (M. H. V. Van Regenmortel, J. P. Briand, S. Muller, and S. Plaué, Eds.) Vol. 19, pp 95–127, Elsevier, Amsterdam, The Netherlands.
- Peeters, J. M., Hazendonk, T. G., Beuvery, E. C., and Tesser, G. I. (1989) Comparison of four bifunctional reagents for coupling peptides to proteins and the effect of the three moieties on the immunogenicity of the conjugates. *J. Immunol. Methods* 120, 133–143.
- Rich, D. H., Gesellchen, P. D., Tong, A., Cheung, A., and Buchner, C. K. (1975) Alkylating derivatives of amino acids and peptides. *J. Med. Chem.* 18, 1004–1010.
- Riddles, P. W., Blakeley, R. L., and Zerner, B. (1979) Ellman's reagent: 5,5'-dithiobis(2-nitrobenzoic acid) a reexamination. *Anal. Biochem.* 94, 75–81.
- Shearwater Polymers Inc. *Catalog* (1995) March issue pp 44–45, Huntsville, AL.
- Slama, J. S., and Rando, R. R. (1980) Lectin-mediated aggregation of liposomes containing glycolipids with variable hydrophilic spacer arms. *Biochemistry* 19, 4595–4600.
- Weygand, F., Hoffmann, D., and Wünsch, E. (1966) Peptidsynthesen mit Dicyclohexylcarbodiimid unter Zusatz von *N*-Hydroxysuccinimid. *Z. Naturforsch.* 21B, 426–428.
- Witter, A., and Tuppy, H. (1960) *N*-(4-dimethylamino-2,5-dinitrophenyl)maleimide: a coloured sulfhydryl reagent. *Biochim. Biophys. Acta* 45, 429–442.
- Zalipsky, S. (1995) Functionalized poly(ethylene glycol) for preparation of biologically relevant conjugates. *Bioconjugate Chem.* 6, 150–165.

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Enzyme-Linked Immunosorbent Assay Detection of Pyrrolizidine Alkaloids: Immunogens Based on Quaternary Pyrrolizidinium Salts

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Polyclonal antibody-based enzyme-linked immunosorbent assays (ELISAs) were developed for the detection of retrorsine (**1**, 351 g/mol), monocrotaline (**2**, 325 g/mol), and retronecine (**3**, 155 g/mol) in the parts per billion (ppb) range. A set of three bifunctional linking arms (**6–8**) was synthesized. By N-alkylation of pyrrolizidine alkaloids (PAs) retrorsine, monocrotaline, and retronecine acetonide (**9**), six haptens (**6.1**, **6.2**, **7.1**, **7.2**, **7.9**, and **8.9**) were synthesized and used to generate rabbit antisera. The resulting anti-retrorsine antiserum gave a 50% inhibition (I_{50}) value of 0.9 ± 0.2 ppb for retrorsine with detection limits of 0.5–10 ppb. The same ELISA system also detected isatidine (**4**, retrorsine *N*-oxide) dihydrate (403 g/mol) with an I_{50} of 1 ppb and senecionine (**5**, 352 g/mol) with an I_{50} of 100 ppb. A second monocrotaline-based ELISA detected monocrotaline with an I_{50} of 36 ± 9 ppb **2** with detection limits of 5–500 ppb and shows no cross-reactivity with **1** or **5**; this ELISA demonstrates the potential for the substrate-specific detection method. A third retronecine-based ELISA detects **3** with an I_{50} of 3000 ± 600 ppb (3 ± 0.6 ppm) and detection limits of 600–10000 ppb. None of these ELISAs cross-react with the structurally similar swainsonine (**10**) or lupinine (**11**) alkaloids. PAs were detected in extracts of *Senecio vulgaris* and *Crotalaria retusa*, but not in *Lupinus* spp., as a demonstration of the ELISA's usefulness.

INTRODUCTION¹

Pyrrolizidine alkaloids [cf. retrorsine (**1**), monocrotaline (**2**), and senecionine (**5**)] constitute a class of secondary plant metabolites of wide geographical and botanical (including Boraginaceae, Compositae, Gramineae, Leguminosae, Orchidaceae, Rhizophoraceae, Santalaceae, and Saptoaceae) distribution which are toxic to both humans and animals (Smith & Culvenor, 1981). Culvenor (1980) estimates that as many as 6000 species may be PA-producing species, accounting for 3% of all flowering plants. Many plants produce more than one PA, and these often co-occur with varying amounts of their corresponding *N*-oxide [cf. isatidine (**4**)]. Structural similarities in the >250 known PAs are striking; for example, the necine base retronecine (**3**) is found in alkaloids from 6 botanical families and 26 genera (Robins, 1982; Bull et al., 1968; McLean, 1970; Peterson & Culvenor, 1983). Collectively, PAs exhibit a broad range of cytotoxic/pathological actions, including hepatotoxic (Mattocks & Bird, 1983; Segall et al., 1985), pneumotoxic (Chesney et al., 1974), embryotoxic (Mattocks, 1986), mutagenic (Ames et al., 1973), carcinogenic (Kuhara et al., 1980; Allen et al., 1975; Svoboda & Reddy, 1972), and teratogenic (Keeler, 1983) effects. Chronic gastrointestinal (Hooper, 1974), cardiopulmonary (Chesney, 1973), and central nervous system (Hooper, 1974) disorders are

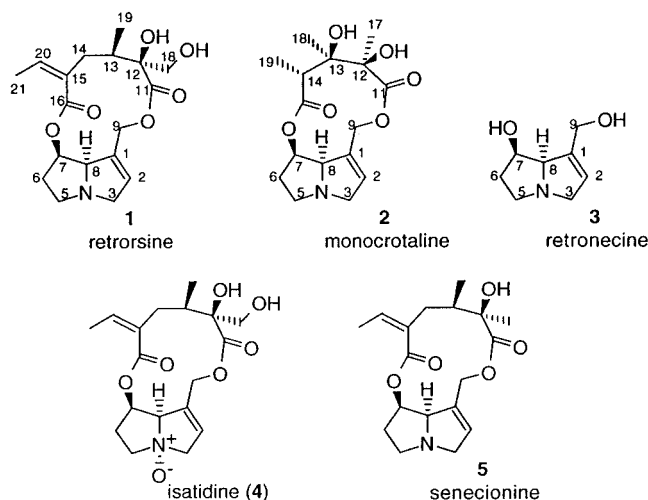


Figure 1. PAs used as analytes in this study.

further manifestations of PA poisoning. Although the toxic response depends on both the chemical nature of the PA and the animal species involved, the development of liver disease (veno-occlusive) is a common occurrence.

Because of these issues, there is an urgent need for a reliable, rapid, and sensitive method for identification/quantification of PAs and PA metabolites in biological/environmental samples. In previous reports, we detailed several strategies for detection of PAs. The first and more speculative strategy involved direct detection of the retronecine substructure in intact parent PAs (Bober et al., 1990; Kurth et al., 1992), while the second strategy involved a somewhat less direct assay but one which capitalized on the fact that hydrolysis of any retronecine-based macrocyclic diester PA liberates the necine base retronecine (e.g., saponification of monocrotaline yields monocrotalic acid and retronecine). This "class-specific" PA ELISA detects retronecine at an I_{50} value of 11 ± 3 ppb, with detection limits of 1.0–100 ppb (Kurth et al., 1989; Roseman et al., 1992).

Free base PAs are readily N-alkylated by methyl iodide in ethereal solvent to produce the N-methylated salt as

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¹ Abbreviations: ELISA, enzyme-linked immunosorbent assay; ppb, parts per billion; PAs, pyrrolizidine alkaloids; I_{50} , 50% inhibition; Tris, tris(hydroxymethyl)aminomethane; IgG-HRP, goat anti-rabbit IgG antibody-horseradish peroxidase conjugate; OPD, *o*-phenylenediamine; BSA, bovine serum albumin; OVA, chicken egg ovalbumin; KLH, keyhole limpet hemocyanin; DCC, dicyclohexylcarbodiimide; NHS, *N*-hydroxysuccinimide; DME, 1,2-dimethoxyethane; DIBAL-H, diisobutylaluminum hydride; PCC, pyridinium chlorochromate; TLC, thin-layer chromatography; PBST, phosphate-buffered saline-Tween.

a precipitate (Leonard, 1960). In this paper, we report that primary functionalized alkyl halides can be used to N-alkylate PAs and that the resulting quaternary ammonium alkaloids can be conjugated to a carrier protein. The development of ELISAs based on these N-alkylated quaternary ammonium PAs is reported.

EXPERIMENTAL SECTION

Equipment. ELISAs were carried out on 96-well Immulon 2 microtiter plates (Dynatech) and read with a UVmax microtiter plates reader (Molecular Devices) at 490 nm. Nuclear magnetic resonance spectra were taken on a QE-300 NMR spectrometer (General Electric) with ^1H spectra at 300 MHz and ^{13}C spectra at 75.6 MHz.

Materials. Senecionine, *Senecio vulgaris* whole plant, *Crotalaria retusa* seeds, and *Lupinus* spp. whole plant samples were a gift from Dr. H. J. Segall (Veterinary Medicine Pharmacology and Toxicology, University of California, Davis). (–)-Lupinine hydrochloride was purchased from Research Plus. Monocrotaline was purchased from Trans World Chemicals. Solvents and inorganic reagents were purchased from Fisher Scientific. Swainsonine, isatidine (retrorsine *N*-oxide), Tween-20, Tris base, citric acid, goat anti-rabbit IgG antibody–horseradish peroxidase conjugate, *o*-phenylenediamine, Freund's complete and incomplete adjuvants, bovine serum albumin, chicken egg ovalbumin, and keyhole limpet hemocyanin were purchased from Sigma Chemical Co. Retrorsine, dicyclohexylcarbodiimide, and *N*-hydroxysuccinimide were purchased from Aldrich Chemical Co. Dialysis tubing was purchased from Spectrum Medical Industries. 1,2-Dimethoxyethane was distilled under a nitrogen atmosphere from sodium/potassium benzophenone ketyl immediately prior to use.

Retronecine (3). In a 250 mL round bottom flask equipped with a reflux condenser and a magnetic stirrer, monocrotaline (6.00 g, 18.4 mmol), $\text{Ba}(\text{OH})_2 \cdot 8\text{H}_2\text{O}$ (15.00 g, 47.55 mmol), and water (125 mL) were combined and heated at reflux for 3 h (with constant bubbling of N_2 through the solution). The mixture was cooled to room temperature and saturated with solid carbon dioxide. The barium carbonate was removed by suction filtration through Celite, and the clear filtrate was concentrated under vacuum to 25% of its original volume. The aqueous concentrate was basified (pH 10) with Na_2CO_3 , saturated with NaCl, and continuously extracted with ether for 72 h. The organic extract was concentrated under reduced pressure and evacuated at 1 Torr overnight to give a light brown solid. Decolorization with activated carbon and recrystallization from acetone gave retronecine (2.05 g, 13.2 mmol, 72%) as white prisms: mp 120–121 °C; ^1H NMR (300 MHz, CDCl_3) δ 1.89–2.02 (2H, complex, NCH_2CH_2), 2.75 (1H, m, NCHHCH_2), 3.22 (1H, m, NCHHCH_2), 3.42 (1H, m, $\text{NCHHCH}=\text{}$), 3.85 (1H, m, $\text{NCHHCH}=\text{}$), 4.06–4.15 (2H, complex, NCH and CHHOH), 4.27–4.31 (2H, complex, CHOH and CHHOH), 5.70 (1H, s br, vinyl); IR (KBr) 3321 (OH) cm^{-1} .

***N*-[(4-Bromocrotonyl)oxy]succinimide (6).** The NHS ester of 4-bromocrotonic acid was formed by the method of Anderson et al. (1964). Dry 3-bromocrotonic acid (0.3025 g, 1.83 mmol) was dissolved in freshly distilled DME (2.6 mL) in an oven-dried 10 mL round bottom flask equipped with a flea-size stirring bar, a septum, and a nitrogen inlet. The mixture was chilled in an ice bath, and a DME solution of DCC (0.24 M in DCC, 8.6 mL, 2.0 mmol) (Aldrich; caution, sensitizer!) was injected. After standing overnight at 4 °C, the dicyclohexylurea was removed by filtration (medium glass frit with Celite). The remaining liquid was reduced by rotary evaporation to reveal a yellow crystalline solid, which after drying *in vacuo*, afforded the crude NHS ester

(0.49 g) which recrystallized from 2-propanol (10 mL) to yield pale yellow needles (0.328 g, 1.25 mmol, 68%) of NHS ester **6**: mp 129–131 °C; ^1H NMR (300 MHz, CDCl_3) δ 2.86 (s, 4H, $\text{COCH}_2\text{CH}_2\text{CO}$), 4.06 (d, 2H, $J = 7$ Hz, CH_2Br), 6.26 (d, 1H, $J = 15$ Hz, CHCHCO), 7.31 (m, CHCHCO); ^{13}C NMR (75 MHz, $\text{CDCl}_3 + \text{DMSO}-d_6$) δ 24.4 ($\text{COCH}_2\text{CH}_2\text{CO}$), 27.7 (BrCH_2), 117.3 (CHCHCO_2), 146.5 (CHCHCO_2), 159.5 (CHCHCO_2), 168.2 ($\text{COCH}_2\text{CH}_2\text{CO}$); IR (KBr) 1642 ($\text{NC}=\text{O}$), 1734 ($\text{OC}=\text{O}$) cm^{-1} . Anal. Calcd for $\text{C}_8\text{H}_8\text{BrNO}_4$: C, 36.67; H, 3.08; Br, 30.49; N, 5.34. Found: C, 36.60; H, 3.01; Br, 30.52; N, 5.28.

***N*-[[*p*-(α -Bromomethyl)benzoyl]oxy]succinimide (7).** *N*-Hydroxysuccinimide (4.33 g, 37.6 mmol) and *p*-(α -bromomethyl)benzoic acid (8.04 g, 37.4 mmol) were combined in a 500 mL round bottom flask, and the vessel was charged with N_2 . These solids were dissolved in dry DME (250 mL), and the solution was chilled in an ice bath. A DME solution (30 mL) of DCC (7.90 g, 38.3 mmol) was added by cannula, and the resulting solution was stirred overnight at 5 °C under N_2 . The solution was then warmed to room temperature, and the dicyclohexylurea was removed by filtration. The DME was removed under reduced pressure, and the crude solid was recrystallized from 2-propanol to provide rhombic white crystals (10.81 g, 34.6 mmol, 93%): mp 160–162 °C; ^1H NMR (300 MHz, CDCl_3) δ 2.86 (s, 4H, $\text{COCH}_2\text{CH}_2\text{CO}$), 4.59 (s, 2H, ArCH_2Br), 7.60 (d, 2H, $J = 8.2$ Hz, meta), 8.08 (d, 2H, $J = 8.2$ Hz); ^{13}C NMR (75 MHz, $\text{CDCl}_3 + \text{CD}_3\text{CN}$) δ 26.3 ($\text{COCH}_2\text{CH}_2\text{CO}$), 32.6 (ArCH_2Br), 125.5 (ipso), 130.3 (ortho), 131.3 (meta), 141.6 (para), 162.2 (ArCO_2N), 170.5 ($\text{COCH}_2\text{CH}_2\text{CO}$); IR (KBr) 1732 ($\text{C}=\text{O}$) cm^{-1} . Anal. Calcd for $\text{C}_{12}\text{H}_{10}\text{BrNO}_4$: C, 46.18; H, 3.23; Br, 25.60; N, 4.49. Found: C, 45.99; H, 3.12; Br, 25.43; N, 4.40.

4-(Bromomethyl)benzaldehyde (8). DIBAL-H (140 mL, 140 mmol) was added under argon over 10 min to a -70 °C solution of ethyl α -bromotoluate (12.07 g, 49.7 mmol) in toluene (20 mL). The reaction mixture was stirred for 1 h at -70 °C and at room temperature for 2 h, and then the reaction was quenched with the addition of 1 M HCl (10 mL). The mixture was taken up in ether (50 mL) and washed with 1 M HCl (3×30 mL), and the aqueous washes were extracted with ether (3×90 mL). The combined organic layers were dried over sodium sulfate and concentrated under reduced pressure. Drying under high vacuum delivered oily, low melting translucent plates of 4-(bromomethyl)benzyl alcohol (8.08 g, 40.2 mmol, 81%): mp 76–77 °C; ^1H NMR (300 MHz, CDCl_3) δ 3.24 (1H, s, OH), 4.43 (2H, s, CH_2Br), 4.50 (2H, s, CH_2OH), 7.20 (2H, ABq, $J = 8.1$ Hz), 7.30 (2H, ABq, $J = 8.1$ Hz); ^{13}C NMR (75 MHz, CDCl_3) δ 33.3 (CH_2Br), 64.2 (CH_2OH), 127.1 (Ar), 129.0 (Ar), 126.8 (CH_2Br), 141.0 (CH_2OH); IR (neat) 3250 (OH) cm^{-1} .

A slurry of PCC (4.37 g, 20 mmol, 1 equiv) in CH_2Cl_2 (40 mL) was added in 5 mL portions over 5 h to 4-(bromomethyl)benzyl alcohol (6.06 g, 30 mmol) in CH_2Cl_2 (100 mL) under argon. The reaction was monitored by silica TLC (20:80 EtOAc:hexane), and after 2 h, the mixture was filtered through Celite in a fritted glass funnel. Flash column chromatography gave **8** as a white crystalline material which sublimated on standing (used without further purification): mp 93.5–95 °C; ^1H NMR (300 MHz, CD_2Cl_2) δ 4.51 (CH_2Br), 7.56 (2H, ABq, $J = 8$ Hz), 7.86 (2H, ABq, $J = 8.2$ Hz), 10.01 (CHO); ^{13}C NMR (75 MHz, CD_2Cl_2) δ 32.6 (CH_2Br), 130.1 (CH, Ar), 130.4 (CH, Ar), 136.8 (CCH_2Br), 144.8 (CHO), 191.8 (CHO); FTIR (NaCl plates) 1693 ($\text{C}=\text{O}$) cm^{-1} .

***N*-[(Succinimid-3-yloxycarbonyl)ethenyl]methyl-retrorsine, Bromide Salt (6.1).** Retrorsine (408 mg, 1.16 mmol) was dissolved in dry DME (12 mL) and treated with a DME (10 mL) solution of bromoester **6** (355 mg, 1.35 mmol). After 2 min, stirring was discon-

tinued, and the solution was allowed to stand overnight. A fine white powder of **6.1** appeared at the bottom of the vessel which was collected by filtration and washed with fresh DME (6 mL), dried under high vacuum in a heated vessel, and used without further purification (670 mg, 1.09 mmol, 94%): mp 115–120 °C dec; ^1H NMR (300 MHz, CD_3CN) δ 0.84 (3H, d, J = 6.5 Hz, H-19 CH_3), 1.73 (1H, m, H-13 CHCH_3), 1.79 (1H, m, H-14b CHH), 1.85 (3H, dd, $J_{\text{vic}} = 7.1$ Hz, $J_{\text{allyl}} = 1.3$ Hz, H-21 CH_3), 2.27 (1H, d, $J_{\text{gem}} = 13.2$ Hz, H-14a CHH), 2.80 (2H, m, H-6 CH_2), 2.87 (4H, s, 3''- and 4''- $\text{COCH}_2\text{CH}_2\text{CO}$), 3.65 (2H, s br, H-18 CH_2), 3.84 (1H, m, H-5b CHH), 4.92 (1H, m, H-5a CHH), 4.30 (1H, d, $J_{\text{gem}} = 12.3$ Hz, H-9b CHH), 4.51 (1H, d, J = 9 Hz, H-3b NCHH), 4.53 (1H, m, H-3a NCHH), 4.54 (2H, s br, $\text{NCH}_2\text{CH}=\text{CHCO}_2$), 5.25 (1H, m, H-8 NCH), 5.46 (1H, d, $J_{\text{gem}} = 12.1$, H-9a CHH), 5.48 (1H, m, H-7 OCH), 5.90 (1H, dq, $J_{\text{q}} = 6.7$ Hz, $J_{\text{allyl}} = 0.8$ Hz, H-20 CHCH_3), 6.33 (1H, s br, H-2), 6.77 (1H, d, J = 15.5 Hz, H-2' $\text{CH}_2\text{CH}=\text{CHCO}_2$), 7.28 (1H, dt, $J_{\text{d}} = 15.5$ Hz, $J_{\text{t}} = 7.2$ Hz, H-3' $\text{CH}_2\text{CH}=\text{CHCO}_2$); IR (KBr) 1559 ($\text{NC}=\text{O}$), 1653 ($\text{CC}=\text{O}$), 3746 (OH) cm^{-1} . Anal. Calcd for $\text{C}_{26}\text{H}_{33}\text{BrN}_2\text{O}_{10}$: C, 50.91; H, 5.42; Br, 13.03; N, 4.57. Found: C, 50.80; H, 5.40; Br, 12.58; N, 4.57.

N-[[2-(Succinimid-3-yloxycarbonyl)ethenyl]methyl]monocrotaline, Bromide Salt (6.2). Monocrotaline **2** (253.7 mg, 0.7806 mmol) was dissolved in dry DME (8 mL) and treated with a DME (6 mL) solution of bromo ester **6** (224.2 mg, 0.8558 mmol). After 2 min, stirring was discontinued, and the solution was allowed to stand overnight. A fine white powder of **6.2** appeared at the bottom of the vessel which was collected by filtration and washed with fresh DME (4 mL), dried under high vacuum in a heated vessel, and used without further purification (403.3 mg, 0.6871 mmol, 88%): mp 120–123 °C dec; ^1H NMR (300 MHz, CD_3CN) δ 1.21 (3H, d, J = 7.3 Hz, H-17), 1.30 (3H, s, H-18), 1.44 (3H, s, H-14), 2.40 (1H, m, H-6 trans), 2.68 (1H, m, H-6 cis), 2.86 (4H, s, H-3'' and H-4''), 3.23 (1H, q, J = 7.1 Hz, H-19), 3.89 (1H, m, H-5 trans), 4.47 (1H, m, H-5 cis), 4.55 (2H, m, H-4'), 4.59 (1H, m, H-3 trans), 4.74 (1H, m, H-3 cis), 4.90 (2H, m, H-8 and H-9 cis), 5.19 (1H, d br, J = 7.4 Hz, H-7), 5.36 (1H, q br, J = 6.8 Hz, H-9 trans), 6.28 (1H, s br, H-2), 6.71 (1H, d, J = 16 Hz, H-2'), 7.28 (1H, dt, $J_{\text{vinyl}} = 15$ Hz, $J_{\text{vicinal}} = 7.1$ Hz, H-3'); IR (KBr) 1655 ($\text{NC}=\text{O}$), 1730 ($\text{CC}=\text{O}$), 3391 (OH) cm^{-1} . Anal. Calcd for $\text{C}_{24}\text{H}_{31}\text{BrN}_2\text{O}_{10}$: C, 49.07; H, 5.32; Br, 13.60; N, 4.77. Found: C, 48.82; H, 5.32; Br, 13.08; N, 4.62.

N-[[2-(Succinimid-4-yloxycarbonyl)phenyl]methyl]retorsine, Bromide Salt (7.1). Retorsine (396 mg, 1.13 mmol) was dissolved in dry DME (12 mL) and treated with a DME (10 mL) solution of bromo ester **7** (530 mg, 1.31 mmol). After 2 min, stirring was discontinued, and the solution was allowed to stand overnight. A fine white powder of **7.1** appeared at the bottom of the vessel which was collected by filtration and washed with fresh DME (6 mL), dried under high vacuum in a heated vessel, and used without further purification (688 mg, 0.912 mmol, 81%): mp 170–174 °C dec; ^1H NMR (300 MHz, CD_3OD) δ 0.82 (3H, d, J = 6.5 Hz, H-19), 1.71 (1H, m, H-13), 1.82 (1H, m, H-14b), 1.85 (3H, dd, $J_{\text{vicinal}} = 7.0$ Hz, $J_{\text{homallylic}} = 1$ Hz, H-21), 2.22 (1H, d, J = 12.7 Hz, H-14a), 2.68 (2H, m, H-6), 2.93 (4H, s, H-3'' and H-4''), 3.65 (2H, s, H-18), 3.85 (1H, m, H-5a), 4.20 (1H, d, $J_{\text{gem}} = 12$ Hz, H-9b), 4.23 (1H, m, H-3b), 4.46 (1H, d, J = 17 Hz, H-3a), 4.80 (1H, s, H-6' NCH_2Ar), 4.83 (1H, d, J = 8 Hz, H-7), 5.17 (1H, d, $J_{\text{gem}} = 12$ Hz, H-9a), 5.43 (2H, dt, $J_{\text{d}} = 10$ Hz, H-8), 5.90 (1H, dq, $J_{\text{vicinal}} = 6.7$ Hz, $J_{\text{allylic}} = 0.6$ Hz, H-20), 6.12 (1H, s br, H-2), 7.95 (2H, ABq, J = 8.3 Hz, H-4'), 8.23 (2H, ABq, J = 8.3 Hz, H-3'); IR (KBr) 1740 ($\text{C}=\text{O}$), 1772 ($\text{C}=\text{O}$), 3377 (OH) cm^{-1} . Anal. Calcd

for $\text{C}_{30}\text{H}_{35}\text{BrN}_2\text{O}_{10}$: C, 54.31; H, 5.32; Br, 12.04; N, 4.22. Found: C, 53.83; H, 5.28; Br, 11.81; N, 4.05.

N-[[2-(Succinimid-4-yloxycarbonyl)phenyl]methyl]monocrotaline, Bromide Salt (7.2). Monocrotaline **2** (411 mg, 1.26 mmol) was dissolved in dry DME (12 mL) and treated with a DME (10 mL) solution of bromo ester **7** (533 mg, 1.32 mmol). After 2 min, stirring was discontinued, and the solution was allowed to stand overnight. A fine white powder of **7.2** appeared at the bottom of the vessel which was collected by filtration and washed with fresh DME (6 mL), dried under high vacuum in a heated vessel, and used without further purification (751 mg, 1.03 mmol, 82%): mp 177–179 °C dec; ^1H NMR (300 MHz, CD_3OD) δ 1.21 (3H, d, J = 7.0 Hz, 3H, C-19 Me), 1.29 (3H, s, C-17), 1.43 (3H, s, C-18), 2.38 (1H, m, H-6b), 2.66 (1H, m, H-6a), 2.90 (4H, s, H-3'' and H-4'' $\text{COCH}_2\text{CH}_2\text{CO}$), 3.23 (1H, q, J = 7.2 Hz, H-14), 3.82 (1H, m, H-5a), 3.94 (1H, m, H-5b), 4.61 (1H, d, J = 12.1 Hz, H-9a), 4.34 (1H, d, J = 12.1 Hz, H-9b), 4.45 (1H, d, J = 17.2 Hz, H-3b), 4.71 (1H, d, J = 16.6 Hz, H-3a), 4.87 (2H, s, H-6 NCH_2Ar), 5.36 (1H, t, J = 7.4 Hz, H-8), 5.41 (1H, q, J = 7.5 Hz, H-7), 6.11 (1H, s br, H-2), 7.93 (2H, d, J = 8.3 Hz, H-4' Ar, meta), 8.23 (2H, d, J = 8.3 Hz, H-3' Ar, ortho); IR (KBr) 1739 ($\text{C}=\text{O}$), 1779 ($\text{C}=\text{O}$), 3370 (OH) cm^{-1} . Anal. Calcd for $\text{C}_{28}\text{H}_{33}\text{BrN}_2\text{O}_{10}$: C, 52.76; H, 5.22; Br, 12.53; N, 4.39. Found: C, 52.35; H, 5.23; Br, 12.23; N, 4.32.

Retronecine Acetonide (9). Retronecine (209 mg, 1.35 mmol) was partially dissolved in acetone (4.7 g, 81 mmol), and 2,2-dimethoxypropane (12.6 g, 121 mmol, predistilled from sodium) and methanesulfonic acid (180 mg, 1.87 mmol, 1.4 equiv) were added. The mixture was stirred overnight under argon and the reaction quenched with potassium carbonate (990 mg, 7.15 mmol). Acetone and dimethoxypropane were removed under reduced pressure, and the resulting crude product was taken up in water (120 mL), extracted with equal volumes of ether (3 $\times$ 120 mL), and dried over MgSO_4 . Concentration under reduced pressure and then high vacuum delivered **9** as a pale yellow oil (190.2 mg, 975 μmol , 72%): ^1H NMR (300 MHz, CDCl_3) δ 1.29 (3H, s, CH_3), 1.32 (3H, s, CH_3), 1.72 (1H, dd, J = 13 Hz, J = 5 Hz, NCH_2CHH), 1.88 (1H, m, NCH_2CHH), 2.50 (1H, m, NCHHCH_2), 3.02 (1H, t, J = 8 Hz, NCHHCH_2), 3.37 (1H, m, $\text{NCHHCH}=\text{}$), 3.64 (1H, m, $\text{NCHHCH}=\text{}$), 4.21 (1H, d, $J_{\text{gem}} = 15$ Hz, $\text{CHHOC}(\text{CH}_3)_2$), 4.30 (2H, s br, $\text{CHOC}(\text{CH}_3)_2$), 4.38 (2H, t, J = 5 Hz, NCH), 4.47 (1H, d, $J_{\text{gem}} = 15$ Hz, $\text{CHHOC}(\text{CH}_3)_2$), 5.32 (1H, s br, vinyl); FTIR (NaCl plates) 507, 515, 521, 542, 580, 617, 668, 733, 768, 876, 885, 951, 995, 1283, 1314, 1447, 1619, 3054 cm^{-1} .

N-[[2-(Succinimid-4-yloxycarbonyl)phenyl]methyl]retronecine Acetonide, Bromide Salt (7.9). Acetonide **9** (100 mg, 0.513 mmol) was dissolved in dry DME (2 mL), and bromo ester **7** (161 mg, 0.516 mmol) in DME (2 mL) was injected with rapid stirring. After 2 min, stirring was discontinued, and the solution was allowed to stand overnight. Fine white needles appeared at the bottom of the vessel which were collected by filtration, washed with fresh DME (2 mL), and dried under high vacuum in a heated vessel to give **7.9** (248 mg, 0.489 mmol, 95%) (used without further purification): mp 142–147 °C dec; ^1H NMR (300 MHz, CD_3CN) δ 1.33 and 1.38 (2C, CH_3CCH_3), 2.21 (2H, s), 2.50 (1H, septet, J = 7.0 Hz), 2.86 (2H, d, J = 0.9 Hz), 3.28 (2H, d, J = 1.4 Hz), 3.45 (2H, d, J = 1.3 Hz), 3.93 (1H, dt, J = 0.9 Hz, J = 0.9 Hz), 4.19 (2H, m), 4.46 (1H, d, J = 16 Hz), 4.66 (1H, dm, J = 15 Hz), 4.90 (1H, m), 4.98 (2H, s), 5.39 (1H, s br), 5.50 (1H, d, J = 5.4 Hz), 7.92 (2H, ABq, J = 7.5 Hz, Ar, meta), 8.20 (2H, ABq, J = 7.3 Hz, Ar, ortho); IR (KBr) 1738 ($\text{NC}=\text{O}$), 1771 ($\text{CC}=\text{O}$) cm^{-1} .

N-[(4-Formylphenyl)methyl]retronecine Acetonide, Bromide Salt (8.9). Acetonide **9** (300 mg, 1.53 mmol) was dissolved in dry DME (3 mL), and (bromo-methyl)benzaldehyde **8** (379 mg, 1.90 mmol) in DME (3 mL) was injected with rapid stirring. After 2 min, stirring was discontinued, and the solution was allowed to stand overnight. Fine white needles appeared at the bottom of the vessel which were collected by filtration, washed with fresh DME (2 mL), and dried under high vacuum in a heated vessel to give **8.9** (248 mg, 0.489 mmol, 95%) (used without further purification): mp 187–190 °C; ^1H NMR (300 MHz, CDCl_3) δ 1.33 and 1.40 (6H, s, CH_3CCH_3), 2.06 (2H, complex, NCH_2CH_2), 2.75 (1H, m, NCHHCH_2), 3.55 (1H, m, NCHHCH_2), 4.20 (1H, d, $J_{\text{gem}} = 17$ Hz, $\text{CHHOC}(\text{CH}_3)_2$), 4.21 (1H, s br, $\text{CHOC}(\text{CH}_3)_2$), 4.40 (1H, d, $J_{\text{gem}} = 16$ Hz, $\text{CHHOC}(\text{CH}_3)_2$), 5.01 (2H, m, $\text{NCH}_2\text{CH}=\text{}$), 5.35 (1H, s br, vinyl), 5.57 and 5.58 (2H, s br, ArCH_2), 5.61 (1H, d, $J = 5$ Hz, NCH), 7.85 (2H, d, $J = 8.2$ Hz, Ar, meta), 7.98 (2H, d, $J = 8.2$ Hz, Ar, ortho), 10.01 (1H, s, CHO); IR (KBr) 1742 ($\text{C}=\text{O}$) cm^{-1} .

Protein Conjugation (General Procedure). Protein was dissolved in phosphate-buffered saline (PBS, pH 7.4) in an oven-baked round bottom flask equipped with a stirring bar. The protein solution was cooled in an ice bath, quaternary ammonium ester was added and the mixture stirred overnight at 5 °C. The solution was dialyzed (molecular mass cutoff 100 000 kDa) repeatedly against PBS (1000 mL) at 5 °C to remove any uncoupled hapten. Dialysate aliquots of hapten–protein conjugate were stored in glass vials at –20 °C until required for immunochemical studies. Following this general procedure, retrorsine–BSA conjugate (**6.1**^{bsa}) was prepared from BSA (60 mg), PBS (12 mL), and quaternary ammonium ester **6.1** (65 mg, 106 μmol), monocrotaline–BSA conjugate (**6.2**^{bsa}) was prepared from BSA (40 mg), PBS (12 mL), and quaternary ammonium ester **6.2** (45 mg, 76.3 μmol), retrorsine–OVA conjugate (**7.1**^{ova}) was prepared from OVA (120 mg), PBS (12 mL), and quaternary ammonium ester **7.1** (82 mg, 109 μmol), and monocrotaline–OVA conjugate (**7.2**^{ova}) was prepared from OVA (30 mg), PBS (12 mL), and quaternary ammonium ester **7.2** (78.7 mg, 108 μmol).

Retronecine–KLH Conjugate (7.3^{klh}). KLH (15 mg) was weighed into an oven-dried 10 mL round bottom flask equipped with an oven-dried stir bar and dissolved in 0.2 M pH 8.5 borate buffer (5 mL). Quaternary salt **7.9** (25 mg, 49 μmol) was dissolved with gentle stirring, and the reaction was quenched after 5 h by the dropwise addition of 0.1 M pH 3.0 phosphate buffer. The pH was adjusted to 3 with 0.1 M HCl, and the reaction mixture was stirred at room temperature for 5 h. Equilibration to pH 7 with 0.1 N NaOH and dialysis at 5 °C in a 100 kDa molecular mass cutoff dialysis tubing against PBS (9 $\times$ 1000 mL, 8 h each) produced the **7.3**^{klh} protein conjugate which was removed (8 mL, 1.9 mg/mL), aliquotted, and stored in a freezer for later use in immunochemical studies.

Retronecine–OVA Conjugate (8.3^{ova}). OVA (50 mg) was weighed into an oven-dried 10 mL round bottom flask equipped with an oven-dried stir bar and dissolved in PBS (3 mL) plus methanol (1 mL). Quaternary salt **8.9** (21 mg, 55 μmol) was dissolved with gentle stirring, and the mixture was stirred for 5 h at room temperature. NaBH_4 (4 mg, 100 μmol) was added and the mixture stirred for 1 h, at which time the reaction was quenched with the dropwise addition of 0.1 M pH 3.0 phosphate buffer. Stirring was continued at room temperature at pH 3 for 5 h. The mixture was equilibrated to pH 7 with 0.1 N NaOH and dialyzed in a 100 kDa molecular mass cutoff dialysis tubing against PBS (9 $\times$ 1000 mL, 8 h each) at 5 °C. Protein conjugate **8.3**^{ova} was removed (6.5

mL, 3.2 mg/mL), aliquotted, and stored in a freezer for later use in immunochemical studies.

Rabbit Immunization. Three-month-old New Zealand white rabbits were immunized subcutaneously with 100 μg of immunogens **6.1**^{bsa} (rabbits 1323 and 2206), **6.2**^{bsa} (rabbits 2203 and 2205), or **7.3**^{klh} (rabbits 2376 through 2380) emulsified in Freund's complete adjuvant (1:1), followed later by three injections of 100 μg of immunogen in Freund's incomplete adjuvant at 3-week intervals. Blood samples were collected 10 days after each booster injection and evaluated by ELISA titration against a homologous coating and analyte. Serum was stored at –20 °C.

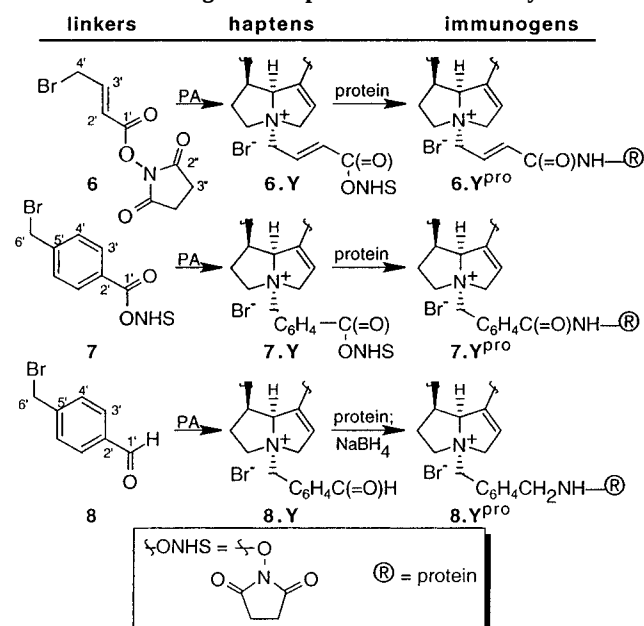
General ELISA. Basic solid phase immunoassay principles were followed (Laurent, 1988) in performing ELISAs in 96-well microtiter plates (Immulon 2). Thus, microtiter plates were coated with OVA conjugates [100 μL /well of a 0.05 M carbonate buffer (pH 10) solution containing 1 $\mu\text{g}/\text{mL}$ of **7.1**^{ova}, **7.2**^{ova}, or **8.3**^{ova}] by storing the plates at 4 °C for 2 h. The plates were washed (4 $\times$) with washing buffer (9 g/L NaCl and 0.5 mL/L Tween-20 with 2 mM pH 7.4 phosphate buffer) and blotted dry. Each washing was accomplished with a hand-held 12-channel plate washer. Appropriate antisera dilutions in PBST (PBS containing 120 mM NaCl, 2.7 mM KCl, and 10 mM inorganic phosphate at pH 7.4 plus 0.045% v/v Tween-20) were prepared from previously frozen samples of rabbit antisera, and after preincubation with standards at 20 °C for 2 h, 50 μL aliquots were pipetted into each well and the plates were allowed to stand covered at 20 °C for 2 h. Washing 4 times with washing buffer was followed by the addition of 50 μL /well of commercial IgG–HRP conjugate (1.0 mg/mL) diluted 1:2000 with PBST. The plates were then incubated for 1 h at 20 °C and washed with washing buffer.

A 50 μL /well solution consisting of OPD (1 mg/mL) and hydrogen peroxide (30% diluted 1:1000) in a Tris-citrate buffer (24.2 g/L of Tris base and 12.6 g/L of citric acid monohydrate adjusted to pH 6.0) base was added to the plate. The color change was monitored with an automated plate reader. When using the kinetic format, the rate of color change (mOD/min) at 450 nm was recorded over a 15 min period. When employing the end point format, the reaction was stopped after 5 min by the addition of 50 μL /well 2 N H_2SO_4 and the final optical density (OD_{490}) was measured at 490 nm. Some initial screenings were performed with the kinetic format, while final characterizations were performed with the end point format.

Nonspecific Binding Controls. As a control, each microtiter plate had 12 wells free of coating antigen, having contained only the coating buffer during the coating procedure. To the first 3 wells was added only OPD, to the second 3 wells were added both IgG–HRP and OPD, and to the last six wells were added antisera, IgG–HRP, and OPD.

Screening of Antisera by Testing Their Titers: Checkerboard Titrations. Optimum dilution factors for both the antiserum and coating antigen used in each assay were determined by checkerboard titrations. Microtiter plates were coated by overnight incubation with coating antigen solutions (**7.1**^{ova}, **7.2**^{ova}, or **8.3**^{ova} at 100 μL /well) ranging from 0.1 to 18.85 $\mu\text{g}/\text{mL}$. Next, solutions of antisera (50 μL /well) with dilution factors ranging from 1:250 to 1:32000 were added to each well. Those coating and antisera concentrations giving optical densities at 490 nm (OD_{490} 's) between 0.9 and 1.3 after 5 min were selected for use in initial competitive ELISA screens.

Competitive ELISA with PA Standards. For standard curves with less sensitive antisera (serum numbers 1323 and 2205), a primary standard of 10.00 mg/mL of

Scheme 1. Antigens Prepared in This Study^a

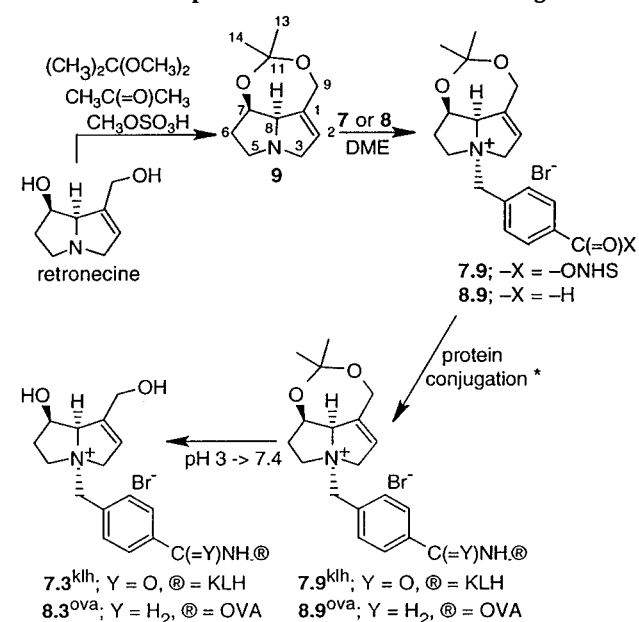
^a For a note regarding compound numbering, see footnote 2.

PAs was used to make a series of diluted standards in PBST buffer with concentrations ranging from 500 $\mu\text{g/mL}$ (500 ppm) to 0.1 ng/mL (0.1 ppb). For more sensitive antisera (2203 and 2206), PA stock solutions in 10 mM PBST were diluted from 100 000 to 0.01 ppb in 1:10 dilution steps. An antisera solution of an appropriate dilution for the particular coating antigen to be used was prepared by dilution in PBST. Samples of each PA standard (1 part) were diluted 1:10 with antisera solution (9 parts) in new, uncoated microtiter plates and preincubated at room temperature for 2 h. One standard, containing only antisera and buffer (1:10), was used as the control to determine the maximum kinetic or end point OD reading. Another sample containing only buffer was used as a blank. Inhibition curves were analyzed by a four-parameter logarithmic curve-fitting procedure which calculated I_{50} values (inhibitor concentration giving 50% inhibition) and the slope of the linear portion of the curve. Below 5% solvent content, methanol did not show any effect on antibody activity.

Comparative Inhibition Calculations. Three coating antigens (7.1^{ova}, 7.2^{ova}, or 8.3^{ova}) and six antisera combinations were evaluated in assays using PAs 1–5 as analytes. Initial screens were performed using PA standards decreasing by factors of 10 from a concentration value of 100 000 ng/mL (100 ppm) to 0.1 ng/mL (0.1 ppb). For those combinations where retronecine showed a promising inhibition range, additional standards were prepared and run in the range where inhibition curves displayed nearly linear behavior. I_{50} values were determined for these systems.

Detection of PAs in Plant Samples. *S. vulgaris* and *C. retusa* were selected as potential positive samples with *Lupinus* spp. selected as a negative control. Whole *Senecio* and *Lupinus* plants where ground in a blender with methanol and refluxed for 1 h with nitrogen bubbled through. *Crotalaria* seeds were pulverized and refluxed with methanol as above. Solids were removed by filtration through glass wool, and the methanolic extract was dialyzed through a 10 000 molecular mass cutoff tubing into 2 volumes of PBS. The dialysates were assayed with the ELISA systems A–C.

Cross-Reactivity Studies with Swainsonine and Lupinine. Retronecine standard curves were also run in the presence of commercially prepared swainsonine

Scheme 2. Preparation of Retronecine Antigens^a

^a Protein conjugation conditions: (i) 7.9 $\rightarrow$ 7.9^{klh}; KLH + borate buffer, pH 8.5; (ii) 8.9 $\rightarrow$ 8.9^{ova}; OVA + PBS, pH 7.4, 4 h, 20 °C; 2 equiv of NaBH₄, MeOH.

and lupinine hydrochloride at working concentrations of 2500 ppb 10 or 11.

RESULTS AND DISCUSSION

Hapten Preparation. The N-4 free base of the necine-based PA is a good nucleophile and has been used in the past to form methiodides (Leonard, 1960). While N-4 is unreactive toward straight chain primary halides such as 6-iodohexanoate in DME, we find that necine-based PAs are reactive toward allylic and benzylic bromides as well as α -bromo esters in DME. Thus, treating a PA with an active electrophile such as 6, 7, or 8 in DME produces a pyrrolizidinium salt as a fine white (floculant) precipitate which is easily recovered on a fine frit (Scheme 1).

Crotyl NHS ester 6 was selected as our first linker target for two reasons. First, 4-bromocrotonate 6 proved to be sufficiently reactive to N-alkylate PAs. Second, hapten carrier tether length is an important variable in small molecule immunoreactivity since it can profoundly affect the hapten's presentation to the immune system. In this regard, the four-carbon crotyl tether² in immunizing antigen 6.Y^{pro} seemed ideal (Goodrow et al., 1990; Harrison et al., 1990). However, in order to avoid "tether recognition" in the ELISA, benzoate hapten 7.Y, the N-alkylation product from reaction of NHS ester 7 with PAs, was targeted because the resulting coating antigen 7.Y^{pro} would present a nonhomologous tether (methylbenzoate) from that of immunogen 6.Y^{pro} (crotyl). Like crotyl NHS ester 6, NHS ester 7 proved sufficiently reactive to N-alkylate the parent PAs.

² Note regarding compound numbering. Composite compound numbers presented as X·Y are used to convey the fact that a linker moiety (X = linker compound number) and a pyrrolizidine alkaloid (Y = PA compound number) are covalently attached via N-4 of the PA. Thus, for example, 7.9 is the hapten derived from linker 7 and PA analogue 9. Likewise, superscripted pro, bsa, klh, or ova following a composite compound number X·Y^{zzz} conveys the linker (X), PA (Y), and carrier protein (zzz; protein, bovine serum albumin, keyhole limpet hemocyanin, or chicken egg ovalbumin, respectively).

Table 1. ^{13}C NMR Data for PA Systems^a

carbon	retrorsines			monocrotalines			retronecines			
	1 ^b	6.1	7.1	2 ^b	6.2	7.2	3 ^b	9	7.9	8.9
1	132.4	125.8	127.7	132.85	122.5	127.8	138.4	140.8	140.3	139.7
2	134.7	132.4	134.3	134.39	132.6	132.9	126	118.2	115.1	113.6
3	62.7	72	70.9	60.58	70	68.7	59.5	60.4	72.3	69.9
5	52.9	64.4	67	53.7	64.4	66.3	53.6	53.8	64.8	64.1
6	37.9	38.5	38.5	33.57	31.2	30.7	36.3	32.8	31.3	30.7
7	75	74	74.3	75.15	77	73.3	71.5	71.3	71.3	70.2
8	77.4	87.6	86.5	76.88	86.2	85.3	77.3	77.3	87.6	86.6
9	61	63.6	63.5	61.37	62	60.8	62.3	60.5	62.4	61.5
10	—	—	—	—	—	—	—	101.3	103.3	102.5
11	175.7	175.3	175.6	173.56	174.2	174.2	—	23.7	24.1	23.4
12	81.3	82.3	82.7	78.79	80.2	80.2	—	24.9	25.2	24.7
13	35.7	36.5	36.6	76.8	72.9	76.9	—	—	—	—
14	34.7	33.3	33.5	44.38	43.3	43.2	—	—	—	—
15	131.2	130.7	130.9	174.04	175.6	158.8	—	—	—	—
16	167.3	167	167.3	—	—	—	—	—	—	—
17	—	—	—	21.96	22.4	22.4	—	—	—	—
18	66.9	67.6	67.9	17.71	17.8	17.8	—	—	—	—
19	11.6	11.7	11.8	13.66	14.2	14.3	—	—	—	—
20	136.6	137	136.4	—	—	—	—	—	—	—
21	14.9	15.5	15.5	—	—	—	—	—	—	—
1'	—	58.5	59	—	58.9	59.2	—	—	162.5	191.3
2'	—	140.6	137.7	—	140.1	136.5	—	—	127.5	134.9
3'	—	131.9	131.6	—	126.3	131.6	—	—	134.1	133.1
4'	—	161.2	134.4	—	161.4	134.1	—	—	131.5	129.9
5'	—	—	131.4	—	—	131	—	—	137.5	137.2
6'	—	—	162.5	—	—	162.5	—	—	58.8	58.8
2''	—	170.8	171.1	—	170.9	171.1	—	—	26.6	—
3''	—	26.3	26.5	—	26.4	26.5	—	—	171.2	—

^a See Figure 1 and Schemes 1 and 2 for numbering. Solvent $\text{CD}_3\text{CN}/\text{DMSO}-d_6$. ^b Literature values [CDCl_3 solvent, see Jones et al. (1990) and Roeder (1990)].

Table 2. Haptens and Immunogens Prepared

pyrrolizidine alkaloid	linker 6	linker 7	linker 8
1	hapten 6.1	hapten 7.1	—
↓	↓	↓	
retrorsine immunogens	6.1 ^{bsa}	7.1 ^{ova}	
2	hapten 6.2	hapten 7.2	—
↓	↓	↓	
monocrotaline immunogens	6.2 ^{bsa}	7.2 ^{ova}	
3 → 9	—	hapten 7.9	hapten 8.9
↓		↓	↓
retronecine immunogens		7.3 ^{klh}	8.3 ^{ova}

NHS-activated crotyl ester **6** was prepared by DCC-mediated condensation of 4-bromocrotonic acid³ and NHS in DME. NHS-activated benzoate ester **7** was prepared in a like manner from α -bromo-*p*-toluic acid and NHS. Subsequent PA N-alkylation resulted in DME insoluble pyrrolizidine salts **6.Y** and **7.Y**, respectively, which were easily isolated by filtration. Unreacted reagents were removed by open air washing with DME. Evaporative removal of solvent from these pyrrolizidine salts delivered water, methanol, and DMSO soluble haptens which were recrystallized from acetonitrile to give analytical samples characterized by ^1H (D_2O or CD_3OD solutions) and ^{13}C NMR ($\text{CD}_3\text{CN} + \text{DMSO}-d_6$ solution) analysis.

Benzaldehyde **8** was prepared as a third linker alternative from ethyl *p*-(α -bromomethyl)benzoate. Attempts at DIBAL-H reduction of this ester to **8** were frustrated by the production of *p*-(α -bromomethyl)benzyl alcohol and recovery of unreacted starting material. In light of this,

reduction with 2 equiv of DIBAL-H (Bookser & Bruice, 1991) yielded *p*-(α -bromomethyl)benzyl alcohol as a low melting yellow solid which was immediately subjected to PCC oxidation in dichloromethane over 4 Å molecular sieves (Herscovic et al., 1982). Column chromatography produced *p*-(α -bromomethyl)benzaldehyde as a white, crystalline solid which sublimates upon standing and readily oxidizes in air to α -bromo-*p*-toluic acid. Benzaldehyde **8** was used to prepare hapten **8.Y** from which coating antigen **8.Y**^{pro} was prepared.

Model quaternary ammonium salts of retronecine were obtained by N-alkylation of **3** with the methyl esters of linkers **6** and **7**. Unfortunately, the fine flocculant powders obtained dissolved into viscous pools upon contact with air, and neither compound delivered a recognizable NMR spectrum in acetonitrile- d_3 .

Since the two free hydroxyls of retronecine no doubt contribute to the hygroscopic nature of these salts, retronecine was protected as acetone **9** by treatment with 2,2-dimethoxypropane in acetone (plus ≈ 1.1 equiv of methanesulfonic acid, Scheme 2). Unlike retronecine which is practically insoluble in ether, acetone **9** was easily extracted into ether. Subsequent N-alkylation of **9** with linker **7** produced stable hapten **7.9** which was crystalline, readily characterizable, and yet freely soluble in aqueous buffer. A ^1H NMR time course of **7.9** in D_2O containing deuterated phosphate buffer⁴ established that the acetone moiety was hydrolyzed within a few hours time in aqueous buffer of $\text{pH} \leq 3$. Hapten **8.9** was also prepared from acetone **9** by N-alkylation with linker **8**.

^{13}C NMR spectral resonances for quaternary ammonium PAs observed in CD_3CN plus $\text{DMSO}-d_6$ (Table 1) closely match published values (Jones et al., 1990;

³ Methyl 4-bromocrotonate was purchased from Aldrich and hydrolyzed in 6 M HCl, THF, and acetone for 2 h. The mixture was evaporated and extracted into ether, and white crystals of 4-bromocrotonic acid were obtained upon evaporation of the ether.

⁴ Deuterated phosphate buffer was formed most inexpensively by quenching a small amount of P_2O_5 in D_2O , adjusting the pH with drops of K_2CO_3 dissolved in D_2O , and monitoring solution pH with pH paper.

Table 3. Homologous Immunochemical Systems: Screening of Antibodies

	system		
	A	B	C
immunizing antigen	6.1 ^{bsa}	6.2 ^{bsa}	7.3 ^{klh}
coating antigen	7.1 ^{ova}	7.2 ^{ova}	8.3 ^{ova}
analyte	retrorsine	monocrotaline	retronecine
serum no.	1323	2203	2380
antiserum titer	980	1200	3480
antiserum dilution	2000	1000	1000
<i>I</i> ₅₀ (ppb)	0.9 ± 0.2	39 ± 9	3000 ± 600
slope ^a	0.7 ± 0.1	0.59 ± 0.03	1.0 ± 0.1

^a The slope *b* and *I*₅₀ were determined from the best-fit four-parameter curve according to the equation $y = (a - d)/[1 + (x/c)^b] + d$; see text for a detailed explanation.

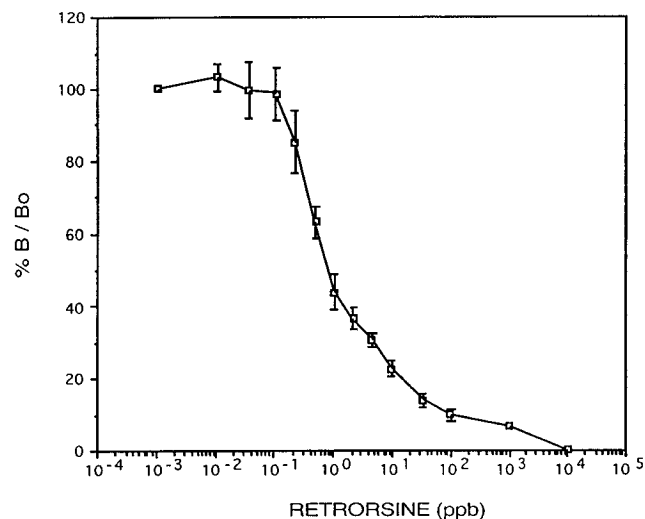


Figure 2. System A retrorsine standard curve. Antibody inhibition is expressed as %*B*/*B*₀, the percentage of bound antibody relative to the observed maximum, versus known retrorsine concentration. The composite standard curve of eight runs reveals a 50% inhibition (*I*₅₀) of 0.9 ± 0.2 ppb in a linear range of 0.5–10 ppb. The error bars reflect the standard deviation in %*B*/*B*₀ between eight runs. The curve has a slope of 0.7 ± 0.1 within the linear range.

Roeder, 1990) for their corresponding free bases taken in CDCl₃ except for those carbon atoms attached to N-4. These carbons are shifted upfield by about 10 ppm. ¹H NMR spectra taken in CD₃OD are somewhat ambiguous for macrocyclic PAs and were assigned with the aid of published spectra (Segall & Dallas, 1983). ¹³C NMR spectral assignments for quaternary compounds were made with reference to published spectra for the parent free base alkaloids (Jones et al., 1990; Roeder, 1990).

The water solubility of the haptens listed in Table 2 obviated the need for cosolvents in the protein conjugation step. Thus, BSA conjugates **6.1**^{bsa} and **6.2**^{bsa} were prepared by treating the appropriate hapten (retrorsine hapten **6.1** or monocrotaline hapten **6.2**) with BSA in phosphate-buffered saline (PBS)⁵ at pH 7.4. The corresponding OVA conjugates **7.1**^{ova}, **7.2**^{ova}, and **8.3**^{ova} were prepared from OVA plus retrorsine hapten **7.1**, monocrotaline hapten **7.2**, and retronecine hapten **8.9**, respectively.

Since the hygroscopic nature of free (unprotected diol moiety) retronecine pyrrolizidinium salts precluded characterization, we were pleased to find that retronecine antigens were available from haptens **7.9**^{klh} and **8.9**^{ova}. Stirring these protein–hapten conjugates in a pH 3

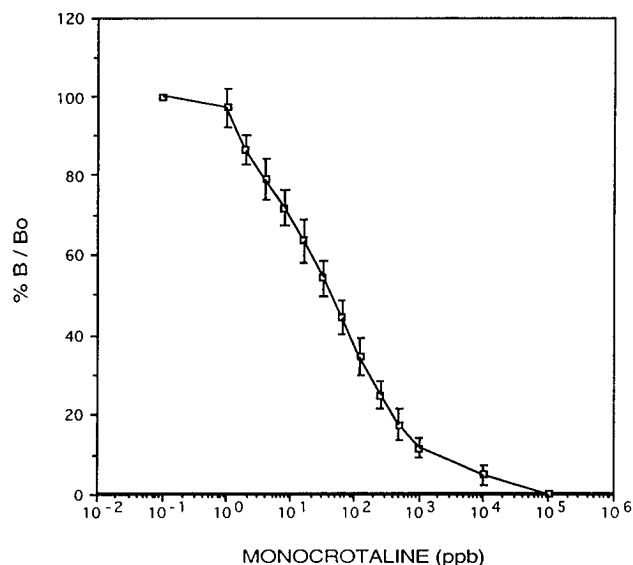


Figure 3. System B monocrotaline standard curve. Antibody inhibition is expressed as %*B*/*B*₀, the percentage of bound antibody relative to the observed maximum, versus known monocrotaline concentration. The composite standard curve of ten runs reveals a 50% inhibition (*I*₅₀) of 36 ± 9 ppb in a linear range of 5–500 ppb. The error bars reflect the standard deviation in %*B*/*B*₀ between ten runs. The curve has a slope of 0.59 ± 0.03 within the linear range.

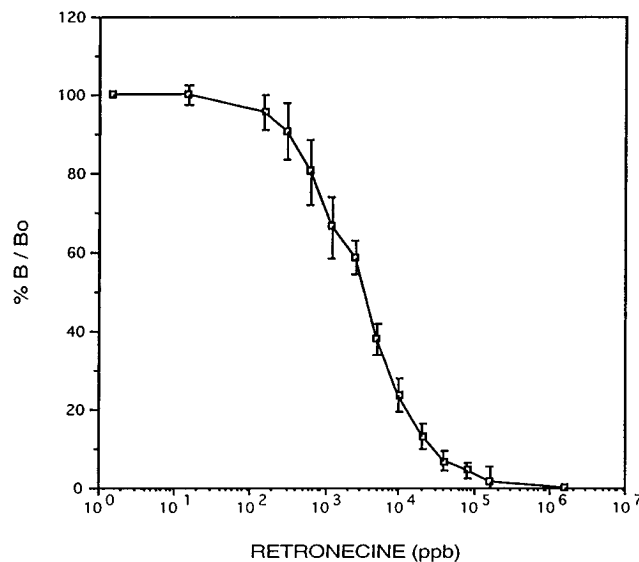


Figure 4. System C retronecine standard curve. Antibody inhibition is expressed as %*B*/*B*₀, the percentage of bound antibody relative to the observed maximum, versus known retrorsine **5** concentration. The composite standard curve of ten runs reveals a 50% inhibition (*I*₅₀) of 3000 ± 600 ppb in a linear range of 600–10000 ppb. The error bars reflect the standard deviation in %*B*/*B*₀ between ten runs. The curve has a slope of 1.0 ± 0.1 within the linear range.

phosphate buffer presumably (*vide infra*) removes the acetonide moiety, and subsequent dialysis in a pH 7.4 buffer provides the targeted immunogens. In this way, KLH conjugate **7.3**^{klh} was prepared from hapten **7.9**^{klh} (Li et al., 1991) and OVA conjugate **8.3**^{ova} was prepared by reductive amination of hapten **8.9**^{ova}.

With these immunizing (BSA or KLH) and coating (OVA) antigens in hand, three-month-old New Zealand white rabbits were immunized with conjugates **6.1**^{bsa}, **6.2**^{bsa}, or **7.3**^{klh}. Sera were collected 10 days following each booster injection and titrated against the appropriate coating antigens **7.1**^{ova}, **7.2**^{ova}, or **8.3**^{ova}, respectively.

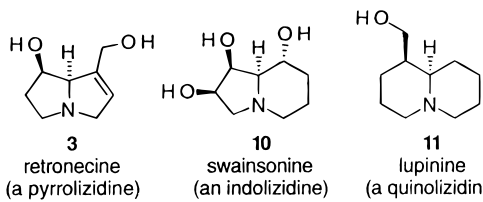
⁵ PBS was used, although a borate buffer at pH 8.5–9 is preferable.

Table 4. Competitive ELISA for PA Detection in Homologous Systems A–C^a

	system A		system B		system C	
	<i>I</i> ₅₀ (ppb) ^b	slope ^c	<i>I</i> ₅₀ (ppb) ^b	slope ^c	<i>I</i> ₅₀ (ppb) ^b	slope ^c
retrorsine 1	0.9 ± 0.2	0.7 ± 0.1	+++	≈0	+++	≈0
monocrotaline 2	3 × 10 ⁵	1.52	36 ± 9	0.59 ± 0.03	+++	≈0
senecionine 3	113	0.37	+++	≈0	+++	≈0
isatidine 4	0.6	0.2	+++	≈0	+++	≈0
retronecine 5	420	0.64	10.9	0.62	3000 ± 600	1.0 ± 0.1

^a PAs were serially diluted and assayed. ^b The notation +++ indicates that meaningful *I*₅₀'s were not available at these slopes. ^c The slope *b* and *I*₅₀ were determined from the best-fit four-parameter curve according to the equation $y = (a - d)/[1 + (x/c)^b] + d$; see text for a detailed explanation.

Table 5. Reagent Grade Alkaloids Tested for Cross-Reactivity with Systems A and B

					
3 retronecine (a pyrrolizidine) 10 swainsonine (an indolizidine) 11 lupinine (a quinolizidine)					
	system A		system B		
	<i>I</i> ₅₀ (ppb)	slope	<i>I</i> ₅₀ (ppb)	slope	
PA standard ^a	0.61	1.1	30	0.44	
2500 ppb swainsonine ^b	0.95	0.66	29	0.64	
2500 ppb lupinine ^c	1.65	0.76	11	0.50	

^a PA standards used were as follows. Retrorsine was used for system A; monocrotaline was used for system B. ^b PA standard spiked to 2500 ppb with reagent grade swainsonine. ^c PA standard spiked to 2500 ppb with reagent grade lupinine.

The cycle of booster shots and small bleeds was repeated until adequate titers and PA detection were obtained.

ELISA Assay Development. Checkerboard titration screening of the resulting antisera was used to establish coating conditions (typically 2 μg/mL coating protein) and serum dilution (typically 1:1000 working concentration). As one might rationally expect, system A (immunogen **6.1**^{bsa} + coating antigen **7.1**^{ova}) has excellent sensitivity for retrorsine and system B (immunogen **6.2**^{bsa} + coating antigen **7.2**^{ova}) for monocrotaline (see Table 3). System C (immunogen **7.3**^{klh} + coating antigen **8.3**^{ova}) showed parts per million level detection for retronecine, sensitivity less than that found with our previously reported retronamine method (Roseman et al., 1992).

Anti-retrorsine antibodies (anti-**6.1**^{bsa} sera 2203, diluted 1:1000) assayed against coating **7.1**^{ova} (immunogen/microtiter plate coating system A, Figure 2) using retrorsine as the analyte afforded the most sensitive anti-retrorsine titer of *I*₅₀ = 0.9 ± 0.2 ppb. System A had a useful linear range of 0.5–10 ppb as defined by the *B/B*₀ range within 15–80% of the maximum control *B*₀. Likewise, anti-monocrotaline antibodies (anti-**6.2**^{bsa} sera 1323, diluted 1:1000) assayed against coating **7.2**^{ova} (immunogen/microtiter plate coating system B, Figure 3) using monocrotaline as the analyte afforded the most sensitive anti-monocrotaline titer of *I*₅₀ = 36 ± 9 ppb with a useful linear range of 5–500 ppb. Finally, anti-retronecine antibodies (anti-**7.3**^{klh} sera 2380, diluted 1:1000) assayed against coating **8.3**^{ova} (immunogen/microtiter plate coating system C, Figure 4) using retronecine as the analyte afforded an anti-retronecine titer of *I*₅₀ = 3000 ± 600 ppb with a useful linear range of 600–10000 ppb.

Color development in these assays was measured using an end point optical density (OD₄₉₀) format on a microtiter plate reader and plotted as a function of PA concentration. A best-fit four-parameter curve was calculated by Softmax software according to the equation $y = (a - d)/[1 + (x/c)^b] + d$, where *a* and *d* are the upper and lower asymptotes, respectively, *b* is the slope of the linear portion of the curve, and *c* is the midpoint of the linear portion of curve, interpreted as the *I*₅₀. The software calculated PA concentration values for individual wells on the basis of optical density with reference to this four-parameter standard curve; hence, unknowns must be diluted or concentrated so they fall within this working range.

Table 6. Detection of PAs in Plant Samples^a

	system A ^b (ppb, w/w)	system B ^c (ppb, w/w)	system C ^d (ppb, w/w)
<i>S. vulgaris</i> (stem, leaf, and flower)	10000	8800	0
<i>C. retusa</i> (seed)	64000	35000	0
<i>Lupinus</i> spp. (seeds)	1900	0	0

^a The plant material was pulverized in methanol, filtered, and dialyzed into PBS. *S. vulgaris* is known to be rich in senecionine. *C. retusa* seeds are rich in monocrotaline. *Lupinus* spp. plants are thought to be PA-free but do contain lupinine. ^b Determined using retrorsine as the standard. ^c Determined using monocrotaline as the standard. ^d Determined using retronecine as the standard.

With the objective of developing “PA-specific” immunoassays, senecionine and isatidine were also serially diluted and assayed with all three systems. Senecionine and isatidine are structurally quite similar to retrorsine, and as a consequence, system A detects these two PAs at comparably low levels. In contrast, monocrotaline is structurally quite distinct from retrorsine and senecionine and is uniquely detected by system B. That both ELISA systems A and B provide very good retronecine detection is apparently a consequence of *in vivo* macrocycle diester hydrolysis of immunogens **6.1**^{bsa} and **6.2**^{bsa} to a retronecine-presenting immunogen. As a consequence, anti-retronecine antibodies were present in all antisera obtained in this study. Attempts at creating nonhomologous systems using the serum from system A with the coating from system B to detect alkaloids **1–5** were largely unsuccessful. These results are presented in Table 4.

Biological samples tested using the assays reported here would no doubt contain non-PA alkaloids (such as indolizidine and quinolizidine alkaloids) which could potentially cross-react with the PA antiserum to give a false “PA positive”. To probe this issue, swainsonine (**10**) and lupinine (**11**) were selected as representative alkaloids for cross-reactivity studies. Lupine seeds which contain lupine alkaloids, including lupinine, were also tested for cross-reactivity. Reassuringly, relatively large concentrations (2500 ppb) of swainsonine and lupinine had no measurable effect on the *I*₅₀ of system A or system B assays (Table 5) and hence do not impede their ability to monitor biological samples for PAs.

The utility of our competitive ELISA for detection of PAs in actual plant samples was demonstrated by extracting *S. vulgaris* and *C. retusa* samples with refluxing methanol and assaying (Table 6). *C. retusa* seeds are

rich in monocrotaline **2** and hence yield a positive ELISA result. Although the PAs in *Senecio* occur largely as *N*-oxides, these too can be detected by this assay since *Senecio* contains senecionine. In contrast, lupine extracts contain lupinine and lupinidine alkaloids and do not result in a positive assay.

CONCLUSIONS

Sensitive, compound-specific ELISA methods have been developed for the detection of the pyrrolizidine alkaloids retrorsine and monocrotaline. These assays are comparable in sensitivity to GC methods [reported 0.1 ppm sensitivity: see Deinzer et al. (1982) and Wiedenfeld (1981)] and have the potential advantages of low cost, fast and easy performance, and portability. The linear ranges of inhibition curves using system A for the detection of retrorsine is 0.5–100 ppb with an I_{50} of 0.9 ± 0.2 ppb. Using system A, good cross-reactivity was observed toward monocrotaline ($I_{50} = 760$ ppb), senecionine ($I_{50} = 100$ ppb), isatidine (retrorsine *N*-oxide, $I_{50} = 1$ ppb), and retronecine ($I_{50} = 420$ ppb). The linear ranges in inhibition curves using system B for the detection of monocrotaline is 5–5000 ppb with an I_{50} of 36 ± 9 ppb. Retronecine is detected at 10.9 ppb, and little cross-reactivity is observed toward other PAs. Indeed, system B holds great potential as a compound-specific detection method. System C detected retronecine at 600–10000 ppb with an I_{50} of 3000 ± 600 ppb [less sensitive than that previously reported by our laboratory (Roseman et al., 1992); little cross-reactivity was observed toward other PAs]. All three systems described were capable of detecting retronecine at some level, and none of these systems show cross-reactivity with either swainsonine or lupinine.

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Supporting Information Available: ^1H and ^{13}C NMR data for compounds **7.9**, **8.9**, and **9** (6 pages). Ordering information is given on any current masthead page.

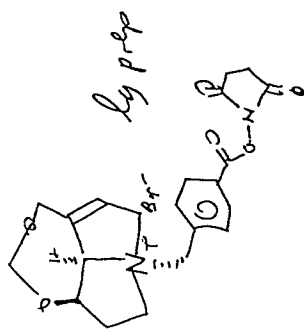
LITERATURE CITED

- Allen, J. R., Hsu, I. C., and Carsten, L. A. (1975) Dehydro-retronecine-induced rhabdomyosarcomas in rats. *Cancer Res.* 35, 997–1002.
- Ames, B. N., Durston, W. E., Yamasaki, E., and Lee, F. D. (1973) Carcinogens are mutagens. Simple test system combining liver homogenates for activation and bacteria for detection. *Proc. Natl. Acad. Sci. U.S.A.* 70, 2281–2285.
- Anderson, G. W., Zimmerman, J. E., and Callahan, F. M. (1964) *J. Am. Chem. Soc.* 86, 1839–1842.
- Bober, M. A., Kurth, M. J., Milco, L. A., Roseman, D. M., Miller, R. B., and Segal, H. J. (1990) A pyrrolizidine alkaloid enzyme-linked immunosorbent assay detection strategy. *ACS Symp. Ser.* 451, 176–183.
- Bookser, B. C., and Bruice, T. C. (1991) Syntheses of Quadruply Two- and Three-Atom, Aza-Bridged, Cofacial Bis(5,10,15,20-tetraphenylporphyrins). *J. Am. Chem. Soc.* 113, 4208–4218.
- Bull, L. B., Culvenor, C. C. J., and Dick, A. T. (1968) in *The Pyrrolizidine Alkaloids*, p 133, North-Holland, Amsterdam.
- Chesney, C. F., Allen, J. R., and Hsu, I. C. (1974) Right ventricular hypertrophy in monocrotaline pyrrole treated rats. *Exp. Mol. Pathol.* 20, 257–268.
- Culvenor, C. C. J. (1980) in *Toxicology in the Tropics* (R. L. Smith and E. A. Bababunmi, Eds.) Taylor and Francis, London.
- Deinzer, M. L., Arbogast, B. L., and Buhler, D. R. (1982) Gas Chromatographic Determination of Pyrrolizidine Alkaloids in Goat's Milk. *Anal. Chem.* 54, 1811–1814.
- Goodrow, M. H., Harrison, R. O., and Hammock, B. D. (1990) Hapten Synthesis, Antibody Development, and Competitive Inhibition Enzyme Immunoassay for s-Triazine Herbicides. *J. Agric. Food Chem.* 38, 990–996.
- Harrison, R. O., Goodrow, M. H., Gee, S. J., and Hammock, B. D. (1990) Hapten synthesis for pesticide immunoassay development *ACS Symp. Ser.* 451, 14–27.
- Herscovic, J., Egron, M.-J., and Antonakis, K. (1982) New oxidative systems for alcohols: molecular sieves with chromium(VI) reagents. *J. Chem. Soc., Perkin Trans. 1*, 1967–1973.
- Hooper, P. T. (1974) Pathology of *Senecio jacobaea* poisoning of mice. *J. Pathol.* 113, 227–230.
- Jones, A. A., Culvenor, C. C. J., and Smith, L. W. (1982) Pyrrolizidine alkaloids – a carbon-13 NMR study. *Aust. J. Chem.* 35, 1173–1184.
- Keeler, R. F. (1983) Plant metabolites that are teratogenic in offspring and toxic in the dam. *Toxicol. (Suppl. 3)*, 221–225.
- Kuhara, K., Takanashi, J., Hirono, I., Furuya, T., and Asada, Y. (1980) Carcinogenic activity of clivorine, a pyrrolizidine alkaloid isolated from *Ligularia dentata*. *Cancer Lett.* 10, 117–122.
- Kurth, M. J., Milco, L. A., Bober, M. A., Miller, R. B., Mount, M. E., and Wicks, B. (1989) A competitive enzyme-linked immunosorbent assay (ELISA) to detect retronecine and monocrotaline in vitro. *Toxicol.* 27, 1059–1064.
- Kurth, M. J., Milco, L. A., and Miller, R. B. (1992) Trifunctional reagents for substrate-protein conjugation: application to pyrrolizidine alkaloid analogs. *Tetrahedron* 48, 1407–1416.
- Laurent, P., Magne, L., De Palmas, J., Bignon, J., and Jaurand, M.-C. (1988) Quantitation of elastin in human urine and rat pleural mesothelial cell matrix by a sensitive avidin-biotin ELISA for desmosine. *J. Immunol. Methods* 107, 1–11.
- Leonard, N. J. (1960) in *The Alkaloids* (R. H. F. Manske, Ed.) Vol. 6, p 35, Academic Press, New York.
- Li, Q. X., Zhao, M. S., Gee, S. J., Kurth, M. J., Seiber, J. N., and Hammock, B. D. (1991) Development of Enzyme-Linked Immunosorbent Assays for 4-Nitrophenol and Substituted 4-Nitrophenols. *J. Agric. Food Chem.* 39, 1685–1692.
- Mattocks, A. R. (1986) in *Chemistry and Toxicology of Pyrrolizidine Alkaloids* pp 191–219, 147–150, 322–323, pp 213–217, Academic Press, London.
- Mattocks, A. R., and Bird, I. (1983) Pyrrolic and *N*-oxide metabolites formed from pyrrolizidine alkaloids by hepatic microsomes in vitro: relevance to in vivo hepatotoxicity. *Chem.-Biol. Interact.* 43, 209–222.
- McLean, E. K. (1970) Toxic actions of pyrrolizidine (Senecio) alkaloids. *Pharmacol. Rev.* 22, 429–483.
- Peterson, J. E., and Culvenor, C. C. J. (1983) in *Handbook of Natural Toxins* (R. F. Keeler, and A. T. Tu, Eds.) Vol. 1, p 637, Dekker, New York.
- Robins, D. J. (1982) The pyrrolizidine alkaloids. *Prog. Chem. Org. Nat. Prod.* 41, 115–203.
- Roeder, E. (1990) Carbon-13 NMR spectroscopy of pyrrolizidine alkaloids. *Phytochemistry* 29, 11–29.
- Roseman, D. M., Wu, X., Milco, L. A., Bober, M., Miller, R. B., and Kurth, M. J. (1992) Development of a Class-Specific Competitive Enzyme-Linked Immunosorbent Assay for the Detection of Pyrrolizidine Alkaloids in Vitro. *J. Agric. Food Chem.* 40, 1008–1014.
- Segall, H. J., and Dallas, J. L. (1983) Proton NMR spectroscopy of pyrrolizidine alkaloids. *Phytochemistry* 22, 1271–1273.
- Segall, H. J., Wilson, D. W., Dallas, J. L., and Haddon, W. F. (1985) trans-4-Hydroxy-2-hexenal: a reactive metabolite from the macrocyclic pyrrolizidine alkaloid senecionine. *Science* 229, 472–475.
- Smith, L. W., and Culvenor, C. C. J. (1981) Plant sources of hepatotoxic pyrrolizidine alkaloids. *J. Nat. Prod.* 44, 129–152.
- Svoboda, D. J., and Reddy, J. K. (1972) Malignant tumors in rats given lasiocarpine. *Cancer Res.* 32, 908–913.
- Wiedenfeld, H., Pastewka, U., Stengl, P., and Röder, E. (1981) On the gas-chromatographic determination of the pyrrolizidine alkaloids of some *Senecio*-species. *Planta Med.* 41, 124–128.

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¹H-NMR spectrum for 7.9

pages 2-5M



GE NMR
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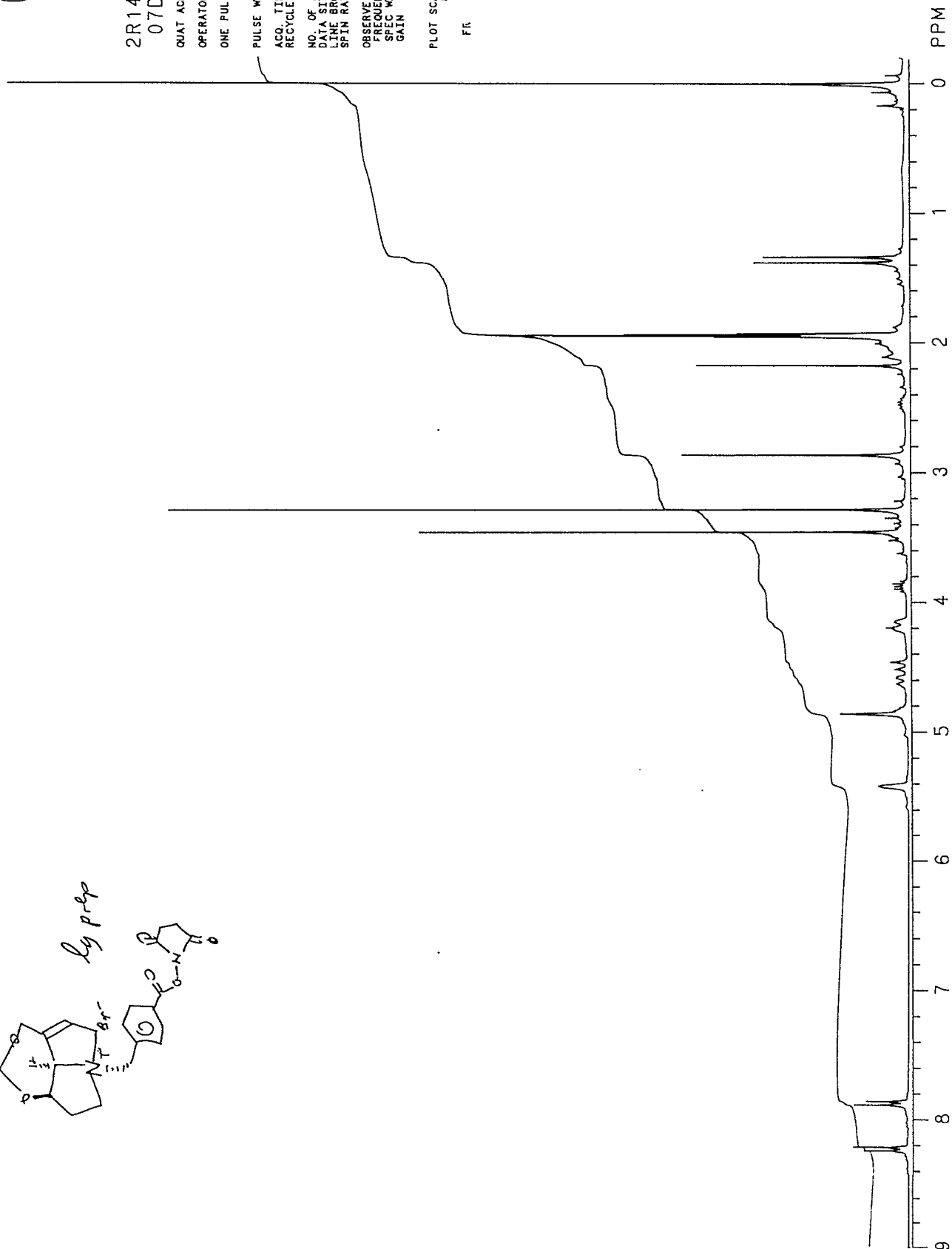
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GAIN = 53 ± 1

PLOT SCALE:
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2706 PPM/CM

FR



¹H-NMR spectrum for 8.9

pages 3*SM



GE NMR
QE PLUS

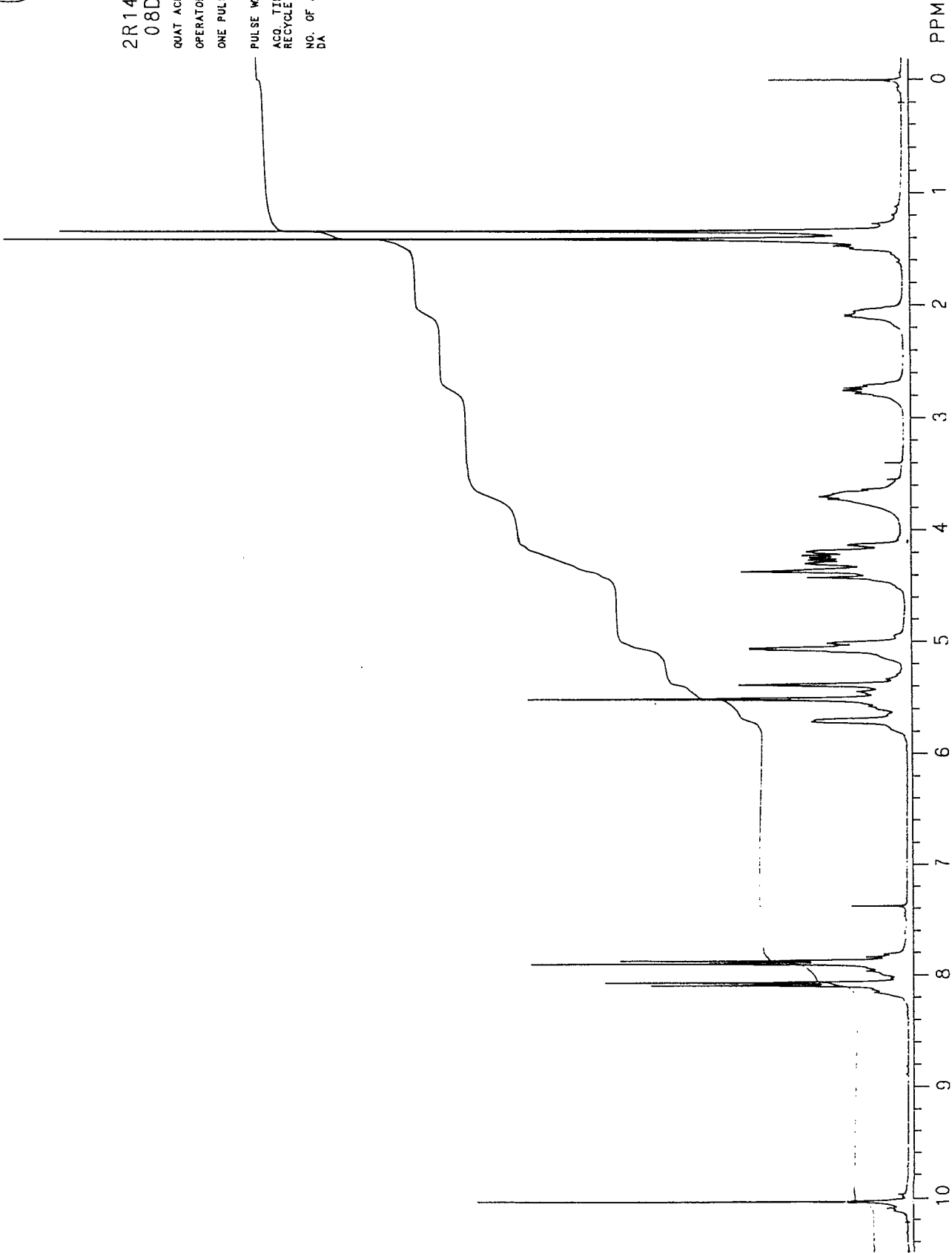
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08DEC92

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OPERATOR: DMR

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O-195-3

¹H-NMR spectrum for 9

pages 4-5M

GE NMR
QE PLUS2DR75B.001
13JUN92

RA ACETONIDE/CDCL3

OPERATOR: DMR

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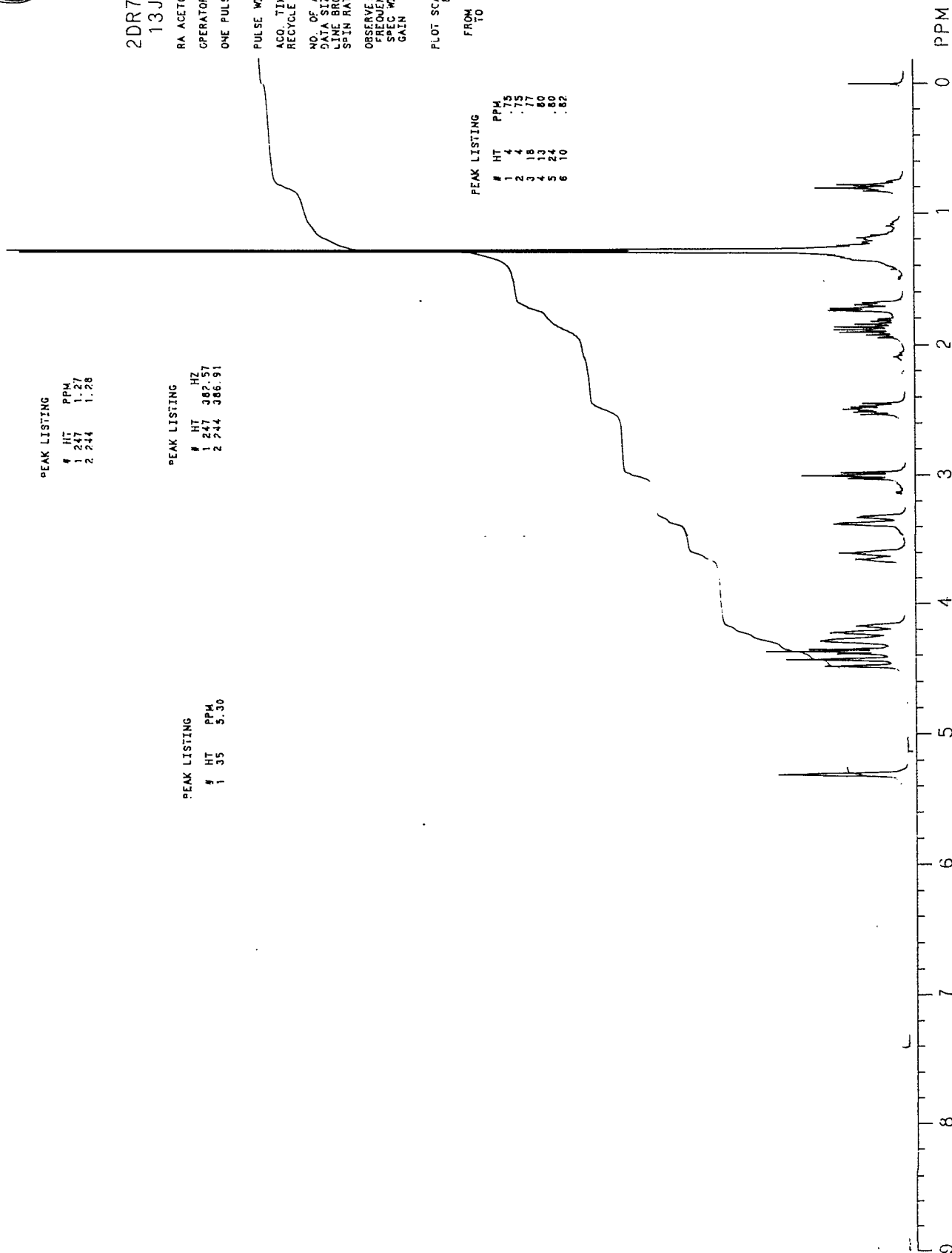
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TO 0.19 PPM

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pages 5*SM

¹³C-NMR spectrum for 7.9GE NMR
QE-3002DR79A.100
17 JUN 93

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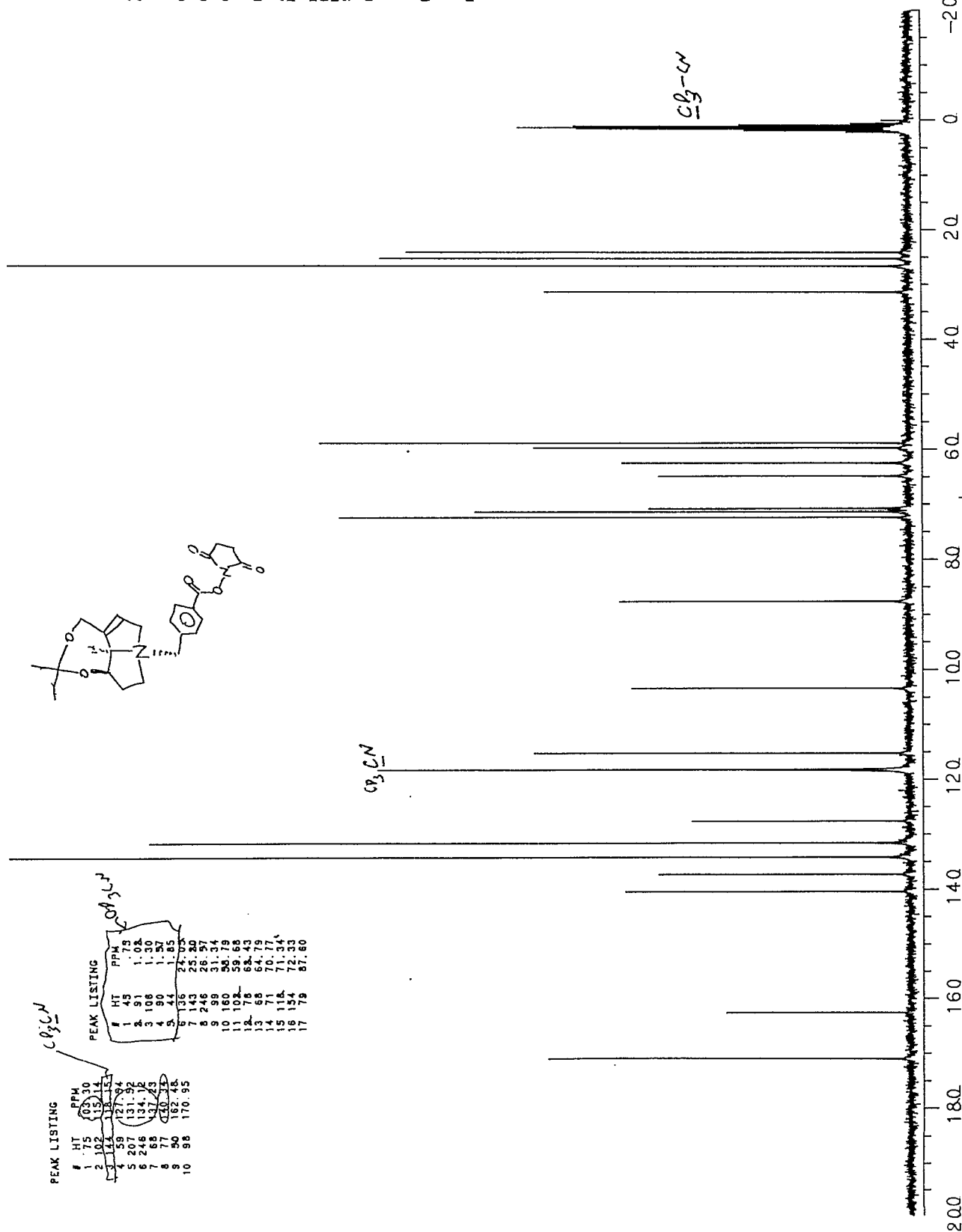
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 TO -19.99 PPM



¹³C-NMR spectrum for 8.9

pages 6-5M

GE NMR
QE PLUS2R141A.101
08DEC92QUAT ACRA BENCHO/CDCL₃-C13

OPERATOR: DMR

ONE PULSE SEQUENCE

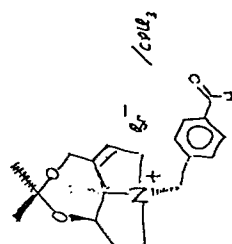
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8	101	70.21
9	235	78.57
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11	222	77.42
12	96	86.55

CDCl₃

PEAK LISTING

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-CHO

M₂

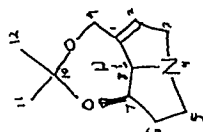
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O-195-6

¹³C-NMR spectrum for 9

pages 7•SM



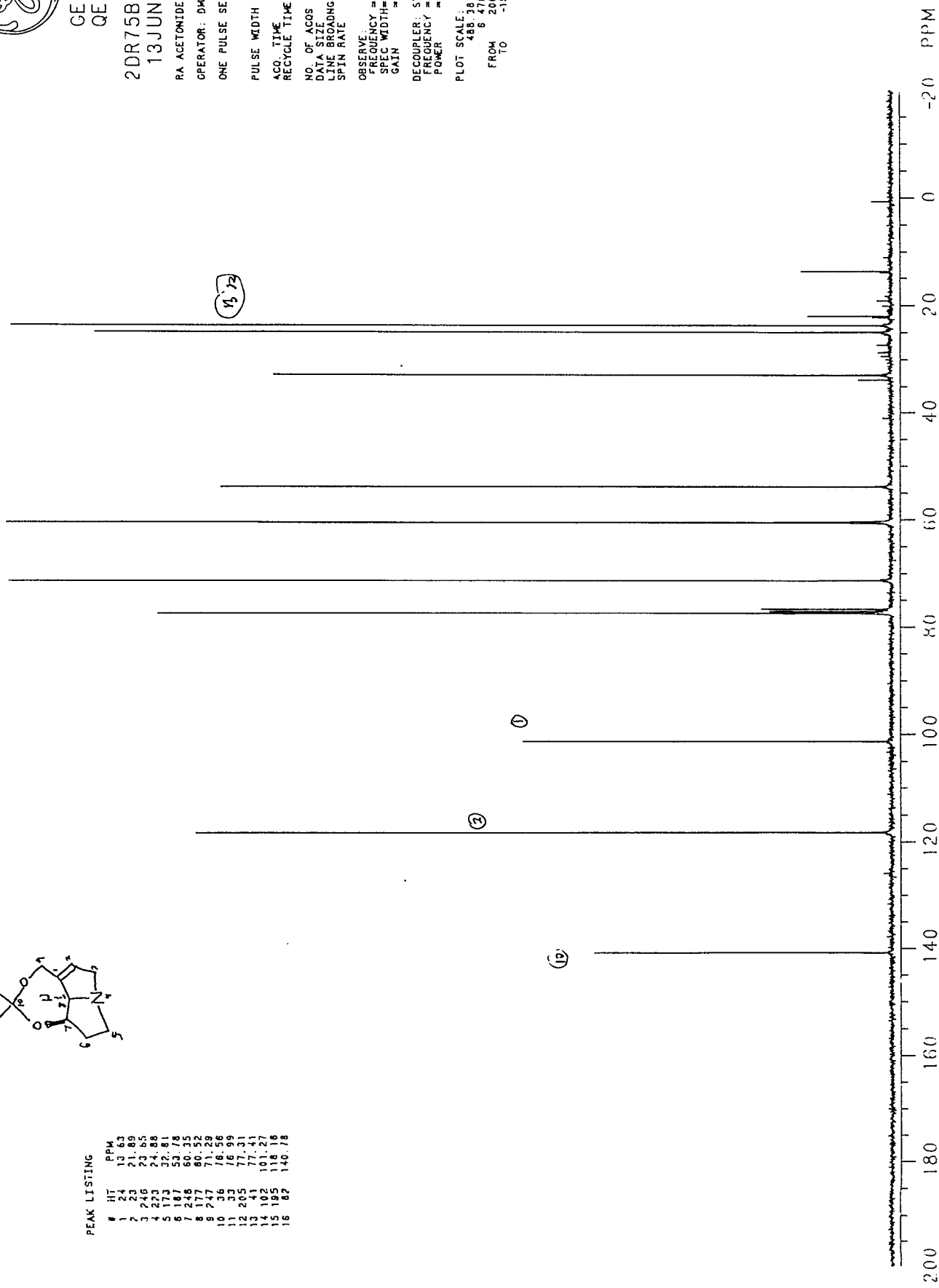
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9	177	78.58
10	36	78.99
11	33	77.31
12	205	77.31
13	41	77.41
14	102	101.27
15	195	118.18
16	82	140.78

GE NMR
QE PLUS2DR75B.100
13JUN92RA ACETONIDE/CDCL₃

OPERATOR: DMR

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Labeling Cyclic Glycoprotein IIb/IIIa Receptor Antagonists with ^{99m}Tc by the Preformed Chelate Approach: Effects of Chelators on Properties of [^{99m}Tc]Chelator–Peptide Conjugates

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Several cyclic GPIIb/IIIa receptor antagonists were labeled with ^{99m}Tc by the preformed chelate approach using chelators such as **H₄L1** [4,5-bis(mercaptoacetamido)pentanoic acid], **H₄L2** [3,4-bis(mercaptoacetamido)benzoic acid], **H₃L3** [2-(mercapto)ethylaminoacetyl-L-cysteine], **H₄L4** [*N*-(mercaptoacetyl)glycylglycylglycine], **H₄L5** [*N*-[2-(mercapto)propionyl]glycylglycylglycine], and **H₄L6** [*N*-[2-(mercapto)propionyl]glycylglycyl- γ -aminobutyric acid]. In this approach, the [^{99m}Tc]chelator complexes are formed first, followed by the activation of the carboxylic group on the complex by formation of its tetrafluorophenol (TFP) ester and the conjugation of the TFP ester with an amino group of a cyclic GPIIb/IIIa receptor antagonist. The ^{99m}Tc -labeled cyclic GPIIb/IIIa receptor antagonists were characterized by radio-HPLC (high-performance liquid chromatography); differences in lipophilicity of the [^{99m}Tc]chelator–peptide conjugate are attributable to the effects of both the cyclic peptide and the chelator.

INTRODUCTION

Venous and arterial thrombus formations are common and potentially life-threatening events. Existing diagnostic modalities are inadequate for diagnosis and determination of the morphology of the evolving thrombus (1). Thus, the development of agents that will not only detect the location but, in addition, determine the age of the thrombi is a critical unmet need in diagnostic nuclear medicine.

We have been actively pursuing a research program to develop a thrombus-imaging agent by labeling a platelet GPIIb/IIIa receptor antagonist with ^{99m}Tc (2, 3). Cyclic peptides containing the RGD (Arg-Gly-Asp) sequence have been shown to be high-affinity antagonists for the GPIIb/IIIa receptor (4). Since the GPIIb/IIIa receptor is expressed only on activated platelets (5), the ^{99m}Tc -labeled cyclic GPIIb/IIIa receptor antagonists should only be bound to platelets intimately involved in the thromboembolic event. ^{99m}Tc is the preferred radionuclide for diagnostic nuclear medicine because of its ideal physical properties (e.g. 6 h half-life, 140 keV, γ -emission), easy availability, and low cost.

A large number of radiolabeling techniques have been developed in the last decade (6–10). These include the direct- and indirect-labeling approaches and the preformed chelate approach. The direct-labeling approach uses a reducing agent (such as stannous chloride) to convert disulfide linkages in a protein into free thiols, which bind strongly to the Tc. This approach is easy to carry out, but very little is known about the coordination chemistry of the Tc. There is little control over the stability of the ^{99m}Tc complex or the nonspecific binding. This method applies only to proteins or their fragments because many small peptides do not have any disulfide bonds or in some cases the disulfide bond is too critical

for maintaining their biological properties to be reduced. In the indirect-labeling approach, a bifunctional chelator is first attached to the peptide or protein to form a chelator–peptide (protein) conjugate. The radiolabeling can be achieved either by direct reduction of $^{99m}\text{TcO}_4^-$ in the presence of chelator–peptide (protein) conjugate or by ligand exchange with an intermediate ^{99m}Tc complex such as [^{99m}Tc]glucoheptonate. The preformed chelate approach involves formation of the [^{99m}Tc]chelator complex and conjugation of the [^{99m}Tc]chelator complex to a peptide or protein in a separate step on the tracer level. In this approach, the chemistry is well-defined, and the peptide or protein is not exposed to the sometimes forcing conditions used to prepare the complex.

Fritzberg and co-workers have used an N_2S_2 diamidedithiol (**H₄L1**) and an N_3S triamidethiol (MAG₂-gaba, mercaptoacetyl-glycylglycyl- γ -aminobutyric acid) in labeling antibodies and their fragments with ^{99m}Tc (11–13) and ^{186}Re (14, 15) by the preformed chelate approach. The attachment of the chelator and the ^{99m}Tc or ^{186}Re does not significantly affect the physical and biological properties of the antibodies or their fragments, since the radiolabeling occurs only on a small portion of these macromolecules.

We have applied the preformed chelate approach to label several cyclic GPIIb/IIIa receptor antagonists with ^{99m}Tc using chelators (Figure 1) such as N_2S_2 diamidedithiols (**H₄L1** and **H₄L2**), an N_2S_2 monoamide–monoaminedithiol (**H₃L3**), and N_3S triamidethiols (**H₄L4**–**H₄L6**). We found that chelators have significant effects on properties (such as lipophilicity) of the [^{99m}Tc]chelator–peptide conjugates. Chelators in their protected forms were used for the ^{99m}Tc labeling in the preformed chelate approach. Synthesis and properties of several [^{99m}Tc]chelator–peptide conjugates are described in this report. The biological evaluation of these conjugates in two canine thrombosis models will be described in a separate communication (16).

EXPERIMENTAL SECTION

Materials. Chemicals were purchased from Aldrich Chemical Co. and were used as received. ^1H NMR

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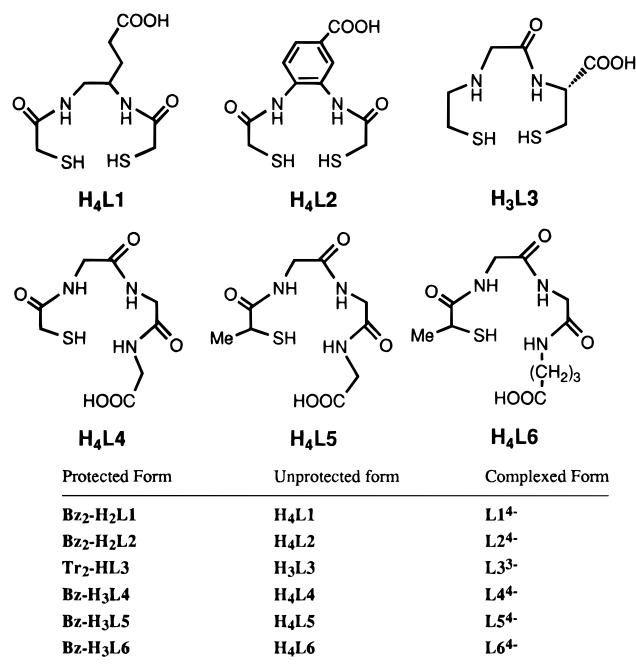


Figure 1. Chelators for ^{99m}Tc labeling of cyclic GPIIb/IIIa receptor antagonists.

spectra were collected on a Bruker 270 MHz FT-NMR spectrometer. The NMR data are reported as δ (parts per million) relative to tetramethylsilane (TMS). Infrared spectra were recorded as KBr disks in the range of 4000–400 cm^{-1} on a Nicolet 5DXB IR spectrophotometer. FAB-MS and elemental analyses were performed by Oneida Research Services, Inc., Whitesboro, NY. NH_3 -DCI mass spectra were recorded on a Finnigan MAT 8230 mass spectrometer. $\text{Na}^{99\text{m}}\text{TcO}_4$ was obtained from a commercial DuPont ⁹⁹Mo/^{99m}Tc generator, North Billerica, MA. Deionized water was obtained from a Millipore MilliQ Water System and was of >18 M Ω quality. The benzoyl-protected chelators, Bz₂-H₂L1, Bz₂-H₂L2, Bz-H₃L4, and Bz-H₃L6, were prepared as previously reported (17, 18). Synthesis of the cyclic GPIIb/IIIa antagonists will be reported elsewhere (19).

The high-performance liquid chromatography (HPLC) method used a Hewlett-Packard Model 1050 instrument and a Vydac C₁₈ column (4.6 mm $\times$ 25 cm) at a flow rate of 1 mL/min with a gradient mobile phase from 100% A (10 mM phosphate buffer, pH 6) to 30% B (acetonitrile) at 15 min and 75% B at 25 min.

Synthesis of the Trityl-Protected Ethyl Ester of H₃L3. *S*-Trityl-L-cysteine Ethyl Ester (2). To a solution of L-cysteine ethyl ester (1) hydrochloride (18.6 g, 100 mmol) in 200 mL of trifluoroacetic acid (TFA) was added triphenylmethanol (52 g, 200 mmol). The resulting dark brown solution was allowed to stir for 2 h at room temperature under nitrogen. The solvent was removed in vacuo. To the residue were added ethanol (100 mL) and 1.0 M sodium ethoxide solution (50 mL). The reaction mixture was stirred for 90 min while the solution turned cloudy. The mixture was filtered, and the filtrate was concentrated in vacuo to give an oily residue. Flash column chromatography using ethyl acetate/hexane (1:3) gave the desired product. A small amount of ethyl acetate in the product is difficult to remove. The yield was 37.5 g (96%). IR (cm^{-1}): 3500–2500 (br, $\nu_{\text{N-H}}$), 1750, 1685 (vs, $\nu_{\text{C=O}}$). NH_3 -DCI MS: m/z 409 ($\text{M} + \text{NH}_4^+$), 392 ($[\text{M} + \text{H}]^+$). ¹H NMR (CDCl_3): 1.15 (t, 3H, CH₃, $J = 7.1$ Hz), 2.60–2.95 (m, 2H, CH₂), 3.05 (m, 1H, CH), 4.08 (m, 2H, CH₂), 7.15–7.45 (m, 15H, aromatic hydrogens).

N-(Bromoacetyl)-*S*-trityl-L-cysteine Ethyl Ester (3). A solution of *S*-trityl-L-cysteine ethyl ester (18 g, 46 mmol) and triethylamine (6.4 mL, 46 mmol) in dry tetrahydrofuran (THF) (250 mL) under nitrogen was cooled to 0 °C. A solution of bromoacetyl bromide (9.28 g, 46 mmol) in dry THF (60 mL) was added dropwise, during which time the solution turned cloudy. After it was stirred at 0 °C for 1 h and room temperature for an additional 1 h, the reaction mixture was filtered. The filtrate was concentrated in vacuo to give an oil, which was partitioned between methylene chloride and water (60 mL each). The organic layer was washed with 5% HCl and saturated sodium bicarbonate solution and dried over anhydrous magnesium sulfate. The solution was filtered, and volatiles were removed under reduced pressure to give the desired product. The yield was 16.3 g (69%). IR (cm^{-1}): 3310 (s, $\nu_{\text{N-H}}$), 2900–3100 (m, $\nu_{\text{C-H}}$), 1750, 1715, 1660 (vs, $\nu_{\text{C=O}}$). NH_3 -DCI MS: m/z 531 ($\text{M} + \text{NH}_4^+$), 514 ($[\text{M} + \text{H}]^+$). ¹H NMR (CDCl_3): 1.26 (t, 3H, CH₃, $J = 7.0$ Hz), 2.60–2.95 (m, 2H, CH₂), 3.82 (s, 2H, CH₂), 4.20 (q, 2H, CH₂, $J = 7.0$ Hz), 4.50 (m, 1H, CH), 6.95 (d, 1H, NH, $J = 6.0$ Hz), 7.20–7.45 (m, 15H, aromatic hydrogens).

2-[[[*S*-Tritylmercapto]ethyl]amino]acetyl]-*S*-trityl-L-cysteine Ethyl Ester (4). To a solution of *N*-(bromoacetyl)-*S*-trityl-L-cysteine ethyl ester (1.0 g, 1.98 mmol) and triethylamine (0.4 mL, 2.9 mmol) in methylene chloride (20 mL) was added *S*-trityl-2-aminoethanethiol (0.64 g, 2.0 mmol). The reaction mixture was stirred at room temperature for 7 days. To the mixture was added water (10 mL). The organic layer was separated, washed with saturated sodium bicarbonate solution (2 $\times$ 10 mL), water (2 $\times$ 10 mL), and brine (10 mL), and dried over anhydrous magnesium sulfate. After filtration, the filtrate was concentrated in vacuo to give a foamy product. Flash chromatography using ethyl acetate/hexane (3:1) gave the expected product. The yield was 0.56 g (38%). IR (cm^{-1}): 3500–2500 (br, $\nu_{\text{N-H}}$), 1745, 1615 (vs, $\nu_{\text{C=O}}$). FAB-MS: m/z 751 ($[\text{M} + \text{H}]^+$). ¹H NMR (CDCl_3): 1.25 (m, 3H, CH₃), 2.30–2.80 (m, 6H, CH₂), 3.10 (s, 2H, CH₂CO), 4.15 (m, 2H, CH₂), 4.54 (m, 1H, CH), 7.20–7.50 (m, 30H, aromatic hydrogens), 7.75 (d, 1H, NH, $J = 6.0$ Hz). Anal. Calcd (found) for C₄₇H₄₆N₂O₃S₂: C, 75.17 (74.15), H, 6.17 (6.07), N, 3.73 (3.68), S, 8.54 (8.57).

Synthesis of Benzoyl-Protected H₄L5. *N*-[2-(Benzoylmercapto)propionyl]glycine (6). Sodium hydroxide (4.5 g, 109 mmol) and *N*-(2-mercapto)propionylglycine (5) (8.20 g, 50 mmol) were dissolved in a mixture of water (40 mL) and toluene (30 mL). The temperature was lowered to 5–15 °C using an ice bath. Benzoyl chloride (4.6 mL, 51 mmol) in toluene (10 mL) was added dropwise with vigorous stirring. After addition, the mixture was stirred at 5–15 °C for another 30 min and then at room temperature for 2 h. The organic layer was separated, washed with H₂O (2 $\times$ 20 mL), and discarded. Aqueous fractions were combined and acidified to pH ~1.5 using concentrated HCl while a white precipitate formed. The solid was collected by filtration, washed with H₂O and a small amount of ethanol, and dried under vacuum. The yield was 13.0 g (97%). IR (cm^{-1}): 3375 (s, $\nu_{\text{N-H}}$), 3200–2500 (br, $\nu_{\text{O-H}}$), 1745 (vs, thio ester $\nu_{\text{C=O}}$), 1663, 1625 (vs, amide and carboxylic $\nu_{\text{C=O}}$). FAB-MS: m/z 268 ($[\text{M} + \text{H}]^+$). ¹H NMR ($\text{DMSO}-d_6$): 1.47 (d, 3H, CH₃, $J = 7.0$ Hz), 3.79 (d, 2H, CH₂, $J = 5.9$ Hz), 4.40 (q, 1H, CH, $J = 7.0$ Hz), 7.53 (m, 2H, =CH), 7.69 (m, 1H, =CH), 7.90 (dd, 2H, =CH, $J = 7.0$ Hz), 8.59 (t, 1H, NH, $J = 5.8$ Hz), 12.6 (bs, 1H, COOH). Anal. Calcd (found) for C₁₂H₁₃NO₄S: C, 53.90 (53.89), H, 4.90 (4.81), N, 5.24 (5.22).

N-[2-(Benzoylmercapto)propionyl]glycine Succinimide Ester (7). To a suspension of *N*-[2-(benzoylmercapto)propionyl]glycine (13.5 g, 50 mmol) and *N*-hydroxysuc-

cinimide (5.75 g, 50 mmol) in THF (200 mL) was added 1-cyclohexyl-3-(2-morpholineethyl)carbodiimide metho-*p*-toluenesulfonate (25 g, 59 mmol), in a mixture of THF (100 mL) and water (100 mL). The reaction mixture was stirred for 24 h at room temperature. Upon addition of acetic acid, the mixture was stirred for another 2 h. The solid was separated by filtration and discarded. The filtrate was evaporated to dryness to give a white solid, which was collected, washed with diethyl ether, and dried in air. The yield was 10.5 g (57.5%). IR (cm^{-1}): 3290 (s, $\nu_{\text{N-H}}$), 1820 (m, succinimide $\nu_{\text{C=O}}$), 1785 (m, ester $\nu_{\text{C=O}}$), 1735 (vs, thio ester $\nu_{\text{C=O}}$), 1600 (vs, amide $\nu_{\text{C=O}}$). FAB-MS: m/z 365 ($[\text{M} + \text{H}]^+$). ^1H NMR (CDCl_3): 1.57 (d, 3H, CH_3 , $J = 7.0$ Hz), 2.79 (s, 4H, CH_2), 4.33 (q, 1H, CH, $J = 7.0$ Hz), 4.39 (m, 2H, CH_2), 7.00 (t, 1H, NH, $J = 5.8$ Hz), 7.44 (m, 2H, =CH), 7.59 (m, 1H, =CH), 7.93 (dd, 2H, =CH, $J = 7.0$ Hz). Anal. Calcd (found) for $\text{C}_{16}\text{H}_{16}\text{N}_2\text{O}_6\text{S}$: C, 52.72 (52.70), H, 4.43 (4.21), N, 7.69 (7.69).

***N*-[2-(Benzoylmercapto)propionyl]glycylglycylglycine (**Bz-H₃L5**).** *N*-[2-(Benzoylmercapto)propionyl]glycine succinimide ester (1.82 g, 5 mmol) and glycylglycine (0.66 g, 5 mmol) were suspended in a mixture of ethanol (150 mL) and water (30 mL). The mixture was heated to reflux for 5 h, during which time the cloudy mixture became a clear solution. The solution was then cooled to room temperature and was kept stirring overnight. Evaporation of solvents under reduced pressure gave the expected product. The crude product was washed with water. Recrystallization from 50% aqueous ethanol solution produced the pure product. The yield was 1.58 g (63%). IR (cm^{-1}): 3380, 3300 (s, $\nu_{\text{N-H}}$), 3100–2500 (br, $\nu_{\text{O-H}}$), 1738 (vs, thio ester $\nu_{\text{C=O}}$), 1680, 1660 (vs, amide $\nu_{\text{C=O}}$). FAB-MS: m/z 382 ($[\text{M} + \text{H}]^+$). ^1H NMR ($\text{DMSO}-d_6$): 1.48 (d, 3H, CH_3 , $J = 7.0$ Hz), 3.78 (m, 4H, CH_2), 3.85 (d, 2H, CH_2 , $J = 6.0$ Hz), 4.41 (m, 1H, CH), 7.52 (m, 2H, =CH), 7.70 (m, 1H, =CH), 7.90 (m, 2H, =CH), 8.15 (t, 1H, NH, $J = 3.0$ Hz), 8.51 (t, 1H, NH, $J = 3.0$ Hz), 8.80 (t, 1H, NH, $J = 3.0$ Hz). Anal. Calcd (found) for $\text{C}_{16}\text{H}_{19}\text{N}_3\text{O}_6\text{S}$: C, 50.39 (50.59), H, 5.02 (5.78), N, 11.02 (10.70).

Deprotection of 2-[[[(*S*-Tritylmercapto)ethyl]amino]acetyl]-*S*-trityl-L-cysteine Ethyl Ester. 2-[[[(*S*-Tritylmercapto)ethyl]amino]acetyl]-*S*-trityl-L-cysteine ethyl ester (10 mg) was dissolved in 2 mL of TFA to give a bright yellow solution. The mixture was stirred at room temperature for 5 min. Upon addition of triethylsilane (1 mL), the reaction mixture became clear. TFA and excess triethylsilane were removed under vacuum to give a white solid residue. To the residue were added 0.5 mL of 5 N NaOH solution and 1.5 mL of THF. The mixture was heated on a boiling water bath for 5 min. To the mixture was added 0.5 M phosphate buffer (1.5 mL, pH 12), and the pH was adjusted to 10–11 using 5 N HCl. The solution was filtered, and the filtrate was diluted to 5 mL using 0.5 M phosphate buffer (pH 12). The freshly prepared solution was used immediately for $^{99\text{m}}\text{Tc}$ labeling.

General Procedure for $^{99\text{m}}\text{Tc}$ Labeling of Cyclic GPIIb/IIIa Receptor Antagonists. *Chelation.* To a 10 mL vial was added 0.5 mL of $^{99\text{m}}\text{TcO}_4^-$ solution (200–400 mCi/mL in saline), 0.5 mL of ligand solution (2 mg/mL in 0.5 M phosphate buffer, pH 12), 0.15–0.20 mL of sodium dithionite solution (5 mg/mL in 0.5 M phosphate buffer, pH 12) for **H₄L1–H₃L3**, or $\text{SnCl}_2 \cdot 2\text{H}_2\text{O}$ solution (5 mg/mL in 1.0 N HCl) for **H₄L4–H₄L6**. The pH was adjusted to 11 (8–10 for **H₃L3**), and the mixture was heated at 100 °C for 30 min. The reaction mixture was analyzed by radio-HPLC.

Activation. To the reaction mixture were added 0.2 mL

Table 1. HPLC Chromatographic Data for $^{99\text{m}}\text{Tc}$ Complexes

complex ^a	retention time (min) ^a	labeling yield (%)
$^{99\text{m}}\text{TcO}(\text{L1})^{2-}$	9.5/10.0	≥90
$^{99\text{m}}\text{TcO}(\text{L1-TFP})^-$	23.0	≥90
$^{99\text{m}}\text{TcO}(\text{L1-III})^-$	15.0	60
$^{99\text{m}}\text{TcO}(\text{L1-IV})^-$	15.7	45
$^{99\text{m}}\text{TcO}(\text{L1-V})^-$	16.2	50
$^{99\text{m}}\text{TcO}(\text{L2})^{2-}$	14.5	85
$^{99\text{m}}\text{TcO}(\text{L2-TFP})^-$	25.0	80
$^{99\text{m}}\text{TcO}(\text{L2-III})^-$	18.1	33
$^{99\text{m}}\text{TcO}(\text{L2-IV})^-$	17.8	21
$^{99\text{m}}\text{TcO}(\text{L2-V})^-$	19.1	30
$^{99\text{m}}\text{TcO}(\text{L3})^-$	7.4/8.7	≥75
$^{99\text{m}}\text{TcO}(\text{L3-TFP})^-$	24.6	≥90
$^{99\text{m}}\text{TcO}(\text{L3-III})^-$	14.8	35
$^{99\text{m}}\text{TcO}(\text{L3-IV})^-$	16.2	34
$^{99\text{m}}\text{TcO}(\text{L4})^{2-}$	7.2	≥90
$^{99\text{m}}\text{TcO}(\text{L4-TFP})^-$	20.8	≥80
$^{99\text{m}}\text{TcO}(\text{L4-III})^-$	13.2	50
$^{99\text{m}}\text{TcO}(\text{L5})^{2-}$	8.1/8.4	90
$^{99\text{m}}\text{TcO}(\text{L5-TFP})^-$	21.9	≥80
$^{99\text{m}}\text{TcO}(\text{L5-III})^-$	13.5	55
$^{99\text{m}}\text{TcO}(\text{L6})^{2-}$	10.8/11.0	98
$^{99\text{m}}\text{TcO}(\text{L6-TFP})^-$	22.8	≥90
$^{99\text{m}}\text{TcO}(\text{L6-III})^-$	14.5	47
$^{99\text{m}}\text{TcO}(\text{L6-IV})^-$	14.9	35
$^{99\text{m}}\text{TcO}(\text{L6-V})^-$	15.5	70

^aUnder the experimental conditions described in the Experimental Section, the carboxylate groups in complexes $^{99\text{m}}\text{TcO}(\text{L})^{n-}$ (**L** = **L1–L6**) are deprotonated.

of 1.0 N HCl, 0.5 mL of 2,3,5,6-tetrafluorophenol (TFP) solution (100 mg/mL in 90% aqueous CH_3CN), and 0.5 mL of 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (WS-CDI) solution (100 mg/mL in 90% aqueous CH_3CN). The pH was adjusted to 6, and the reaction mixture was heated at 45–50 °C for 30 min. Radio-HPLC showed ≥85% of the activated ester.

Conjugation. To the mixture was added 2–3 mg of peptide in 0.5 mL of 0.5 M phosphate buffer (pH 9). After the pH was adjusted to 9.0–10.0, the reaction mixture was incubated at 40 °C for 30 min and analyzed by radio-HPLC. The conjugation yields ranged from 30 to 60%, depending on the identity and the amount of the peptide. The yields and retention times for the $^{99\text{m}}\text{Tc}$ chelator complexes, the TFP–ester complexes, and $^{99\text{m}}\text{Tc}$ chelator–peptide conjugates are listed in Table 1. The products were purified and separated from the unlabeled cyclic peptide before biological evaluation.

RESULTS AND DISCUSSION

Selection of Target Molecules. Platelet GPIIb/IIIa is a membrane protein that mediates the aggregation of platelets. In response to stimulation by a variety of agonists, including thromboxane, thrombin, or epinephrine, this protein undergoes a conformational change, allowing it to bind to fibrinogen (4). Multiple GPIIb/IIIa molecules appear to bind to a single molecule of fibrinogen, which leads to platelet aggregation and formation of a platelet-rich thrombus. Thus, inhibition of fibrinogen binding to the GPIIb/IIIa receptor blocks platelet aggregation and thrombus formation.

As with many members of the integrin superfamily of adhesion molecules, the GPIIb/IIIa receptor recognizes proteins and peptides bearing the tripeptide sequence Arg-Gly-Asp (RGD). A number of RGD-containing small molecules, including peptides (4, 20–22) and peptidomimetics (23, 24), have been synthesized and studied for their antithrombotic activities. These studies are directed toward the development of antithrombotic agents

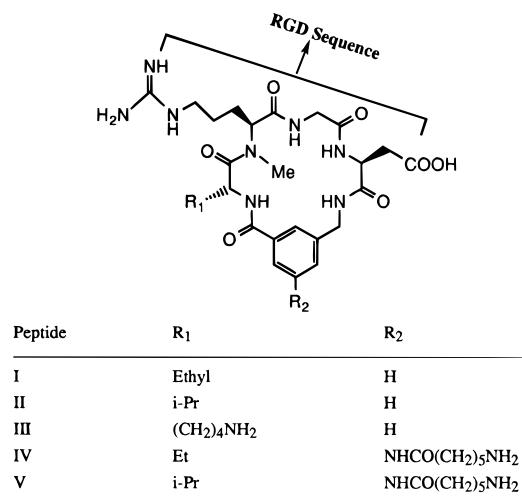


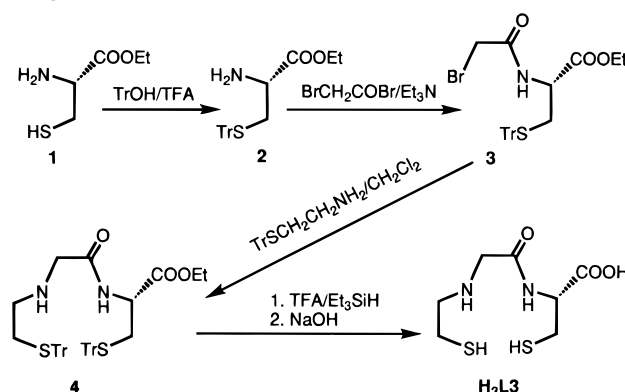
Figure 2. Cyclic GPIIb/IIIa receptor antagonists.

for the treatment of thrombosis. It seems that cyclization of a peptide can give rise to impressive gains in affinity and specificity due to restriction of conformational mobility. Recently, DeGrado (4) and co-workers reported a series of very potent cyclic GPIIb/IIIa receptor antagonists, two (I and II) of which are shown in Figure 2. Both peptides contain a rigid *m*-(aminomethyl)benzoic acid (Mamb) moiety and show very high affinity for the GPIIb/IIIa receptor with IC₅₀ values in the nanomolar range for inhibition of both platelet aggregation and fibrinogen binding (4). This makes them the candidates of choice as target molecules to carry the radionuclide (^{99m}Tc) to thrombi. If the attachment of a chelator and ^{99m}Tc labeling do not block RGD sequence binding, the ^{99m}Tc-labeled cyclic peptides should be able to bind strongly to the GPIIb/IIIa receptor and thus incorporate into rapidly growing thrombi under both arterial and venous conditions. Since peptides are expected to exhibit more rapid blood clearance than the larger proteins, the ^{99m}Tc-labeled cyclic GPIIb/IIIa receptor antagonists should have the potential to permit earlier imaging of thrombi.

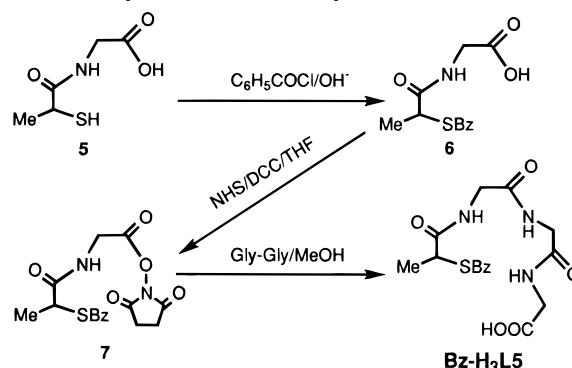
In order to label cyclic GPIIb/IIIa receptor antagonists with ^{99m}Tc, a linker is required to connect the cyclic peptide framework with a Tc-binding group. Since the RGD sequence is vital for maintaining biological activity, we prepared linker-modified cyclic peptides by incorporation of a lysine residue between the arginine residue and the Mamb ring or by attachment of a 6-aminocaproic acid group on the Mamb ring. The use of a 6-aminocaproic acid linker keeps the Tc center far from the RGD tripeptide sequence to minimize the effect of ^{99m}Tc labeling on the binding affinity for the GPIIb/IIIa receptor (19). The amino group can be used for the conjugation of either a chelator by the indirect-labeling approach or a ^{99m}Tc complex by the preformed chelate approach.

Selection of a Chelator. A bifunctional chelator can be divided into three parts: a binding unit, a conjugation group, and a spacer, which connects the binding unit and the conjugation group. There are several requirements for an ideal chelator. First, the binding unit can selectively stabilize an intermediate or lower oxidation state of Tc so that the ^{99m}Tc complex is not subject to redox reactions; oxidation changes are often accompanied by transchelation of ^{99m}Tc from a [^{99m}Tc]chelator-peptide conjugate to the native chelating ligands in biological systems. Second, the chelator forms a ^{99m}Tc complex which is kinetically inert with respect to dissociation. Finally, the conjugation group can be easily attached to the target peptide.

Scheme 1. Synthesis of Trityl-Protected Ethyl Ester of H₃L3



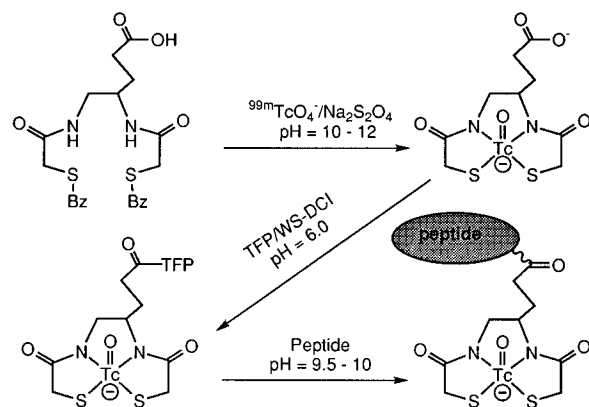
Scheme 2. Synthesis of Benzoyl-Protected H₄L5



Various chelators have been used in labeling proteins and peptides with ^{99m}Tc and ¹⁸⁶Re. These include DTPA (23), N₂S₂ diamidedithiols (11–13, 17), N₃S triamidethiols (14, 15, 17), BATOs (26, 27), N₂S₂ diaminedithiols (28), and hydrazino nicotinamide (HYNIC) (29, 30). The chelators used in this study are chosen because they all form stable anionic or neutral technetium complexes (neglecting charges on the carboxylate group) with the Tc=O core. The coordination chemistry of these three types of chelators with technetium is well-documented (31–35). The use of these chelators allows us to study their effect on the physical and biological properties of the [^{99m}Tc]chelator-peptide conjugates.

Synthesis of Chelators. H₃L3. The *S*-trityl-protected ethyl ester of H₃L3 was prepared (Scheme 1) in three steps from L-cysteine ethyl ester (1). The reaction of L-cysteine ethyl ester hydrochloride with triphenylmethanol in TFA afforded compound 2, which was then allowed to react with bromoacetyl bromide in THF in presence of triethylamine to give compound 3. The reaction of compound 3 with *S*-trityl-2-aminoethanethiol (33) in the presence of Et₃N in dichloromethane produced the *S*-trityl-protected ethyl ester of H₃L3 (4). It was characterized by spectroscopic (IR, NMR, and FAB-MS) methods and elemental analysis. Deprotection of the trityl groups in compound 4 was achieved by the radical cleavage of the C–S bonds. Hydrolysis of the ethyl ester of H₃L3 under basic conditions afforded unprotected H₃L3. The freshly prepared H₃L3 solution was used immediately for ^{99m}Tc labeling.

Benzoyl-Protected H₄L5. The benzoyl-protected H₄L5 was readily prepared (Scheme 2) from commercially available *N*-(2-mercaptopropionyl)glycine (5) in three steps. The thiol group was protected by reaction of compound 5 with benzoyl chloride under basic conditions. Activation of the carboxylic group of compound 6 was achieved by formation of its succinimide ester (7). Con-

Scheme 3. Labeling Cyclic GPIIb/IIIa Peptides by the Preformed Chelate Approach Using H_4L1


jugation of compound **7** with glycylglycine afforded the benzoyl-protected **H₄L5**, which has been characterized by IR, ¹H NMR, and FAB-MS spectroscopy and elemental analysis.

Labeling of Cyclic GPIIb/IIIa Antagonists by the Preformed Chelate Approach. The preformed chelate approach used in this study involves three steps of tracer level (^{99m}Tc) synthesis: chelation of the radionuclide (^{99m}Tc), activation of the pendant carboxylate group on the chelate by formation of its TFP ester, and conjugation of the TFP ester with the amino group of a peptide. The reaction product in each step is characterized by radio-HPLC. The final products, the [^{99m}Tc]chelator-peptide conjugates, are purified by HPLC before biological evaluation.

N₂S₂ Diamidedithiols. Since the coordination chemistry of diamidedithiols with technetium is well-understood, we used chelators, **H₄L1** and **H₄L2**, to label the cyclic I Ib/IIIa antagonists with ^{99m}Tc. We prepared the complex [^{99m}TcO(**L1**)]²⁻ by reducing pertechnetate with sodium dithionite in the presence of **H₄L1** under basic conditions (pH 10–12). Heating at 100 °C for 30 min is necessary for successful radiolabeling. As expected, the coordination of **H₄L1** to the Tc center results in two diastereomers (*syn* and *anti*) due to the relative orientation of the functional group (CH₂CH₂COOH) to the Tc=O core. The carboxylic group was activated by formation of its tetrafluorophenol (TFP) ester, [^{99m}TcO(**L1**-TFP)]⁻. Conjugation of [^{99m}TcO(**L1**-TFP)]⁻ with a cyclic GPIIb/IIIa peptide was carried out at pH 9.5–10.0 to form the complex [^{99m}TcO(**L1**-peptide)]⁻ (Scheme 3). The conjugation yields varied from 50 to 80%, depending upon the amount of cyclic peptide used for conjugation. Total time for radiolabeling was about 3 h. As an example, Figure 3 shows radio-HPLC chromatograms for complexes [^{99m}TcO(**L1**)]²⁻ (A), [^{99m}TcO(**L1**-TFP)]⁻ (B), [^{99m}TcO(**L1**-V)]⁻ (C), and the HPLC-purified [^{99m}TcO(**L1**-V)]⁻ (D). The HPLC-purified [^{99m}TcO(**L1**-V)]⁻ was found to be stable for at least 12 h in saline solution.

H₄L2 is of interest because the carboxylic group is well-separated from the metal center by a rigid aromatic benzene ring so that intramolecular interaction between the carboxylate O and the Tc(V) center is minimized. Unlike chelators functionalized on a saturated carbon, it does not form diastereomers upon coordination to the Tc=O core. We have used **H₄L2** in labeling the cyclic GPIIb/IIIa peptides by a procedure similar to that described for **H₄L1**. It is noted that the conjugation of [^{99m}TcO(**L2**-TFP)]⁻ with the cyclic I Ib/IIIa peptides requires higher pH (10–10.5) than that of [^{99m}TcO(**L1**-TFP)]⁻. The conjugation yields are lower (20–30%) than those obtained using **H₄L1**.

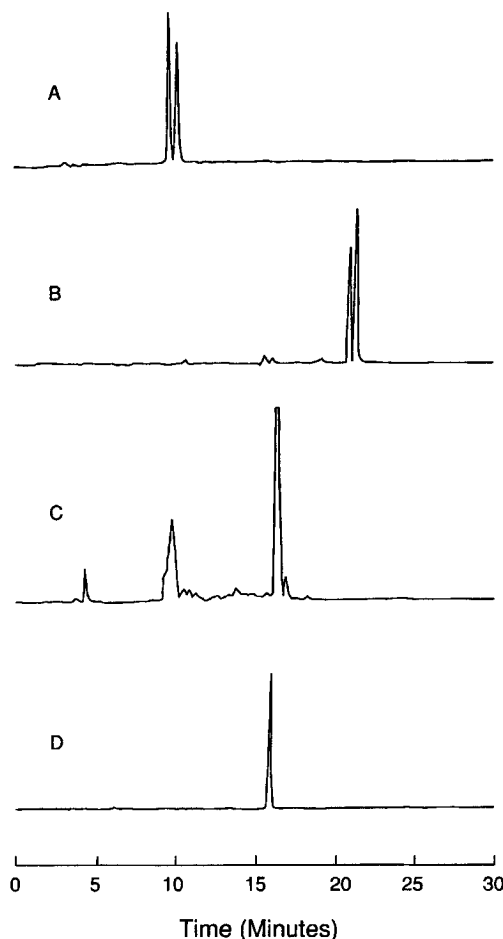


Figure 3. Radio-HPLC chromatograms for [^{99m}TcO(**L1**)]²⁻ (A), [^{99m}TcO(**L1**-TFP)]⁻ (B), [^{99m}TcO(**L1**-V)]⁻ (C), and the HPLC-purified [^{99m}TcO(**L1**-V)]⁻ (D).

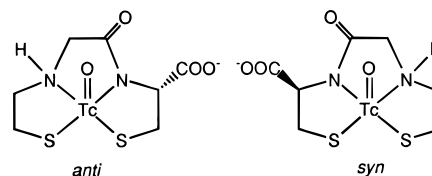


Figure 4. *Syn* and *anti* isomers of [^{99m}TcO(**L3**)]⁻.

Monoamide–Monoaminedithiol. **H₃L3** contains a secondary amine nitrogen, an amide nitrogen, and two thiolate sulfur donors. It acts as a tribasic ligand in binding to the Tc=O core to form a neutral complex, [^{99m}TcO(**L3**)]⁰, providing that the carboxylate group is protonated. We prepared [^{99m}TcO(**L3**)]⁰ by reducing pertechnetate with sodium dithionite in the presence of free ligand **H₃L3** at pH 8–10. Two isomers were observed because of the *anti* and *syn* orientations of the carboxylate group relative to the Tc=O moiety (Figure 4). The carboxylate group in [^{99m}TcO(**L3**)]⁰ was activated by formation of its TFP ester, [^{99m}TcO(**L3**-TFP)]⁰, which was then conjugated with a peptide at pH 9.5–10 to afford [^{99m}TcO(**L3**-peptide)]⁰ conjugates. The conjugation yields were 30–50%. The HPLC-purified [^{99m}TcO(**L3**-V)]⁰ was found to be stable for 24 h in saline solution.

N₃S Triamidethiols. Like N₂S₂ diamidedithiols, N₃S triamidethiols also form stable Tc(V) complexes with the Tc=O core. For **H₄L5** and **H₄L6**, the presence of a methyl group on the ligand backbone results in two diastereomers because of its relative orientation to the Tc=O bond (Figure 5). Stannous chloride was used instead of sodium dithionite as the reducing agent for pertechnetate. The chelation reaction was carried out

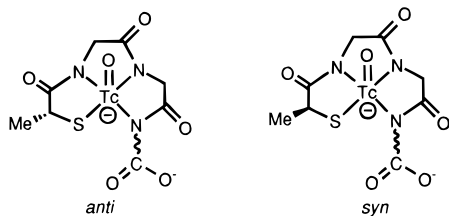


Figure 5. *Syn* and *anti* isomers of $[^{99m}\text{TcO}(\text{L})]^{2-}$ ($\text{L} = \text{L5}$ and L6).

at pH ~ 11 with heating for 30 min at 100 $^{\circ}\text{C}$. The activation of the carboxylic group and conjugation of the TFP ester with a peptide were performed in a fashion similar to that described for **H4L1**. The conjugation yields ranged from 30 to 70%, depending on the amount of cyclic peptide used for conjugation. The ^{99m}Tc -labeled cyclic GPIIb/IIIa antagonists $[^{99m}\text{TcO}(\text{L-peptide})]^{n-}$ ($\text{L} = \text{L6}$, peptide = III and V) were stable for 12 h in saline solution.

HPLC Data for ^{99m}Tc Complexes. Table 1 summarizes radio-HPLC data for all the ^{99m}Tc complexes, which include $[^{99m}\text{TcO}(\text{L})]^{n-}$, $[^{99m}\text{TcO}(\text{L-TFP})]^{n-}$, and $[^{99m}\text{TcO}(\text{L-peptide})]^{n-}$. For the ^{99m}Tc -labeled cyclic GPIIb/IIIa receptor antagonists, $[^{99m}\text{TcO}(\text{L-peptide})]^{n-}$ ($\text{L} = \text{L1}$, **L3**, **L5**, and **L6**), the two isomeric forms are not well-separated using the HPLC method described in the Experimental Section. They are isolated as a mixture of two diastereomers for the *in vivo* animal studies in a canine AV model (16). In general, the conversion of the anionic carboxylate group into its TFP ester results in a dramatic increase in lipophilicity. Conjugation of the TFP ester with a cyclic peptide results in a sharp decrease in lipophilicity. These changes are reflected by the differences in the HPLC retention times and are caused by the change in the molecular charge and the identity of the molecule attached to the carboxylic group. For complexes $[^{99m}\text{TcO}(\text{L-V})]^{n-}$ ($\text{L} = \text{L1-L6}$), the rank order in retention time is the same as that for complexes $[^{99m}\text{TcO}(\text{L})]^{n-}$ ($\text{L} = \text{L1-L6}$). For complexes $[^{99m}\text{TcO}(\text{L1-peptide})]^{n-}$ and $[^{99m}\text{TcO}(\text{L6-peptide})]^{n-}$ (peptide = III-V), the retention time follows the rank order of the peptide. Therefore, both cyclic peptide and the chelator have significant effects on physical properties (such as lipophilicity) of the $[^{99m}\text{Tc}]$ chelator-peptide conjugates. These effects are also seen on biological properties (such as thrombus uptake and the target-to-background ratio) of the conjugates, $[^{99m}\text{TcO}(\text{L-peptide})]^{n-}$ ($\text{L} = \text{L1}$ and **L6**, peptide = III and V), in two canine thrombosis models (16).

CONCLUSIONS

In conclusion, three cyclic GPIIb/IIIa receptor antagonists were labeled with ^{99m}Tc by the preformed chelate approach using chelators such as N_2S_2 diamidedithiols (**H4L1** and **H4L2**), a monoamine-monoamidedithiol (**H3L3**), and N_3S triamidedithiols (**H4L4-H4L6**). The ^{99m}Tc -labeled cyclic GPIIb/IIIa receptor antagonists were formed in moderate to good yields, depending on the amount of the peptide used in the conjugation reaction. They were purified and separated from the unlabeled cyclic peptide before biological evaluation.

Although the preformed chelate approach has been widely used in labeling proteins and their fragments with ^{99m}Tc (11-13, 17, 36) and ^{186}Re (14, 15), there is little information available on the use of this approach in labeling biologically active small molecules such as peptides. The advantage of the approach is that the chemical and biological properties of a series of conjugates with a range of lipophilicities and differing charges

can be determined before extensive efforts are directed at synthesizing chelator-modified peptides. We have synthesized and evaluated conjugates using six different chelators and three different peptides using milligram quantities of the peptides.

For proteins and their fragments, the attachment of the chelator and ^{99m}Tc does not significantly affect their physical and biological properties because these properties are determined mainly by the much larger polypeptide structure. For small molecules such as cyclic GPIIb/IIIa peptides, the ^{99m}Tc complexes constitute a significant portion of the $[^{99m}\text{Tc}]$ chelator-peptide conjugates. The physical and biological properties should be a function of both the peptide and the ^{99m}Tc complex, as seen in this and other (16) studies. However, the use of the appropriate combination of chelator, linker, and peptide can still result in ^{99m}Tc -labeled GPIIb/IIIa receptor antagonists with good biological activity.

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LITERATURE CITED

- (1) Knight, L. (1990) Radiopharmaceuticals for thrombus detection. *Semin. Nucl. Med.* 20, 52-67.
- (2) Barrett, J. A., Heminway, S., Damphousse, D. J., Thomas, J., Looby, R. J., Edwards, D. S., Harris, T. D., Rajopadhye, M., Liu, S., and Carroll, T. R. (1994) Platelet GPIIb/IIIa antagonists in the canine arteriovenous shunt: potential thrombus imaging agents. *J. Nucl. Med.* 35, 52P (abstract 202).
- (3) Harris, T. D., Barrett, J. A., Bourque, J. P., Carroll, T. R., Damphousse, P. R., Edwards, D. S., Glowacka, D., Liu, S., Looby, R. J., Poirier, M. J., Rajopadhye, M., and Yu, K. Design and synthesis of radiolabeled GPIIb/IIIa receptor antagonists as potential thrombus imaging agents. *J. Nucl. Med.* 35, 245P (abstract 1005).
- (4) Jackson, S., DeGrado, W., Dwivedi, A., Parthasarathy, A., Higley, A., Krywko, J., Rockwell, A., Markwalder, J., Wells, G., Wexler, R., Mousa, S., and Harlow, R. (1994) Template-constrained cyclic peptides: design of high-affinity ligands for GPIIb/IIIa. *J. Am. Chem. Soc.* 116, 3220-3230.
- (5) Haskell, E., Adams, S., Feigen, L., Saffitz, J., Gorczyński, R., Sobel, D., and Abendschein, D. (1989) Prevention of reoccluding platelet-rich thrombi in canine femoral arteries with a novel peptide antagonist of platelet glycoprotein IIb/IIIa receptors. *Circulation* 80, 1775-1782.
- (6) Fritzberg, A. R., Berninger, R. W., Hadley, S. W., and Wester, D. W. (1988) Approaches to radiolabeling of antibodies for diagnosis and therapy of cancer. *Pharm. Res.* 5, 325-334.
- (7) Otsuka, F. L., and Welch, M. J. (1987) Methods to label monoclonal antibodies for use in tumor imaging. *Nucl. Med. Biol.* 14, 243-249.
- (8) Hnatowich, D. J. (1990) Antibody radiolabeling, problems and promises. *Nucl. Med. Biol.* 17, 49-55.
- (9) Griffiths, G. L., Goldenberg, D. M., Jones, A. L., and Hansen, H. J. (1992) Radiolabeling of monoclonal antibodies and fragments with technetium and rhenium. *Bioconjugate Chem.* 3, 91-99.
- (10) Eckelman, W. C., Paik, C. H., and Steigman, J. (1989) Three approaches to radiolabeling antibodies with ^{99m}Tc . *Nucl. Med. Biol.* 18, 589-603.
- (11) Fritzberg, A. R., Abrams, P. G., Beaumier, P. L., Kasina, S., Morgan, A. C., Rao, T. N., Reno, J. M., Sanderson, J. A.,

- Srinivasan, A., and Wilbur, D. S. (1988) Specific and stable labeling of antibodies with technetium-99m with a diamide dithiolate chelating agent. *Proc. Natl. Acad. Sci. U.S.A.* 85, 4025–4029.
- (12) Eary, J. F., Schroff, R. W., Abrams, P. G., Fritzberg, A. R., Morgan, A. C., Kasina, S., Reno, J. M., Srinivasan, A., Woodhouse, C. S., Wilbur, D. S., Natale, R. B., Collins, C., Stehlin, J. S., Mitchell, M., and Nelp, W. B. (1989) Successful imaging of malignant melanoma with technetium-99m-labeled monoclonal antibodies. *J. Nucl. Med.* 30, 25–32.
- (13) Kasina, S., Rao, T. N., Srinivasan, A., Sanderson, J. A., Fitzner, J. N., Reno, J. M., Beaumier, P. L., and Fritzberg, A. R. (1991) Development and biological evaluation of a kit for preformed chelate technetium-99m radiolabeling of an antibody Fab fragment using a diamide dimercaptide chelating agent. *J. Nucl. Med.* 32, 1445–1451.
- (14) Breitz, H. B., Weiden, P. L., Vanderheyden, J.-L., Appelbaum, J. W., Bjorn, M. J., Fer, M. F., Wolf, S. B., Ratliff, B. A., Seiler, C. A., Foisie, D. C., Fisher, D. R., Schroff, R. W., Fritzberg, A. R., and Abrams, P. G. (1992) Clinical experience with rhenium-186-labeled monoclonal antibodies for radioimmunotherapy: Results of phase I trials. *J. Nucl. Med.* 33, 1099–1112.
- (15) Schroff, R. W., Weiden, P. L., Appelbaum, J. W., Fer, M. F., Breitz, H. B., Vanderheyden, J.-L., Ratliff, B. A., Fisher, D. R., Foisie, D. C., Hanelin, L. G., Morgan, A. C., Jr., Fritzberg, A. R., and Abrams, P. G. (1990) Rhenium-186 labeled antibody in patients with cancer: Report of a pilot phase I study. *Antibody, Immunoconjugates, Radiopharm.* 3, 99–111.
- (16) Barrett, J. A., Damphousse, D. J., Heminway, S. J., Liu, S., Edwards, D. S., and Carroll, T. R. (1996) Biological evaluation of ^{99m}Tc -labeled cyclic GP-IIb/IIIa receptor antagonists in the canine arteriovenous shunt and deep vein thrombosis models: effects of chelators on biological properties of ^{99m}Tc -chelator-peptide conjugates. *Bioconjugate Chem.* 7, 203–208.
- (17) Fritzberg, A. R., Kasina, S., Eshima, D., and Johnson, D. L. (1986) Synthesis and biological evaluation of Tc-99m MAG_3 as a hippuran replacement. *J. Nucl. Med.* 27, 111–116.
- (18) Liu, S., and Edwards, D. S. (1994) New N_2S_2 diamidedithiol and N_3S triamidethiols as bifunctional chelating agents for labeling small peptides with ^{99m}Tc . *J. Nucl. Biol. Med.* 38, 451.
- (19) Rajopadhye, M., Harris, T. D., Edwards, D. S., and Liu, S. Unpublished results.
- (20) Ali, F. E., Bennett, D. B., Calvo, R. R., Elliott, J. D., Hwang, S. M., Ku, T. W., Lago, M. A., Nichols, A. J., Romoff, T. T., Shah, D. H., Vasko, J. A., Wong, A. S., Yellin, T. O., Yuan, C.-K., and Samanen, J. M. (1994) Conformationally constrained peptides and semipeptides derived from RGD as potent inhibitors of the platelet fibrinogen receptor and platelet aggregation. *J. Med. Chem.* 37, 769–780 (and references therein).
- (21) Cheng, S., Craig, W. S., Mullen, D., Tschopp, J. F., Dixon, D., and Pierschbacher, M. D. (1994) Design and synthesis of novel cyclic RGD-containing peptides as highly potent and selective integrin $\alpha_{\text{IIb}}\beta_3$ antagonists. *J. Med. Chem.* 37, 1–8 (and references therein).
- (22) Teng, W., Rose, J. W., Phillips, D. R., Nannizzi, L., Arsen, A., Campbell, A. M., and Charo, I. F. (1993) Design of potent and specific integrin antagonists. *J. Biol. Chem.* 268, 1066–1073.
- (23) Ku, T. W., Ali, F. E., Barton, L. S., Bean, J. W., Bondinell, W. E., Burgess, J. L., Callahan, J. F., Calvo, R. R., Chen, L.-C., Eggleston, D. S., Gleason, J. G., Huffman, W. F., Hwang, S. M., Jakas, D. R., Karash, C. B., Keenan, R. M., Kopple, K. D., Miller, W. H., Newlander, K. A., Nichols, A., Parker, M. F., Peishoff, C. E., Samanen, J. M., Uzinskas, I., and Ven-slavsky, J. W. (1993) Direct design of a potent non-peptide fibrinogen receptor antagonist based on the structure and conformation of a highly constrained cyclic RGD peptide. *J. Am. Chem. Soc.* 115, 8861–8862.
- (24) Zablocki, J. A., Miyano, M., Garland, R. B., Pireh, D., Schretzman, L., Rao, S. N., Lindmark, R. J., Panzer-Knodle, S. G., Nicholson, N. S., Taite, B. B., Salyers, A. K., King, L. W., Campion, J. G., and Feigen, L. P. (1993) Potent *in vitro* and *in vivo* inhibitors of platelet aggregation based upon the Arg-Gly-Asp-Phe sequence of fibrinogen. A proposal on the nature of the binding interaction between the Arg-guanidine of RGD mimetics and the platelet GP IIb/IIIa receptor. *J. Med. Chem.* 36, 1811–1919.
- (25) Lanteingne, D., and Hnatowich, D. J. (1984) The labeling of DTPA coupled proteins. *Int. J. Radiat. Isot.* 35, 617–621.
- (26) Linder, K. E., Wen, M. D., Nowotnik, D. P., Malley, M. F., Gougoutas, J. Z., Nunn, A. D., and Eckelman, W. C. (1991) Technetium labeling of monoclonal antibodies with functionalized BATOs. 1. $\text{TcCl}(\text{DMG})_3\text{PITC}$. *Bioconjugate Chem.* 2, 160–170.
- (27) Linder, K. E., Wen, M. D., Nowotnik, D. P., Ramalingam, K., Sharkey, R. M., Yost, F., Narra, R. K., Nunn, A. D., and Eckelman, W. C. (1991) Technetium labeling of monoclonal antibodies with functionalized BATOs: 2. $\text{TcCl}(\text{DMG})_3\text{CPITC}$ labeling of B72.3 and NP-4 whole antibodies and NP-4 F(ab)_2 . *Bioconjugate Chem.* 2, 407–414.
- (28) Baidoo, K. E., Scheffel, U., Finley, P., Lever, S. Z., and Wagner, H. N., Jr. (1994) No carrier added (NCA) Tc-99m-diaminedithiolate derivatives of chemotactic peptides for imaging inflammation. *J. Nucl. Med.* 35, 19P (abstract 67).
- (29) Abrams, M. J., Juweid, M., tenKate, C. I., Schwartz, D. A., Hauser, M. M., Gaul, F. E., Fuccello, A. J., Rubin, R. H., Strauss, H. W., and Fischman, A. J. (1990) Technetium-99m-human polyclonal IgG radiolabeled via the hydrazino nicotinamide derivative for imaging focal sites of infection in rats. *J. Nucl. Med.* 31, 2022–2028.
- (30) Babich, J. W., Solomon, H., Pike, M. C., Kroon, D., Graham, W., Abrams, M. J., Tompkins, R. G., Rubin, R. H., and Fischman, A. J. (1993) Technetium-99m-labeled hydrazino nicotinamide derivatized chemotactic peptide analogs for imaging focal sites of bacterial infection. *J. Nucl. Med.* 34, 1967–1974.
- (31) Davison, A., Jones, A. G., Orvig, C., and Sohn, M. (1981) A new class of oxotechnetium(5+) chelate complexes containing TcON_2S_2 core. *Inorg. Chem.* 20, 1629–1632.
- (32) Rao, T. N., Adhikesavalu, D., Camerman, A., and Fritzberg, A. R. (1990) Technetium(V) and rhenium(V) complexes of 2,3-bis(mercaptoacetamido)propanoate. Chelate ring stereochemistry and influence on chemical and biological properties. *J. Am. Chem. Soc.* 112, 5798–5804.
- (33) O'Neil, J. P., Wilson, S. R., and Katzenellenbogen, J. A. (1994) Preparation and structural characterization of monoamine-monoamide bis(thiol) oxo complexes of technetium(V) and rhenium(V). *Inorg. Chem.* 33, 319–323.
- (34) Bormans, G., Cleynhens, B., Adriaens, P., De Roo, M., and Verbruggen, A. (1993) Synthesis and labeling characteristics of ^{99m}Tc -mercaptoacetyltri-peptides. *J. Labelled Compd. Radiopharm.* 33, 1065–1078.
- (35) Grommon, G., Rajagopalan, R., Palenik, G. J., Koziol, A. E., and Mosco, D. L. (1995) Synthesis, characterization and crystal structures of technetium(V)-oxo complexes useful in nuclear medicine. 1. Complexes of mercaptoacetyl-glycylglycylglycine (MAG_3) and its methyl ester derivative ($\text{MAG}_3\text{-OMe}$). *Inorg. Chem.* 34, 1764–1772.
- (36) de Bree, R., Roos, J. C., Quak, J. J., den Hollander, W., van den Brekel, M. W. M., van der Wal, J. E., Tobi, H., Snow, G. B., and van Dongen, G. A. M. S. (1994) Clinical imaging of head and neck cancer with technetium-99m-labeled monoclonal antibody E48 IgG or F(ab)_2 . *J. Nucl. Med.* 35, 775–783.

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Biological Evaluation of ^{99m}Tc -Labeled Cyclic Glycoprotein IIb/IIIa Receptor Antagonists in the Canine Arteriovenous Shunt and Deep Vein Thrombosis Models: Effects of Chelators on Biological Properties of ^{99m}Tc Chelator–Peptide Conjugates

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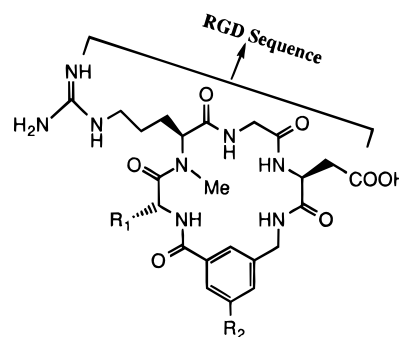
Radiopharmaceuticals Division, The Dupont Merck Pharmaceutical Company, 331 Treble Cove Road, North Billerica, Massachusetts 01862. Received September 18, 1995[⊗]

A series of ^{99m}Tc -labeled cyclic glycoprotein IIb/IIIa receptor antagonists, $^{99m}\text{TcO}(\text{L1–III})^-$, $^{99m}\text{TcO}(\text{L6–III})^-$, $^{99m}\text{TcO}(\text{L1–V})^-$, and $^{99m}\text{TcO}(\text{L6–V})^-$, were evaluated in a canine arteriovenous (AV) shunt model for their potential use as thrombus imaging agents. The thrombus formed consists of a platelet-rich head and a fibrin-rich tail. All four agents were incorporated into the growing thrombus under both arterial (platelet-rich) and venous (platelet-poor) conditions. The rank order for uptake was $^{99m}\text{TcO}(\text{L1–V})^- > ^{99m}\text{TcO}(\text{L6–V})^- > ^{99m}\text{TcO}(\text{L6–III})^- > ^{99m}\text{TcO}(\text{L1–III})^-$ (arterial range, 5.8–0.47 % id/g; venous range, 0.58–0.04 % id/g). The uptakes of both $^{99m}\text{TcO}(\text{L6–III})^-$ and $^{99m}\text{TcO}(\text{L1–III})^-$ under both arterial and venous conditions were not significantly greater than that of ^{99m}Tc -albumin and ^{125}I -fibrinogen. In contrast, the uptakes of both $^{99m}\text{TcO}(\text{L1–V})^-$ and $^{99m}\text{TcO}(\text{L6–V})^-$ were significantly greater than those of ^{99m}Tc -albumin and ^{125}I -fibrinogen and comparable to that of ^{111}In -platelets under both arterial and venous conditions. All four ^{99m}Tc -chelator–peptide conjugates are cleared faster than the controls with the clearance of the conjugates of peptide III faster than that of the conjugates of peptide V. The differences in incorporation are attributable to the effect of both the cyclic peptide and the chelator. The conjugate $^{99m}\text{TcO}(\text{L1–V})^-$ was also studied using a canine DVT (deep vein thrombosis) model. $^{99m}\text{TcO}(\text{L1–V})^-$ was actively incorporated into the growing thrombus with images clearly detectable within 15 min postinjection. At 2 h postinjection, thrombus/blood and thrombus/muscle ratios [region of interest (ROI)/background] were approximately 7/1 and 10/1, respectively. This clearly demonstrated that the conjugate $^{99m}\text{TcO}(\text{L1–V})^-$ has the potential for rapid diagnosis of thrombotic events occurring under both arterial and venous conditions.

INTRODUCTION

Venous and arterial thrombus formation are common and potentially life-threatening events. However, existing diagnostic modalities are inadequate for diagnosis and determination of the morphology of the evolving thrombus (1). Thus, the development of agents which will not only detect the location but also determine the age of the thrombi is a critical unmet need in diagnostic nuclear medicine.

Deep vein thrombus is the result of a hypercoagulable state coupled with a period of stasis occurring in a low-shear environment. The end result is the formation of a fibrin-rich thrombus which also contains some platelets and erythrocytes. In contrast, an arterial thrombus is the result of the rupture of an atherosclerotic plaque occurring under high-shear conditions, resulting in the formation of a platelet-rich thrombus (2). Our approach utilizes a series of radiolabeled platelet glycoprotein IIb/IIIa antagonists. The glycoprotein IIb/IIIa complex (GPIIb/IIIa) is expressed on the membrane surface of activated platelets and plays an integral role in platelet aggregation and thrombus formation (3). It has been demonstrated that the tripeptide sequence Arg-Gly-Asp (RGD) is capable of inhibiting the binding of fibrinogen to the platelet GPIIb/IIIa receptor (4). Cyclic peptides (Figure 1) containing the RGD (Arg-Gly-Asp) sequence



Peptide	R ₁	R ₂
I	Ethyl	H
II	i-Pr	H
III	(CH ₂) ₄ NH ₂	H
IV	Et	NHCO(CH ₂) ₅ NH ₂
V	i-Pr	NHCO(CH ₂) ₅ NH ₂

Figure 1. Cyclic GPIIb/IIIa receptor antagonists.

have been shown to be high-affinity antagonists for the GPIIb/IIIa receptor (3). Since the GPIIb/IIIa receptor is expressed only on activated platelets (5), the ^{99m}Tc -labeled cyclic GPIIb/IIIa receptor antagonists should only be bound to platelets intimately involved in the thromboembolic event. ^{99m}Tc is the preferred radionuclide for diagnostic nuclear medicine because of its ideal physical properties (e.g. 6 h half-life, 140 keV, γ -emission), easy availability, and low cost.

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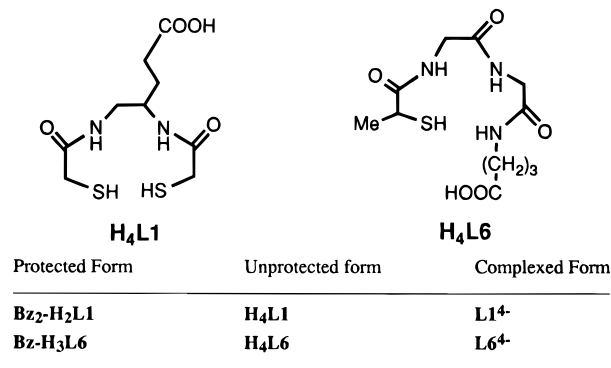


Figure 2. Two chelators used for ^{99m}Tc labeling of cyclic peptides.

In our previous contribution, we described the ^{99m}Tc labeling of several cyclic GPIIb/IIIa receptor antagonists by the preformed chelate approach using various chelators. As a continuation of that study, we evaluated four [^{99m}Tc]chelator-peptide conjugates, [^{99m}TcO(L1-III)]⁻, [^{99m}TcO(L6-III)]⁻, [^{99m}TcO(L1-V)]⁻, and [^{99m}TcO(L6-V)]⁻, in a canine arteriovenous (AV) shunt model (Figure 2). These agents were incorporated into the growing thrombus under both arterial and venous conditions; differences in incorporation are attributable to the effect of both the chelator and the cyclic peptide. We also evaluated the conjugate [^{99m}TcO(L1-V)]⁻ using a canine DVT (deep vein thrombosis) model. It was clearly demonstrated that the conjugate [^{99m}TcO(L1-V)]⁻ has the potential for rapid diagnosis of thrombotic events occurring under both arterial and venous conditions.

EXPERIMENTAL SECTION

Na^{99m}TcO₄ was obtained from a commercial DuPont ⁹⁹Mo/^{99m}Tc generator, North Billerica, MA. [^{99m}Tc]Albumin kits, ¹²⁵I-labeled canine fibrinogen, and [¹¹¹In]oxine kits were purchased from Medi-Physics Inc., Arlington Heights, IL, and were used as directed. Deionized water was obtained from a Millipore MilliQ Water System and was of >18 MΩ quality.

The cyclic GPIIb/IIIa receptor antagonists were labeled with ^{99m}Tc by the preformed chelate approach as described in our previous contribution (6). The products were purified by high-performance liquid chromatography (HPLC). The product peaks were collected, and the volatiles were removed under reduced pressure. The residue was redissolved in 0.9% saline solution and analyzed by radio-HPLC. The [^{99m}Tc]chelator-peptide conjugates were ≥95% pure. Doses for biological evaluation were prepared by dilution with 0.9% saline to the required concentration: 300 μCi/mL for AV shunt and 1.5 mCi/mL for DVT studies.

Canine Arteriovenous Shunt Methodology. Adult beagle dogs of either sex (9–13 kg) were anesthetized with pentobarbital sodium (35 mg/kg, iv) and ventilated with room air via an endotracheal tube (12 strokes/min, 25 mL/kg). For arterial pressure determination, the left carotid artery was cannulated with a saline-filled polyethylene catheter (PE-240) and connected to a Statham pressure transducer (model P23ID, Gould Co., Oxnard, CA). Mean arterial blood pressure was determined via damping the pulsatile pressure signal. Heart rate was monitored using a cardi tachometer (Grass Instrument Inc., Quincy, MA) triggered from a lead II electrocardiogram generated by limb leads. A jugular vein was cannulated (PE-240) for drug administration. Both femoral arteries and femoral veins were cannulated with silicon-treated (Sigmacote, Sigma Chemical Co., St.

Louis, MO), saline-filled polyethylene tubing (PE-200) and connected with a 5 cm section of silicon-treated tubing (PE-240) to form extra corporeal arteriovenous shunts. Shunt patency was monitored using a Doppler flow system (model VF-1, Crystal Biotech Inc., Hopkinton, MA) and a flow probe (2–3.5 mm, Crystal Biotech Inc.) placed proximal to the locus of the shunt. All parameters were monitored continuously on a model 7D polygraph recorder (Grass Instrument Inc., Quincy, MA) at a paper speed of 10 mm/min or 10 mm/s.

Upon completion of a 15 min postsurgical stabilization period, an occlusive thrombus was formed by the introduction of a thrombogenic surface (4-0 braided silk thread, 5 cm in length, Ethicon Inc., Somerville, NJ) into one shunt with the other serving as a control. A 1 h shunt period was employed with the test agent (150 μCi/kg, iv) administered as an infusion over 5 min, beginning 5 min before insertion of the first thrombogenic surface. The thrombus formed was comprised of a platelet-rich component on the thrombogenic surface and a fibrin-rich tail. At the end of the 1 h shunt period, the silk was carefully removed, and the portions were separated and weighed. The percent incorporation was determined via well counting. Thrombus weight was calculated by subtraction of the weight of the silk prior to placement from the total weight of the silk upon removal from the shunt. Arterial blood was withdrawn prior to infusion and every 30 min thereafter for determination of blood clearance, whole blood collagen-induced platelet aggregation, collagen prothrombin time, activated partial thromboplastin time, and fibrinogen and platelet count. Template bleeding time was also performed prior to infusion and every 30 min thereafter.

[¹¹¹In]Platelet Preparation. The isolation and ¹¹¹In labeling of platelets were performed according to the literature method (7) with some modification. Canine arterial blood (40 mL) was withdrawn in 2.5% ACD solution [2.5 g of trisodium citrate, 1.4 g of citric acid, and 2.0 g of dextrose in 100 mL of H₂O (pH 4.5)]. An additional 20 mL of blood was drawn in 3.8% sodium citrate solution which serves as a control. The samples were centrifuged at 1400 rpm for 15 min to form platelet-rich plasma (PRP) and platelet-poor plasma (PPP). The ACD-PRP was pelleted (2800 rpm for 15 min) and washed twice with ACD. The platelet pellet was resuspended in ACD (2 mL), and 60–100 μCi of [¹¹¹In]oxine was added. The suspension was incubated at 37 °C for 5 min, followed by the addition of PPP (5 mL). The suspension was centrifuged at 2800 rpm for 10 min. The supernatant and platelet pellet were counted to assess labeling efficiency (>80% in all cases). The platelet pellet was resuspended in PPP (5 mL) and platelet viability determined by comparison of collagen-induced platelet aggregation using nonlabeled and ¹¹¹In-labeled platelets. Only those ¹¹¹In-labeled platelet preparations which were within 25% of control were injected into the animal.

Canine Deep Vein Thrombosis Methodology. This model incorporates the triad of events (hypercoagulable state, period of stasis, and low-shear environment) essential for the formation of a venous fibrin-rich actively growing thrombus. Adult beagle dogs of either sex (9–13 kg) were anesthetized with pentobarbital sodium (35 mg/kg, iv) and ventilated with room air via an endotracheal tube (12 strokes/min, 25 mL/kg). For arterial pressure determination, the right femoral artery was cannulated with a saline-filled polyethylene catheter connected to a pressure transducer (model P23ID, Gould Co.). Heart rate was monitored using a cardi tachometer (Grass Instrument Inc.) triggered from a lead II electrocardiogram generated by limb leads. The right femoral vein was cannulated for drug administration. For the

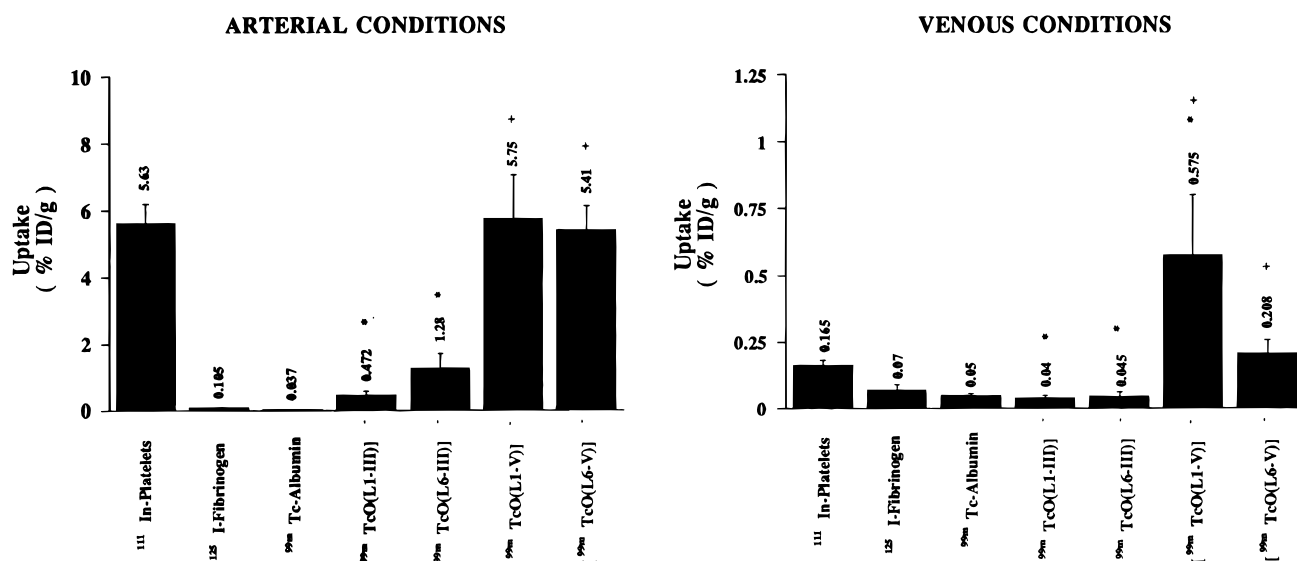


Figure 3. Thrombus uptakes for [¹²⁵I]fibrinogen, [¹¹¹In]platelets, [^{99m}Tc]albumin, [^{99m}TcO(L1–III)][–], [^{99m}TcO(L6–III)][–], [^{99m}TcO(L1–V)][–], and [^{99m}TcO(L6–V)][–] under both arterial (left) and venous (right) conditions. All values are expressed as the mean ± the standard error of the mean. *N* = 10 for platelets and fibrinogen (except for venous conditions where *N* = 5). *N* = 3 for [^{99m}TcO(L1–III)][–]. *N* = 2 for [^{99m}TcO(L6–III)][–], and *N* = 4 for [^{99m}Tc]albumin, [^{99m}TcO(L1–V)][–], and [^{99m}TcO(L6–V)][–]. An * indicates a significant difference between [¹¹¹In]platelets and treatment groups, while a + indicates a significant difference from [¹²⁵I]fibrinogen and [^{99m}Tc]albumin using the Newman–Keuls test, *P* ≤ 0.05.

Table 1. Summary of the Hematological and Hemodynamic Effects of [¹¹¹In]Platelets/[¹²⁵I]Fibrinogen, [^{99m}Tc]Albumin, [^{99m}TcO(L1–III)][–], [^{99m}TcO(L6–III)][–], [^{99m}TcO(L1–V)][–], and [^{99m}TcO(L6–V)][–], 150 μCi/kg, iv in the Canine AV Shunt

treatment	parameter ^a				
	heart rate (bpm)	mean arterial pressure (mmHg)	APTT (s)	platelet count (×10 ³)	aggregation (Ω)
[¹¹¹In]platelets/[¹²⁵I]fibrinogen					
control	135 ± 5	113 ± 8	17 ± 1	272 ± 17	27 ± 3
EOI	128 ± 5	113 ± 7	21 ± 2	264 ± 17	29 ± 2
1 h postinjection	124 ± 5	112 ± 7	17 ± 1	264 ± 19	29 ± 2
[^{99m}Tc]albumin					
control	150 ± 6	124 ± 8	15 ± 0	281 ± 8	24 ± 1
EOI	150 ± 5	124 ± 8	15 ± 1	289 ± 11	27 ± 3
1 h postinjection	147 ± 7	124 ± 10	15 ± 1	284 ± 7	23 ± 2
[^{99m}TcO(L1–III)][–]					
control	170 ± 10	134 ± 12	13 ± 0	302 ± 9	31 ± 7
EOI	164 ± 9	137 ± 10	13 ± 0	312 ± 10	32 ± 6
1 h postinjection	159 ± 10	139 ± 5	13 ± 0	323 ± 8	32 ± 5
[^{99m}TcO(L6–III)][–]					
control	145 ± 17	112 ± 14	14 ± 1	242 ± 37	20 ± 2
EOI	151 ± 13	122 ± 14	14 ± 1	238 ± 27	20 ± 8
1 h postinjection	138 ± 1	115 ± 5	14 ± 0	250 ± 44	14 ± 9
[^{99m}TcO(L1–V)][–]					
control	166 ± 11	130 ± 9	15 ± 1	276 ± 31	26 ± 4
EOI	160 ± 12	132 ± 12	15 ± 1	259 ± 34	22 ± 5
1 h postinjection	150 ± 9	126 ± 13	15 ± 1	282 ± 33	28 ± 5
[^{99m}TcO(L6–V)][–]					
control	150 ± 14	116 ± 11	14 ± 0	263 ± 13	25 ± 4
EOI	142 ± 8	115 ± 6	14 ± 1	239 ± 24	21 ± 5
1 h postinjection	133 ± 7	115 ± 5	15 ± 1	263 ± 20	21 ± 0

^a All values are expressed as the mean ± the standard error of the mean. EOI = end of infusion. *N* = 10 for [¹¹¹In]platelets/[¹²⁵I]fibrinogen. *N* = 3 for [^{99m}TcO(L1–III)][–]. *N* = 2 for [^{99m}TcO(L6–III)][–], and *N* = 4 for [^{99m}Tc]albumin, [^{99m}TcO(L1–V)][–], and [^{99m}TcO(L6–V)][–].

induction of a venous thrombus, a 5 cm segment of both jugular veins was isolated and circumscribed with silk suture. A balloon catheter (3–4 F Baxter Co., McGraw Park, IL) was advanced from the facial vein into the jugular vein. A microthermister probe (Physitemp Co., Clifton, NJ) was placed on the vessel which serves as an indirect measure of venous flow. A period of stasis and hypercoagulability was induced by inflating the balloon and the local administration of 5 u of thrombin (American Diagnostica, Greenwich, CT) into the occluded segment. After an additional 15 min, the balloon was deflated and flow reestablished as verified by the microthermister probe.

The test agent (10 mCi/kg, iv) was administered over 5 min beginning at reflow. Serial images were acquired using a γ-camera (Digital Dyna Camera, Picker International, Cleveland, OH) every 5 min for 2 h and region of interest (ROI) and target/background ratios calculated. Arterial blood was withdrawn prior to administration and every 30 min thereafter for determination of blood clearance, hematology, platelet function, and coagulation status. At the end of the protocol, the animal was euthanized with an overdose of pentobarbital and the vessel excised. The thrombus was removed and weighed, and the amount of incorporation was determined via a γ-well counter (LKB 1282, Wallac Inc., Gaithersburg, MD).

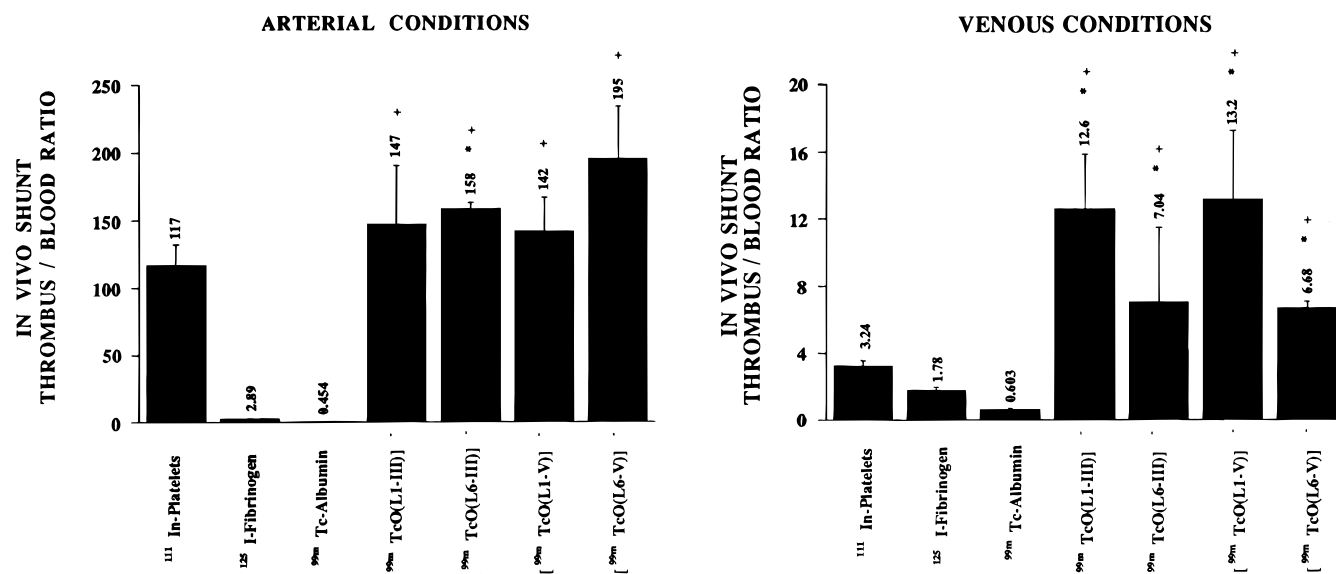


Figure 4. Thrombus/blood ratios for [^{125}I]fibrinogen, [^{111}In]platelets, [$^{99\text{m}}\text{Tc}$]albumin, [$^{99\text{m}}\text{TcO}(\text{L1-III})^-$], [$^{99\text{m}}\text{TcO}(\text{L6-III})^-$], [$^{99\text{m}}\text{TcO}(\text{L1-V})^-$], and [$^{99\text{m}}\text{TcO}(\text{L6-V})^-$] under both arterial (left) and venous (right) conditions. All values are expressed as the mean $\pm$ the standard error of the mean. $N = 10$ for platelets and fibrinogen (except for venous conditions where $N = 5$). $N = 3$ for [$^{99\text{m}}\text{TcO}(\text{L1-III})^-$], $N = 2$ for [$^{99\text{m}}\text{TcO}(\text{L6-III})^-$], and $N = 4$ for [$^{99\text{m}}\text{Tc}$]albumin, [$^{99\text{m}}\text{TcO}(\text{L1-V})^-$], and [$^{99\text{m}}\text{TcO}(\text{L6-V})^-$]. An * indicates a significant difference between [^{111}In]platelets and treatment groups, while a + indicates a significant difference from [^{125}I]fibrinogen and [$^{99\text{m}}\text{Tc}$]albumin using the Newman-Keuls test, $P \leq 0.05$.

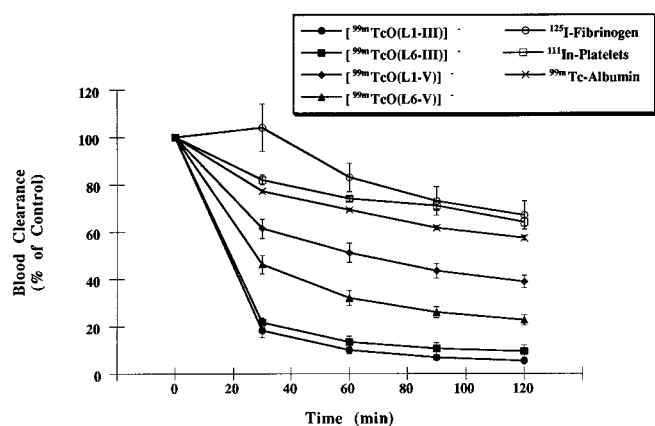


Figure 5. Blood clearance curves for [^{125}I]fibrinogen, [^{111}In]platelets, [$^{99\text{m}}\text{Tc}$]albumin, [$^{99\text{m}}\text{TcO}(\text{L1-III})^-$], [$^{99\text{m}}\text{TcO}(\text{L6-III})^-$], [$^{99\text{m}}\text{TcO}(\text{L1-V})^-$], and [$^{99\text{m}}\text{TcO}(\text{L6-V})^-$]. All values are expressed as the mean $\pm$ the standard error of the mean. $N = 10$ for platelets and fibrinogen (except for venous conditions where $N = 5$). $N = 3$ for [$^{99\text{m}}\text{TcO}(\text{L1-III})^-$], $N = 2$ for [$^{99\text{m}}\text{TcO}(\text{L6-III})^-$], and $N = 4$ for [$^{99\text{m}}\text{Tc}$]albumin, [$^{99\text{m}}\text{TcO}(\text{L1-V})^-$], and [$^{99\text{m}}\text{TcO}(\text{L6-V})^-$].

Hematologic Studies. Platelet, white blood cell (WBC), and red blood cell (RBC) counts and hematocrit determinations were performed on whole blood collected in 2 mg/mL disodium ethylenediaminetetraacetic acid (EDTA) using a Sysmex K1000 (TEA Medical Electronics Co., Los Alamitos, CA). Template bleeding time was assessed via an incision in the lower lip (Surgicutt, Baxter Co.) and the time to formation of a clot monitored. Whole blood platelet aggregation was measured using a lumiaggregometer (Chrono-Log Co., Havertown, PA) by recording the change in impedance (platelet aggregation). Blood samples were collected in 10 mM sodium citrate and diluted 50% with saline supplemented with 0.5 mM Ca. Aggregation was induced with collagen (5 $\mu\text{g}/\text{mL}$, Chrono-Log Co.), and the changes in impedance were recorded over 6 min. Activated partial thromboplastin time (APTT) and prothrombin time (PT) were monitored using a microsample coagulation analyzer (MCA-210, BIO/DATA Co., Horsham, PA).

Data Analysis. All values are expressed as the mean $\pm$ the standard error of the mean. In the DVT studies, the target (thrombus) uptake was calculated by drawing a 4×4 pixel area in the region interest and determining the average intensity. Background values were determined in a similar manner. Blood background was calculated using the jugular vein just distal to the locus of the thrombus. Statistical analysis consisted of a one-way analysis of variance and, when appropriate, a Student's paired t test, a two-tailed probability for assessing differences within treatment, and a Newman-Keuls test for assessing differences between means of treatment groups. Differences were considered significant at $P \leq 0.05$.

RESULTS AND DISCUSSION

In this study, we used a canine AV shunt model to evaluate several $^{99\text{m}}\text{Tc}$ -labeled cyclic GPIIb/IIIa receptor antagonists for their potential use in the detection of rapidly growing arterial and venous thrombi. The thrombus formed in this model was comprised of a platelet-rich head (arterial conditions) and a fibrin-rich tail (venous conditions) which allowed the rapid assessment of an agent under both arterial and venous conditions. To validate this model, the amount of incorporation of [^{111}In]platelets (58 μCi), [^{125}I]fibrinogen (106 μCi), and [$^{99\text{m}}\text{Tc}$]albumin (153 μCi) was assessed via well counting. [^{111}In]Platelets and [^{125}I]fibrinogen were adequately incorporated into the growing thrombus, with [^{111}In]platelets favoring the platelet-rich head (5.63 ± 0.6 % id/g) with lesser amounts of incorporation observed in the fibrin-rich tail (0.17 ± 0.02 % id/g). Similar amounts of [^{125}I]fibrinogen were incorporated under both arterial and venous conditions (0.11 ± 0.02 vs 0.07 ± 0.02 % id/g, respectively), while little incorporation was observed with [$^{99\text{m}}\text{Tc}$]albumin (arterial, 0.05 ± 0.01 % id/g; venous, 0.04 ± 0.001 % id/g) (Figure 3). The administration of [^{111}In]platelets, [^{125}I]fibrinogen, and [$^{99\text{m}}\text{Tc}$]albumin did not alter any of the parameters studied (Table 1). Thus, the deposition on the thrombogenic surface mimics that of arterial conditions, i.e. high-shear and platelet-rich thrombus, and the platelet-poor tail mimics that of a venous thrombus, i.e. platelet-poor and low-shear environment.

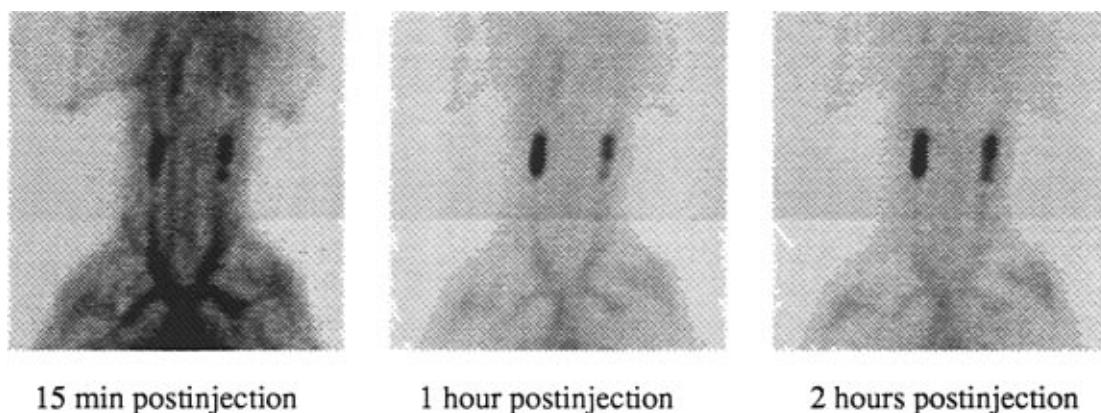


Figure 6. Illustrated is a typical selection of images derived from studies of [$^{99m}\text{TcO}(\text{L6-V})$] $^-$, 1 mCi/kg, iv, in the canine DVT model. Each image represents a 5 min acquisition beginning at the time listed postinjection.

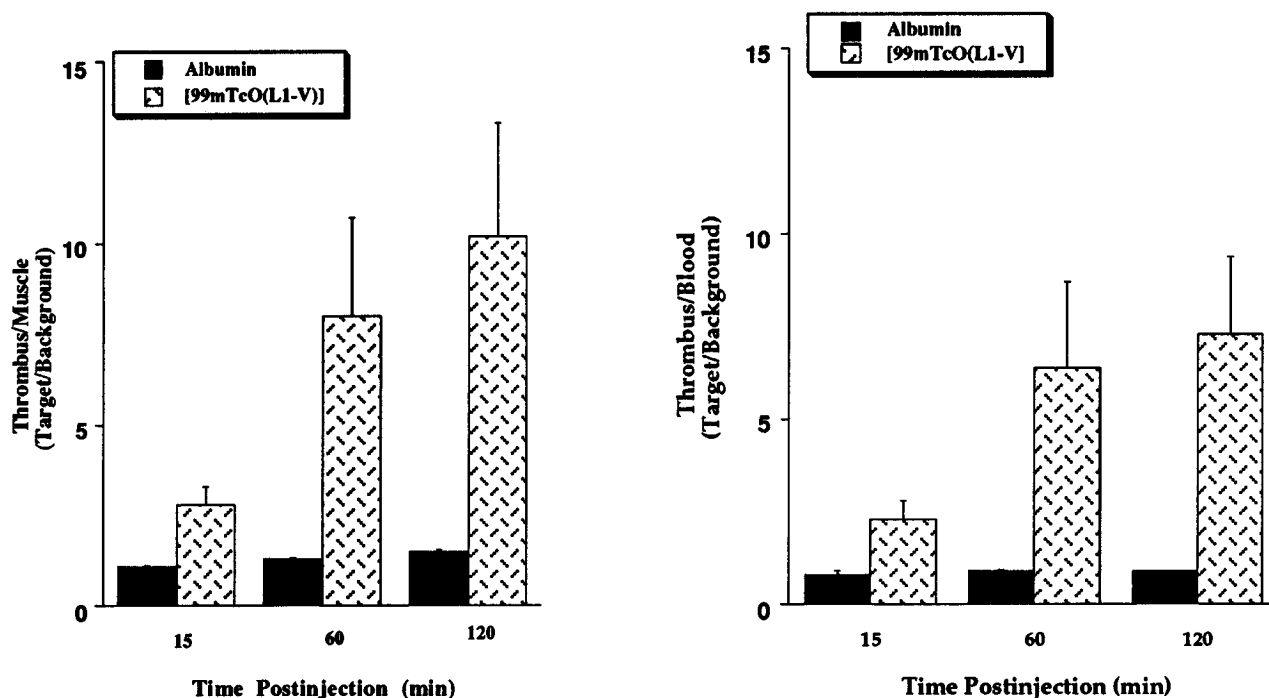


Figure 7. Assessment of [$^{99m}\text{TcO}(\text{L6-V})$] $^-$ and [^{99m}Tc]albumin, 1 mCi/kg, iv, in the canine DVT model. Illustrated in the left panel is the thrombus/muscle ratio, while in the right panel is the thrombus/blood ratio calculated from the image. Each histogram is the mean $\pm$ the standard error of the mean for 4–6 dogs.

Four [^{99m}Tc]chelator–peptide conjugates were assessed in the canine femoral arteriovenous shunt model, and all of them were incorporated into the growing thrombus under both arterial and venous conditions (Figure 3). Greater thrombus uptake occurred under arterial conditions, reflecting a more platelet-rich thrombus. The rank order for uptake was [$^{99m}\text{TcO}(\text{L1-V})$] $^-$ > [$^{99m}\text{TcO}(\text{L6-V})$] $^-$ > [$^{99m}\text{TcO}(\text{L6-III})$] $^-$ > [$^{99m}\text{TcO}(\text{L1-III})$] $^-$ (arterial range, 5.8–0.47 % id/g; venous range, 0.58–0.04 % id/g). Under arterial conditions, the uptake of neither [$^{99m}\text{TcO}(\text{L6-III})$] $^-$ nor [$^{99m}\text{TcO}(\text{L1-III})$] $^-$ was significantly higher than that of [^{99m}Tc]albumin and [^{125}I]fibrinogen. In contrast, the uptakes of both [$^{99m}\text{TcO}(\text{L1-V})$] $^-$ and [$^{99m}\text{TcO}(\text{L6-V})$] $^-$ were significantly greater than the negative controls and comparable to the positive control, [^{111}In]platelets, under both arterial and venous conditions. This is probably related to the stronger affinity of the cyclic peptide V for GPIIb/IIIa than that of the cyclic peptide III (8) and the slower rate of clearance of cyclic peptide V from the blood, resulting in a greater receptor residence time (Figure 5).

In spite of the differences in thrombus uptake, all four [^{99m}Tc]chelator–peptide conjugates show comparable thrombus/blood ratios at 1 h after administration under both arterial and venous conditions (Figure 4). The conjugates with the highest uptake, [$^{99m}\text{TcO}(\text{L1-V})$] $^-$ and [$^{99m}\text{TcO}(\text{L6-V})$] $^-$, have the slower blood clearance rate, while the conjugates with the lower uptake, [$^{99m}\text{TcO}(\text{L6-III})$] $^-$ and [$^{99m}\text{TcO}(\text{L1-III})$] $^-$, have the faster clearance rates. Figure 5 shows the blood clearance curves for the four [^{99m}Tc]chelator–peptide conjugates and the three controls. The rank order for blood clearance was [$^{99m}\text{TcO}(\text{L1-III})$] $^-$ > [$^{99m}\text{TcO}(\text{L6-III})$] $^-$ > [$^{99m}\text{TcO}(\text{L6-V})$] $^-$ > [$^{99m}\text{TcO}(\text{L1-V})$] $^-$. All the [^{99m}Tc]chelator–peptide conjugates are cleared faster than the controls with the clearance of the conjugates of peptide III increasingly faster than the conjugates of peptide V. It is quite clear that both the peptide and the chelator affect the blood clearance of the [^{99m}Tc]chelator–peptide conjugates. The four [^{99m}Tc]chelator–peptide conjugates evaluated did not adversely affect any of the parameters studied (Table 1). Thus, the ^{99m}Tc -labeled GPIIb/IIIa receptor antagonists reported in this study have the ability to be rapidly

incorporated into growing thrombi in vivo under both arterial and venous conditions.

The ability of $[^{99m}\text{TcO}(\text{L1-V})]^-$ (1 mCi/kg, iv) to be actively incorporated into a growing venous thrombus was also studied using a canine DVT reflow model. $[^{99m}\text{TcO}(\text{L1-V})]^-$ was administered at reflow with its rate of uptake and contrast ratio assessed using γ -scintigraphy. $[^{99m}\text{Tc}]$ Albumin was not incorporated into the rapidly growing thrombus. $[^{99m}\text{TcO}(\text{L1-V})]^-$ was actively incorporated into the growing thrombus with images clearly detectable within 15 min postinjection (Figure 6). At 2 h postinjection, thrombus/blood and thrombus/muscle ratios (ROI/background) were approximately 7/1 and 10/1, respectively (Figures 6 and 7). The present study is in agreement with the work of Oster et al. (9), who demonstrated that the 7E3 antibody, directed against the platelet GPIIb/IIIa receptor, was also capable of detecting new thrombi in the carotid, femoral, and pulmonary arteries induced by the insertion of a copper coil. The target/background values ranged between 2/1 and 5/1 at 1.5 h postadministration. More recently, a venom derived from the viper *Bitis arietans* was shown to be effective in detection of venous thrombi. This venom, bitistatin, contains an RGD peptide recognition sequence which has been found to be essential for the binding to the platelet GPIIb/IIIa receptor (10). A ^{99m}Tc -labeled dimer of a cyclic peptide (P280) has also been shown to detect venous thrombi. However, P280 was rapidly cleared ($<2\%$ dose in blood at 4 h) with rather low uptake in the thrombus (0.0068 % id/g) (11). In contrast, $[^{99m}\text{TcO}(\text{L1-V})]^-$ demonstrated a slower rate of clearance from the blood ($39 \pm 3\%$ dose at 2 h) coupled with a much greater uptake in both the AV shunt and DVT models reported here (0.58 ± 0.22 and 1.61 ± 0.2 % id/g, respectively).

In summary, the ^{99m}Tc -labeled RGD-containing cyclic peptides have the ability to detect rapidly growing thrombi under both arterial and venous conditions at concentrations which do not affect hemodynamics, hematology, the coagulation cascade, and platelet function. In addition, the conjugate $[^{99m}\text{TcO}(\text{L1-V})]^-$ was able to detect a rapidly growing venous thrombus within 15 min postinjection. Thus, agents targeted at the platelet GPIIb/IIIa receptor possess the potential for the rapid diagnosis of thromboembolic events occurring under both arterial and venous conditions. Further, this study demonstrates that the addition of ^{99m}Tc to small molecules, such as cyclic RGD peptides, does not alter the agent's ability to interact with its target. Thus, the use

of the appropriate combination of chelator, linker, and peptide results in ^{99m}Tc -labeled GPIIb/IIIa receptor antagonists with good biological activity.

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LITERATURE CITED

- (1) Knight, L. C. (1990) Radiopharmaceuticals for thrombus detection. *Semin. Nucl. Med.* 20, 52–67.
- (2) Fuster, V., Stein, B., Badimon, L., and Chesebro, J. (1988) Antithrombotic therapy after myocardial reperfusion in acute myocardial infarction. *J. Am. Coll. Cardiol.* 12, 78A–84A.
- (3) Plow, E. F., Marguerie, G., and Ginsberg, M. (1987) Fibrinogen, fibrinogen receptors and the peptides that inhibit these interactions. *Biochem. Pharmacol.* 36, 4035–4041.
- (4) Shattil, S. J., Hoxie, J. A., Cunningham, M., and Brass, L. F. (1985) Changes in the platelet membrane glycoprotein I Ib/IIIa complex during platelet activation. *J. Biol. Chem.* 260, 11107–11110.
- (5) Haskel, E., Adams, S., Feigen, L., Saffitz, J., Gorczynski, R., Sobel, D., and Abendschein, D. (1989) Prevention of reoccluding platelet-rich thrombi in canine femoral arteries with a novel peptide antagonist of platelet glycoprotein I Ib/IIIa receptors. *Circulation* 80, 1775–1782.
- (6) Liu, S., Edwards, D. S., Looby, R. J., Poirier, M. J., Rajopadhye, M., Bourque, J. P., and Carroll, T. R. (1996) Labeling cyclic glycoprotein I Ib/IIIa receptor antagonists with ^{99m}Tc by the preformed chelate approach: effects of chelators on properties of $[^{99m}\text{Tc}]$ chelator–peptide conjugates. *Bioconjugate Chem.* 7, 196–202.
- (7) Thakur, M. L., Welch, M. J., Joist, J. H., and Coleman, R. E. (1976) In-111 labeled platelets: studies on preparation and evaluation of in vitro and in vivo function. *Thromb. Res.* 9, 345–357.
- (8) Rajopadhye, M., Harris, T. D., Edwards, D. S., and Liu, S. Unpublished results.
- (9) Oster, Z., Srivastava, S., Som, P., Meinken, G., Scudder, L., Yamamoto, K., Atkins, H., Brill, A., and Coller, B. (1985) Thrombus radioimmunosintigraphy: an approach using monoclonal antiplatelet antibody. *Proc. Natl. Acad. Sci. U.S.A.* 82, 3465–3468.
- (10) Knight, L. C., Maurer, A. H., Romano, J., and Buczala, S. (1993) Preliminary evaluation of labeled disintegrins (snake venom peptides) for thrombus imaging. *J. Nucl. Med.* 34, 66P (abstract 258).
- (11) Knight, L. C., Lister-James, J., Dean, R. T., and Maurer, A. H. (1993) Evaluation of Tc-^{99m} labeled cyclic peptides for thrombus imaging. *J. Nucl. Med.* 34, 17P (abstract 60).

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Hypersensitization of Multidrug Resistant Human Ovarian Carcinoma Cells by Pluronic P85 Block Copolymer

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The chemosensitizing effects of Pluronic P85 block copolymer were studied using two human ovarian carcinoma sublines: the glycoprotein P (P-gp) multidrug resistant (MDR) SKVLB cells and non-MDR SKOV3 cells. The dramatic increase (up to 700 times) in the daunorubicin cytotoxic activity was observed in the presence of 0.01% (22 μ M) to 1% (2.2 mM) copolymer in the case of SKVLB cells. By contrast, the copolymer induced a less than 3-fold increase in the drug activity in SKOV3 cells. As a result, the MDR subline demonstrated much higher response ("hypersensitivity") to the daunorubicin/Pluronic compared to that of the non-MDR cells. The copolymer increased the cytotoxic effects of other MDR type drugs (doxorubicin, epirubicin, vinblastine, and mitomycin C) by a factor of 20–1000 and non-MDR type drugs (methotrexate and cisplatin) by a factor of 2–5.5. The daunorubicin influx in the cytoplasm and nuclei of SKVLB cells was also increased in the presence of the copolymer, while in SKOV3 cells, it remained practically unchanged. However, the hypersensitization of the MDR cells by the copolymer could not be merely explained by the P-gp modulation. Therefore, the possible role of the copolymer in inhibition of non-P-gp drug resistance is hypothesized, which may also explain the sensitization of MDR cells with respect to non-MDR type drugs as well as sensitization of parental cells. The concentration dependence of the IC₅₀ in MDR cells indicates that just the copolymer unimers are responsible for the hypersensitization effect. The results obtained suggest that Pluronic P85 can be used as a delivery system to enhance the activity of antineoplastic agents against MDR tumors.

Multidrug resistance (MDR) is often found in many types of human tumors that have relapsed after an initial favorable response to drug therapy (1, 2). The sensitivity of the MDR tumor cells to antineoplastic agents can decrease significantly (2), which hinders the efficacy of these drugs in tumor therapy. One mechanism for the appearance of MDR is a positive selection of cells with an amplified family of closely related genes encoding glycoprotein P (P-gp), a 170 kDa transmembrane protein that functions as an ATP-dependent drug efflux pump (3–7). In certain cases, up to 100-fold overexpression of P-gp in MDR cells has been observed (8). An increased expression of this protein leads to a lowered net accumulation of the drug in the cell and consequently to a decrease in drug effect toward MDR cells.

One current approach used to overcome MDR in cancer cells is the administration of antineoplastic agents concurrent with agents that can modulate P-gp. Among the known MDR modulatory agents are P-gp inhibitors such as verapamil and SDZ PSC-833 that specifically bind with P-gp and inhibit its function (9). Another class of MDR inhibitors that has recently attracted attention is natural and synthetic surfactants. In particular, the ability of certain liposome formulations to decrease *in vitro* the resistance of MDR cells with respect to liposome-bound drugs has recently been reported (9–15). The nonionic polyethoxylated surfactants, such as Cremophor,

Tween 80, and Solutol HS15, have also been used to modulate MDR (16–18). These reports suggest that various surfactants such as lipids and nonionic detergents have the ability to modulate MDR in cancer cells.

We have previously proposed an approach to enhance the efficacy of certain types of drugs by using micelles of the polymeric surfactants, poly(oxyethylene-*b*-oxypropylene-*b*-oxyethylene) block copolymers (also known as "Pluronic copolymers"), as microcontainers for drug delivery (19). Pluronic micelles have first been used *in vivo* for the targeted delivery of a neuroleptic across the blood brain barrier to the brain (19, 20). Furthermore, Pluronic copolymers could reverse MDR in carcinoma cells *in vitro* (21–24) and were shown to substantially increase the activity of anthracycline antibiotics against drug sensitive and resistant tumors *in vivo* (24, 25). This paper studies the effects of Pluronic P85 block copolymer on the activity of antineoplastic drugs in MDR and sensitive human ovarian carcinoma cells (SKVLB and SKOV3, respectively). The data on the copolymer effect on daunorubicin cytotoxicity, drug-induced DNA damages, intracellular transport, and distribution in SKOV3 and SKVLB cell lines are presented. The copolymer effects on the cytotoxic activity of other MDR type drugs (doxorubicin, epirubicin, vinblastine, and mitomycin C) and non-MDR type drugs (methotrexate and cisplatin) are also explored.

MATERIALS AND METHODS

Cells. Human ovarian carcinoma cell lines (SKVO3 and SKVLB) were cultivated in RPMI-1640 medium supplemented with 10% fetal bovine serum at 37 °C and 5% CO₂. SKVLB cells were obtained from SKVO3 cells by long-time cultivation in the presence of vinblastine (26). To maintain the resistance of SKVLB cells, during each fourth passage, these cells were cultivated in the presence of 400 ng/mL daunorubicin.

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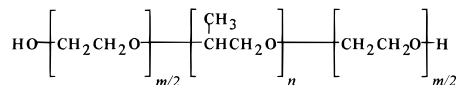
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Anticancer Drugs. Daunorubicin, doxorubicin, vinblastine, mitomycin C, methotrexate, and cisplatin were purchased from Sigma (St. Louis, MO). Epirubicin (fapmarubicin) was purchased from Farmitalia C. Erba (Milan, Italy).

Preparation of the Micellar Form of the Drugs. Pluronic P85 [poly(oxyethylene-*b*-oxypropylene-*b*-oxyethylene) block copolymer] characterized by the general structure formula



was purchased from Serva (FRG) and used without further purification. The copolymer was dissolved at various concentrations (0.01–5%) in RPMI-1640 at 4 °C, and the solutions obtained were sterilized by filtration through a 0.2 μm filter. The drugs were then diluted in these solutions (3 nM to 100 μM) and incubated for 30 min at 37 °C prior to cell experiments.

Characteristics of Pluronic P85 Micelles and Drug Partitioning. The cmc ,¹ the micelle hydrodynamic radius, and the surfactant aggregation number in the absence and presence of daunorubicin were determined using surface tension measurements, quasi-elastic light scattering, and ultracentrifugation as previously described in ref 27. The partitioning of daunorubicin in the Pluronic P85 micelles was studied by fluorescence spectroscopy as previously described (27). Briefly, the daunorubicin was dissolved in Pluronic solutions of various concentrations, and the drug fluorescence spectra ($\lambda_{\text{ex}} = 471 \text{ nm}$) were recorded using a Hitachi F4000 spectrofluorimeter at 20 °C. The fluorescence intensity data at various Pluronic concentrations were used to determine the drug partitioning coefficients (27).

Cytotoxicity Test. The cells were cultured in drug-free medium for a minimum of four passages prior to experimental use. They were subsequently harvested by trypsinization, suspended in fresh medium, plated at 2000–3000 cells/well in 96-well plates (Costar, Cambridge, MA), and cultured over 2 days to allow reattachment. The medium was then replaced with fresh medium (100 μL per well). Drug solutions with or without the copolymer were added to the cells (100 μL per well). Cells were incubated with drugs for various time intervals at 37 °C and 5% CO_2 , then washed three times with fresh medium, and cultured for 4 days. After 4 days, the drug cytotoxic activity was evaluated using the XTT assay (28). Briefly, 50 mL/well of sterile 1 mg/mL XTT (Sigma, St. Louis, MO) in RPMI-1640 containing 5 μL /mL of 1.54 mg/mL phenazine metasulfate (Sigma, St. Louis, MO) in PBS was added to the cells and incubated with the cells for 16 h. The absorbance at 450 nm was determined on a microplate reader. All experimental points were carried out in triplicate. Values shown are mean. The standard error of the mean did not exceed 10% of the mean values ($p < 0.05$).

Drug-Induced DNA Strand Breaks. The DNA damage in drug-treated cells was determined using a direct fluorescence assay (29) modified as reported in ref 30. Briefly, control and drug-treated cells were distributed in three sets of samples each containing four tubes,

each set designated as T, P, and B. Cells in all tubes were lysed with 4.5 M urea. DNA in P tubes was submitted to alkali treatment (29) for 30 min at 0 °C and then 60 min at 15 °C. DNA in B tubes was submitted to alkali treatment for 30 min at 20 °C. DNA in T tubes was not exposed to alkali. After neutralization of the solution in tubes P and B, ethidium bromide was added to each tube and the relative fluorescence values of the samples ($\lambda_{\text{ex}} = 520 \text{ nm}$, $\lambda_{\text{em}} = 590 \text{ nm}$) were determined. The percentage of the double-stranded DNA after alkali treatment (D) was determined as

$$D = (P - B)/(T - B) \times 100\% \quad (1)$$

where P , B , and T represent fluorescence measured in P, B, and T samples, respectively.

Daunorubicin Transport and Distribution in the Cells. The transport and subcellular distribution of free daunorubicin and daunorubicin in the copolymer solution were studied by fluorescent spectroscopy. The cells were cultured in 50 mL flasks until the formation of monolayers. The medium was then replaced with fresh medium, and 10 $\mu\text{g/mL}$ daunorubicin or 10 $\mu\text{g/mL}$ daunorubicin in 1% Pluronic P85 solution was added at 37 °C for various time intervals of 5–120 min. The cells were then transferred to 4 °C, washed three times with cold PBS, and detached by scraper. Daunorubicin accumulation in the cells was assayed by measuring cell fluorescence at $\lambda_{\text{ex}} = 471 \text{ nm}$ and $\lambda_{\text{em}} = 556 \text{ nm}$, corresponding to the maxima in daunorubicin excitation and emission spectra. Under these conditions, the cell fluorescence was mainly due to the portion of the drug localized in the cytoplasm and plasma membrane, while the fluorescence of the nuclear portion of the drug was quenched (31). To analyze daunorubicin binding to the DNA, the cells were treated with 0.1% SDS for 30 min at room temperature. The treatment led to an increase in the fluorescence by a factor of approximately 2–10, which was due to the destruction of the daunorubicin–DNA complex [the model experiment on the complexation of daunorubicin with DNA from salmon sperm demonstrated 95% quenching of the fluorescence in the complex, while after destruction of the complex with 0.1% SDS, fluorescence was restored to the level observed in the absence of DNA; in the absence of DNA, SDS only marginally (by a factor 1.1) increased the fluorescence]. The portions of the drug localized in the cytoplasm and plasma membrane and drug bound to the nuclear DNA were determined from the fluorescence data using the following equations:

$$I_c = I_1 - \gamma I_n \quad \text{and} \quad I_n = \epsilon I_2 - I_c \quad (2)$$

where I_1 and I_2 are the fluorescence intensities measured in the daunorubicin-loaded cells before and after SDS treatment, respectively, I_c and I_n are the portions of the drug fluorescence corresponding to the drug in the cytoplasm and plasma membrane (I_c) and DNA (I_n), γ is the coefficient accounting for the daunorubicin quenching in the DNA ($=0.05$), and ϵ is the coefficient accounting for the change of the daunorubicin quantum yield in the 0.1% SDS solution ($=0.9$).

Efflux Studies. Cell monolayers were incubated for 1 h at 37 °C with 10 $\mu\text{g/mL}$ daunorubicin or 10 $\mu\text{g/mL}$ daunorubicin in 1% Pluronic P85 solution to obtain substantial levels of drug accumulation in the cell. The cells were then transferred to 4 °C to inhibit P-gp-mediated efflux in SKVLB cells, washed three times with cold PBS, and incubated again at 37 °C for various time intervals in drug-free medium. After the incubation, the

¹ Abbreviations: BSA, bovine serum albumin; cmc , critical micelle concentration; HLB, hydrophilic–lipophilic balance; SDS, sodium dodecylsulfate; PBS, phosphate-buffered saline; XTT, 2,3-bis(2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium 5-carboxanilide inner salt.

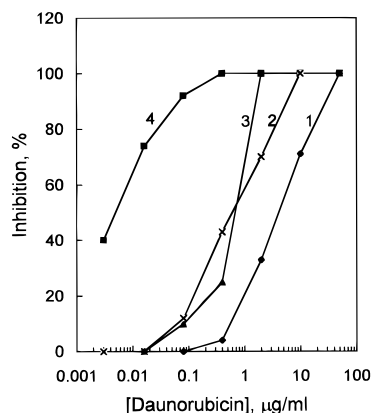


Figure 1. Cytotoxicity of (1 and 2) free daunorubicin and (3 and 4) daunorubicin/Pluronic with respect to (1 and 4) SKVLB and (2 and 3) SKOV3 cells. Cells were incubated with the drugs for 120 min. Pluronic P85 concentration equals 1%. Daunorubicin concentration is varied.

cells were again transferred to 4 °C, washed three times with cold PBS, and detached by scraper. The amount of drug in cytoplasm and plasma membrane and DNA was determined as described above.

RESULTS

Cytotoxicity of Pluronic Copolymers. At concentrations less than 5% and incubation periods less than 120 min, Pluronic P85 alone had no cytotoxic effects in either SKOV3 or SKVLB cells. Under these conditions, cytotoxicity of the copolymer, as determined by the XTT assay, did not exceed 10%. Since higher concentrations of the copolymer or longer incubation times produced some cytotoxicity, we studied below the copolymer concentration and incubation times equal or less than 1% and 120 min, respectively.

Reversion of MDR by Pluronic P85. The cytotoxicity of free daunorubicin and daunorubicin in Pluronic P85 solution (daunorubicin/Pluronic) with respect to SKOV3 and SKVLB cell lines was examined using XTT assay (Figure 1). An about 700-fold decrease in the IC_{50} of daunorubicin in the presence of Pluronic P85 was observed in SKVLB cells. By contrast, the copolymer had a much less significant effect on drug efficacy in the case of SKOV3 cells. The daunorubicin/Pluronic cytotoxicity curves observed in this case became more steep compared to the free drug curves, which was indicative of the amplification of the drug effect. However, the IC_{50} values of daunorubicin/Pluronic observed in a series of repeated measurements (data not presented) were decreased less than 3-fold compared to the free drug IC_{50} values. As a result, the resistant subline demonstrated considerably higher response to the cytotoxic action of the daunorubicin/Pluronic compared to the response in the parental line.

Effect of Incubation Time on Drug Cytotoxicity. The dependence of cytotoxic effects on the time of exposure with SKOV3 and SKVLB cells is shown in Figure 2. The time dependencies of IC_{50} values observed for SKOV3 and SKVLB cells treated with the free drug were parallel (Figure 2a). The IC_{50} values decreased 5–9-fold with an increase in the time of cell exposure to drugs from 5 to 120 min. Therefore, the cell resistance factor did not change appreciably within the time interval studied and approximated a value of 10 (Figure 2b). A dramatically different picture was observed with the cells treated with the daunorubicin/Pluronic. During the time interval, the IC_{50} in SKVLB decreased approximately 400-fold, while in SKOV3 cells, it changed less signifi-

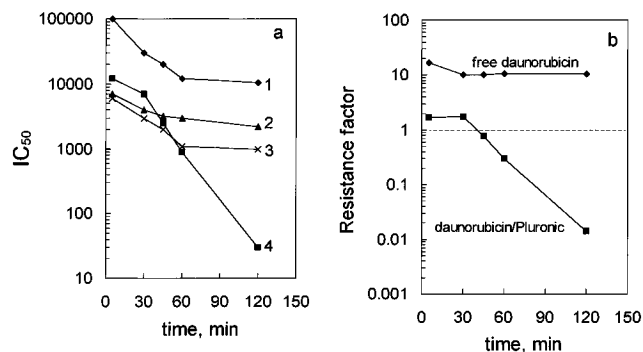


Figure 2. (a) Effect of the duration of the cell exposure to the drug on the cytotoxic effect of (1 and 3) free daunorubicin and (2 and 4) daunorubicin/Pluronic in (1 and 4) SKVLB and (2 and 3) SKOV3 cells. (b) Dependence of the resistance factor (IC_{50} ratio in SKVLB and SKOV3 cells) on the duration of the cell exposure to the free daunorubicin and daunorubicin/Pluronic. Pluronic P85 concentration equals 1%.

Table 1. Effect of Dilution of Pluronic P85 on the Cytotoxicity of the Daunorubicin/Pluronic with Respect to Resistant SKVLB Cells^a

Pluronic concentration % wt	IC_{50} , ng/mL	resistance reversion index, $IC_{50,(-)P85}/IC_{50,(+)P85}$
1	7	714
0.5	10	500
0.1	12	417
0.05	16	312
0.01	20	250
0	5000	—

^a Cells treated with the drug for 120 min.

cantly (Figure 2a). The effects on SKVLB and SKOV3 cells were equivalent after 30–40 min. At longer incubation times, the resistance of the cells with respect to the daunorubicin/Pluronic was inverted. As a result, the index of cell resistance decreased from 1.7 (5 min) to 0.014 (120 min) (Figure 2b).

Effect of Pluronic Concentration. The dependence of the cytotoxic effects on the concentration of the copolymer was examined for SKVLB cells (Table 1). The decrease in Pluronic P85 concentration from 1% (2.2 mM) to 0.01% (22 μ M) was accompanied by only a 3–4-fold increase in IC_{50} , corresponding to a decrease in the resistance reversion index from about 700 to 250. Therefore, even after dilution by a factor of 100, the copolymer preserved the ability to significantly reverse drug resistance in SKVLB cells.

Drug-Induced DNA Strand Breaks in SKOV3 and SKVLB Cells. The free drug significantly increased the level of strand breaks in SKOV3 cells compared to the level of breaks in untreated cells (Figure 3). Approximately the same increase in strand breaks was observed after treatment of these cells with daunorubicin/Pluronic. By contrast, while free daunorubicin did not produce excessive strand breaks in the SKVLB cells, significant DNA damage was observed in SKVLB cells when the drug was administered in the copolymer solution.

Influx and Intracellular Distribution of Free Daunorubicin. The data on the daunorubicin influx were plotted as the kinetic curves of I_c and I_n vs time of cell incubation with the drug for SKOV3 (Figure 4) and SKVLB cells (Figure 5). These curves characterize the kinetics of daunorubicin accumulation in the cytosol and cell membrane (Figures 4a and 5a) and drug binding with the DNA (Figures 4b and 5b). The influx of daunorubicin into the cytoplasm of SKOV3 cells was described by a curve with a maximum; during first 30 min, the I_c value increased, while subsequent incubation of the cells with

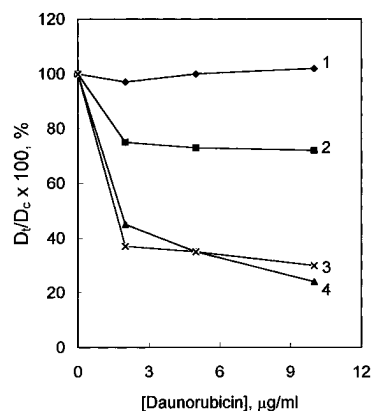


Figure 3. Daunorubicin-induced DNA strand breaks in (1 and 2) SKVLB and (3 and 4) SKOV3 cells treated with (1 and 3) free daunorubicin and (2 and 4) daunorubicin/Pluronic. The ratio of D in drug-treated and untreated cells ($D_t/D_c \times 100\%$) is shown. Pluronic P85 concentration equals 1%.

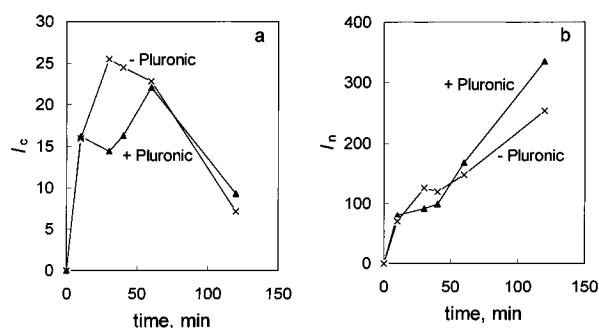


Figure 4. Kinetics of daunorubicin influx in SKOV3 cells in the absence and presence of 1% Pluronic P85: (a) accumulation in cytoplasm and plasma membrane (I_c) and (b) binding with DNA (I_n).

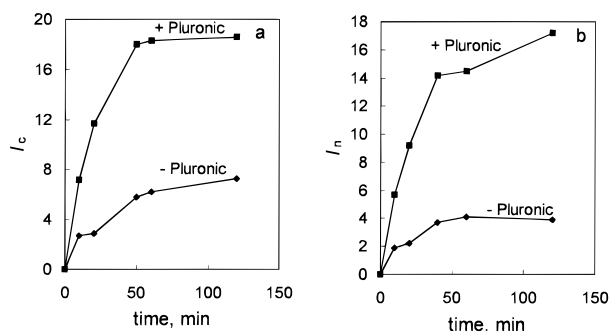


Figure 5. Kinetics of daunorubicin influx in SKVLB cells in the absence and presence of 1% Pluronic P85: (a) accumulation in cytoplasm and plasma membrane (I_c) and (b) binding with DNA (I_n).

daunorubicin led to a decrease in I_c (Figure 4a). The I_n value increased over 120 min and at all times studied was significantly (from 4 to 100-fold) higher than I_c (Figure 4b). This type of kinetic behavior is characteristic of a two-stage process:

drug in external medium $\rightleftharpoons$
 drug in cytoplasm and membrane $\rightleftharpoons$
 fluorescence emitted
stage one
 drug in nucleus
 fluorescence quenched
stage two

The first stage consists of drug transport from the extracellular medium to the cytoplasm and membrane, while the second stage is transport to the DNA, leading

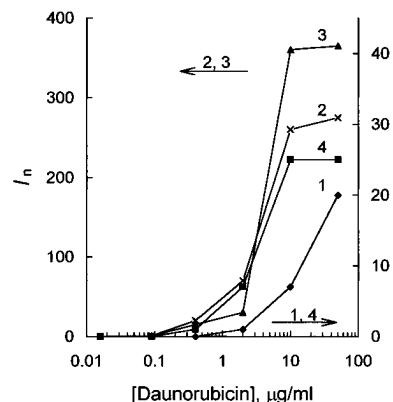


Figure 6. Binding of (1 and 2) free daunorubicin and (3 and 4) daunorubicin/Pluronic with DNA in (1 and 4) SKVLB and (2 and 3) SKOV3 cells. Cells were incubated with the drugs for 120 min. Pluronic P85 concentration equals 1%. Daunorubicin concentration is varied.

to fluorescence quenching. A different picture was observed in the SKVLB cells treated with free daunorubicin. In this case, both I_c and I_n were significantly lower than those in SKOV3 cells. The decreased ability of these cells to accumulate the drug can be explained by the P-gp-mediated efflux of the drug from the cell. No maximum in I_c time dependence was observed in this case over the time period studied. This is consistent with the decreased capacity of the nuclear DNA to bind the drug. In contrast to the case of SKOV3 cells, the drug binding with DNA in SKVLB cells was characterized by a curve indicating saturation (Figure 5b). Furthermore, the concentration dependence of daunorubicin binding with DNA suggests that the binding in SKVLB cells is significantly lower than that in the parental subline (Figure 6). First, the maximal binding of daunorubicin observed in SKVLB cells was about 15 times less than the binding in SKOV3 cells. Second, the apparent binding constant in SKVLB cells was 10 times lower than that in SKOV3 cells.

Effect of Pluronic P85 on the Influx and Intracellular Distribution. No significant changes in I_c and I_n vs time dependence were observed when daunorubicin/Pluronic was added to SKOV3 cells instead of the free drug (Figure 4). However, copolymer caused dramatic changes in the response of SKVLB cells to daunorubicin, resulting in a substantial elevation in the total influx of the drug. In this case, after a 40 min incubation, the I_c values approximated the maximal value observed with the SKOV3 cells (Figure 5a), suggesting that the drug in the presence of Pluronic P85 is efficiently transported into the cytoplasm and membrane. In contrast to the results with SKOV3 cells, the daunorubicin concentration in the cytoplasm of SKVLB cells remained constant after reaching the maximal value (40 min) which is consistent with the lower capacity of the DNA of SKVLB cells to bind daunorubicin compared to that in SKOV3 cells. However, while the binding of the daunorubicin/Pluronic with DNA in SKVLB cells was several-fold lower than the binding observed in SKOV3 cells, it was increased compared to the binding of the free drug in the SKVLB cells (Figure 5b). The concentration dependencies of the drug binding suggest that, although the maximal binding of daunorubicin/Pluronic observed in SKVLB cells was essentially unaffected, the apparent binding constant was decreased at least 10 times compared to the binding of free drug and was equal to that observed for the parental subline (Figure 6).

Efflux of the Free and Micelle-Incorporated Drug. A rapid decline in both I_c and I_n (85 and 95%, respectively,

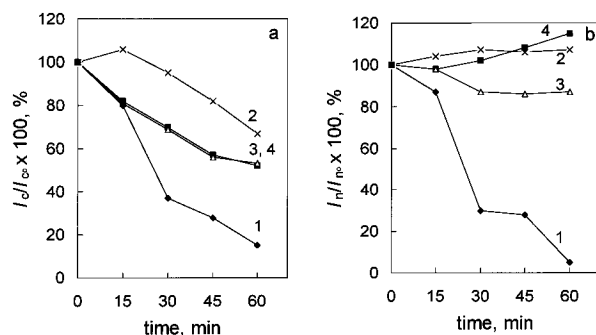


Figure 7. Efflux of (1 and 2) free daunorubicin and (3 and 4) daunorubicin/Pluronic from (a) the cytoplasm and plasma membranes and (b) nuclei of (1 and 4) SKVLB and (2 and 3) SKOV3 cells. The daunorubicin levels in the cytoplasm and membranes and nuclei are expressed as the percentage of the initial levels ($I_c/I_{c0} \times 100$ and $I_n/I_{n0} \times 100$, respectively).

Table 2. Effect of Pluronic P85 on Reversion of Resistance of SKVLB Ovarian Carcinoma Cells with Respect to Various Drugs

drug	IC ₅₀ , ng/mL	
	free drug	drug in 1% pluronic P85
doxorubicin	6000	300
epirubicin ^a	60000	2000
vinblastine	1200	1.1
mitomycin C	800	10
methotrexate	500	90
cisplatin	400	200

^a Cells were incubated with drug for 60 min. In all other cases, incubation time was 120 min.

during the first 60 min) was observed after transfer of the drug-treated SKVLB cells in a drug-free medium (Figure 7). This decline was consistent with the P-gp-mediated efflux of daunorubicin from the cells. In contrast, only a 33% decrease in I_c and no decrease in I_n were observed in the SKOV3 cells treated with the free drug. Both the I_c and I_n components of the efflux were significantly reduced in SKVLB cells treated with the daunorubicin/Pluronic. In this case, the I_c is only decreased by 47% of the initial value and I_n is even slightly increased (=15%) over a 60 min incubation period. This demonstrates that the copolymer provides for an increase in the daunorubicin retention time in the SKVLB cells. Some increase of daunorubicin efflux in the presence of copolymer was observed in the SKOV3 cells. Interestingly, the efflux of daunorubicin/Pluronic from the cytoplasm was exactly the same (=47%) in both SKOV3 and SKVLB cell lines (Figure 7a). It is probable that this efflux component accounts for the passive diffusion of the drug from the cells, facilitated in the presence of the copolymer.

Effect of Pluronic P-85 on the Activity of Other Cytotoxic Drugs. Table 2 compares the cytotoxic effects of several types of anticancer drugs in Pluronic P85 solutions with the effects of the free drugs on SKVLB cells. The cytotoxicity of MDR type drugs, doxorubicin, epirubicin, vinblastine, and mitomycin C, increased 20–1000 times in the presence of 1% Pluronic P85. Less significant changes (only 2–5.5 times) were observed in the IC₅₀ of non-MDR type drugs, cisplatin and methotrexate.

Pluronic Micellar System and Drug Partitioning. To characterize daunorubicin and Pluronic P85 interactions, the micelle formation and daunorubicin partitioning in the micelles have been studied. The Pluronic P85 solutions used in the current study were optically transparent over a wide range of temperatures (18–40 °C) and surfactant concentrations (0.001–1%). A multimolecular

Table 3. Molecular Characteristics of Pluronic P85 and Parameters Characterizing the Multimolecular Micelles Formed by This Copolymer in Aqueous Solution at 37 °C^a

characteristics of the copolymer ^b				characteristics of the micelles ^c	
block lengths		molecular mass	HLB	cmc, %	micelle radius, nm
n	m				
38	51	4500	12–18	0.005	7.3 ± 0.3
					aggregation number
					57 ± 16

^a From ref 27. ^b The hydrophilic–lipophilic balance (HLB) was calculated from Glc relative retention ratios (32). ^c The Critical micelle concentration (cmc), micelle hydrodynamic radius, and aggregation number (i.e. number of copolymer molecules constituting one micelle) were determined using the fluorescent probe technique, quasi-elastic light scattering, and ultracentrifugation (27).

micellar species of the copolymer was formed at copolymer concentrations exceeding the cmc as measured by surface tension, light scattering, and ultracentrifugation. The cmc, the micelle hydrodynamic radius, and the Pluronic P85 aggregation number (i.e. the number of the copolymer molecules in one micelle) are presented in Table 3. Under the conditions studied, these parameters were not affected by the pH (pH 3–9), the ionic strength (0.2 M), or the presence of anticancer drugs (5 mg/mL) or serum proteins (3% BSA) in the system. At concentrations exceeding the cmc, daunorubicin was partitioned between the bulk aqueous phase and the hydrophobic micelle core formed by the oxypropylene chain blocks. Under these conditions, the dynamic exchange of the drug molecules between the bulk phase and micelle microphase takes place. A partitioning model for a drug noncovalently incorporated into the micellar microcontainer has been reported previously (27). According to this model, the fraction of the drug that is incorporated in the micelles is expressed by

$$\alpha = \frac{C_{mic}}{C_o} = \frac{P([Pluronic] - cmc)}{119 + (P - 1)([Pluronic] - cmc)} \quad (3)$$

where C_{mic} and C_o are the bulk concentration of the drug in the copolymer solution and the total bulk concentration of the drug in the system, respectively, [Pluronic] is the copolymer concentration (% wt), and P is the partitioning coefficient that is expressed by

$$P = [drug]_m/[drug]_w \quad (4)$$

where $[drug]_m$ and $[drug]_w$ are the local drug concentrations in the micellar and aqueous phases, respectively. The change in the polarity of the microenvironment after daunorubicin incorporation in the micelle resulted in an increase in the fluorescence of the drug. The daunorubicin partitioning coefficients were determined as previously described for other probes (27) from the dependencies of daunorubicin fluorescence at $\lambda_{ex} = 471$ nm and $\lambda_{em} = 556$ nm on Pluronic P85 concentration using the linear plots (Figure 8),

$$\frac{I_{max} - I}{I - I_o} = \frac{119}{P([Pluronic] - cmc)} - \frac{1}{P} \quad (5)$$

where I_o is the fluorescence intensity in the absence of the copolymer, I is the fluorescence at the given copolymer concentration, I_{max} is the fluorescence at the “saturating” concentration of the copolymer (when fluorescence reaches the maximal value), and 119 is the coefficient that depends on the partial specific volume of the copolymer (27). The value of P approximated 185. Using

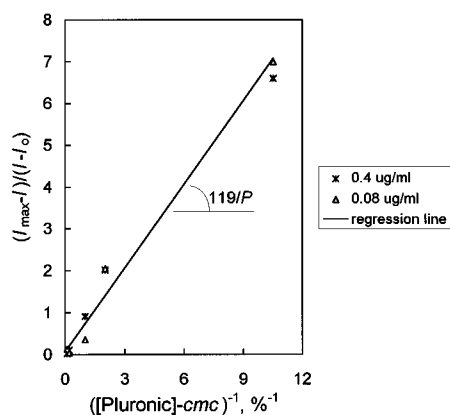


Figure 8. Linear plot for determination of the daunorubicin partitioning coefficient. The data were obtained using two concentrations of doxorubicin (0.4 and 0.08 $\mu\text{g/mL}$) and varying concentrations of Pluronic P85. P value was determined from the slope of the linear regression line using Microsoft Excel 5.0 program.

Table 4. Fractions of Daunorubicin Incorporated into the Micelles at Various Pluronic P85 Concentrations at 37 °C

Pluronic P85 concentration, %	$\alpha \times 100$, %
1	61.0
0.5	44.0
0.1	12.3
0.05	6.5
0.01	0.4

this partitioning coefficient, the fractions of the drug that is incorporated in the micelles were determined from eq 3. The values obtained for various Pluronic P85 concentrations are presented in Table 4.

DISCUSSION

Two sublines of human ovarian carcinoma cells, SKVLB and SKOV3, have been chosen as a model for this study. The SKVLB subline, which has been selected from the parental SKOV3 cell, is known to be an MDR type due to P-gp overexpression (26). The major result of this study is that Pluronic P85 dramatically increased the cytotoxicity of daunorubicin with respect to the MDR cells and much less significantly changed the activity of the drug with respect to the non-MDR subline (Figure 1). The copolymer effect in MDR cells approximated 700-fold (Table 1) and significantly surpassed the MDR-reversing effects of liposomes and polyoxyethylated surfactants that have been previously reported (9–18). Further, the study on daunorubicin transport into the cells suggested that the copolymer enhanced the drug influx into the cytoplasm and its binding with the DNA in the MDR cells (Figures 4–6). Once taken up by the resistant cells, the daunorubicin/Pluronic was retained much longer in the nuclei and cytoplasm of the MDR cells than the free drug which was rapidly eliminated from the cells due to the P-gp-mediated efflux (Figure 7). The drug uptake in the sensitive cells was not significantly altered in the presence of the copolymer, providing further evidence to support an effect of the copolymer on P-gp. The data on the DNA strand breaks suggest that the mechanism of drug action in the SKVLB cells was not changed by the copolymer. The enhanced cytotoxic effect of daunorubicin in the presence of Pluronic P85 correlated with the increase in the drug-induced DNA damage that is presently considered to be a major mechanism of anthracycline cytotoxicity (Figure 3). Furthermore, no significant changes in DNA damage were observed in the presence of the copolymer in the sensitive subline which is also

consistent with the cytotoxicity and transport data. Our data also suggest that the sensitization of MDR cells in the presence of the copolymer is a general phenomenon characteristic for a broad range of MDR type and non-MDR antineoplastic agents (Table 2).

Another important result of this study is that in the presence of Pluronic P85 daunorubicin became significantly more active (“hypersensitive”) with respect to SKVLB cells compared to the parental subline (Figure 2). This result cannot be explained merely by increased drug transport into the MDR subline. Most currently known modulators of MDR (i.e. inhibitors of P-gp) render MDR cells sensitive to MDR type drugs so that the IC_{50} values approach those of sensitive parental cells. It is known that anthracyclines are easily transported across the plasma membrane of the non-MDR cells (31). By contrast, in the absence of the copolymer, free daunorubicin was poorly transported into the SKVLB cells due to the P-gp-mediated efflux. Since the cytoplasm concentration of daunorubicin in SKVLB cells in the presence of the copolymer approximated the drug concentration in the SKOV3 cells (Figures 4a and 5a), we conclude that, once the efflux system for the drug in SKVLB cells is overcome, these copolymer-treated cells become more sensitive to the drug’s cytotoxic action compared with the parental subline. The reason for the hypersensitivity of SKVLB cells with respect to daunorubicin/Pluronic is not yet clear. It cannot be explained by the cytotoxic effect of the copolymer, since under the conditions studied the copolymer did not affect cell viability. Interestingly, data on the daunorubicin influx and intracellular distribution suggest that the DNA of SKVLB cells have a significantly lower capacity with respect to the drug binding compared with the DNA of SKOV3 cells (Figure 6). This result gives independent support of a recent observation that MDR is associated with the changes in intracellular distribution of anthracyclines, specifically with a decreased accumulation of these drugs in the nucleus (33). Furthermore, our binding and cytotoxicity data also suggest that in the presence of the copolymer the lower amounts of the drug that intercalated in the DNA of the MDR cells caused substantially higher cytotoxic effects compared to the non-MDR subline. We hypothesize that in addition to P-gp inhibition the copolymer affects some non-P-gp mechanisms of drug resistance that are yet to be identified in ovarian carcinoma cells. This hypothesis may also give grounds for reconciliation of the sensitization of MDR cells with respect to non-MDR type drugs as well as sensitization of parental cells with respect to daunorubicin. Extensive studies on non-P-gp mechanisms of drug resistance are currently ongoing (34–36). It is worth mentioning in this respect that we have recently observed (data in preparation) several-fold sensitization of parental cells and hypersensitization of MDR cells by Pluronic copolymers on MDR and non-MDR Chinese hamster ovary (CH^r C5 and AUX-B1) and human breast carcinoma (MCF-7 ADR and MCF-7) cell lines.

The results obtained also raise the question about the mechanism of the P-gp-modulatory effects of Pluronic P85. One possible explanation for increased drug influx is an inhibitory effect of Pluronic P85 on the P-gp function in SKVLB cells. Such inhibition may result from a direct interaction of the copolymer molecules with the P-gp or from some copolymer-induced changes in the structure of the plasma membrane that affect P-gp function. However, other mechanisms for the copolymer effect that account for the bypass of the efflux pump, rather than its inhibition, must also be considered. One possible mechanism consists of a copolymer-induced increase in

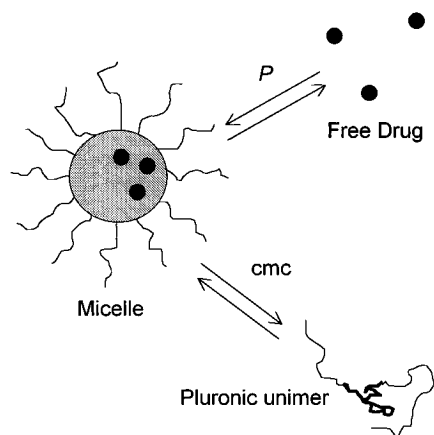


Figure 9. Schematic representation of the dynamic equilibrium in the Pluronic P85 and drug solution. The copolymer exchange between multimolecular micelles and bulk solution is characterized by the *cmc*. The drug exchange between the micelles and bulk solution is characterized by the partitioning coefficient.

the permeability of the cell plasma membrane. The increase in membrane permeability with respect to the drug can result in increased drug influx that compensates for the efflux pump component and leads to a net increase in total uptake. There are previous reports that Pluronic copolymers enhance passive diffusion of low-molecular mass compounds across plasma membranes into cells (37). Our previous work (38) has demonstrated that Pluronic P85-based micellar systems provide for enhanced transport of negatively charged ATP molecules into the cytoplasm that otherwise cannot be transported into a cell. Finally, data obtained in our laboratory (in preparation) also suggest that Pluronic P85 increases the ion permeability of liposome membranes while not affecting the liposome integrity. Interestingly, the increased ion transport in liposomes was observed over the same range of copolymer concentrations that is studied in this work.

Another important problem that is raised by this work is the evaluation of the roles of the multimolecular micellization of the copolymer and drug partitioning in the micellar system in the MDR modulation phenomenon. Originally, the drug delivery concept advanced by us implied that the drug molecules noncovalently incorporate in the core of the Pluronic micelles that serve as carriers for the drug delivery and targeting (19, 20). The validity of this consideration has previously been demonstrated in the experiments on the endocytic uptake of fluorescein incorporated in the Pluronic P85 micelles (39). However, this consideration is not necessarily true for daunorubicin transport in MDR cells reported in this work. Generally, in the daunorubicin/Pluronic systems, both the free and micelle incorporated fractions of the drug and copolymer are present (Figure 9). First, the drug molecules are partitioned and dynamically exchanged between the bulk aqueous solution and the micellar microphase. Therefore, the question is, in what form, micellar or free, are the drug molecules transported into the MDR cells? The answer to this question is obtained in part from the partitioning study performed in this work. Significant MDR reversion is observed at the low copolymer concentration (0.01%) (Table 1) when according to the data of the partitioning study (Table 4) only 0.4% of the total drug is incorporated into the micelles. This result suggests that the effects observed with the MDR cells involve interaction of the free drug form with the cells. We hypothesize therefore that the copolymer interacts with the plasma membrane and enhances the influx of the free drug form presented in

the extracellular media. The study of the validity of this hypothesis is in progress. Second, the copolymer molecules are also partitioned and dynamically exchanged between the multimolecular micelles and the bulk solution (Pluronic unimers). Therefore, the question is, what form of the copolymer, micellar or unimer, is responsible for the effects observed in the MDR cells? The answer to this question can be obtained after the study of MDR modulatory effects of the drug/copolymer system and drug transport in the MDR cells at various concentrations of the copolymer. Some preliminary conclusions can be made on the basis of the cytotoxicity vs concentration dependence presented in this paper (Table 1). The hypersensitization of MDR cells was observed at as low a concentration of the copolymer as 0.01% (22 μ M), which is very close to the *cmc* (27). The increase of the copolymer concentration up to 1% (2.2 mM) leads to a proportional elevation in the micelle concentration, while the unimer concentration remains practically unchanged. Since only 3-fold change in IC_{50} is observed in this concentration range, we hypothesize that just the copolymer unimers are responsible for the hypersensitization effect.

The major practical outcome of this work is that Pluronic P85 can possibly be used as a delivery system for enhancing the activity of antineoplastic agents against MDR tumors. Furthermore, since MDR cells are more susceptible to the cytotoxic effects of daunorubicin/Pluronic than the parental cells, one can assume that it is possible to reduce the development of MDR during chemotherapy by using the copolymer-based drug forms.

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LITERATURE CITED

- (1) Goldstein, L. J., Gottesman, M. M., and Pastan, I. (1991) Expression of the MDR1 gene in human cancers. *Cancer Treat. Res.* 57, 101–119.
- (2) Goldstein, L. J., Gottesman, M. M., and Pastan, I. (1992) Multidrug resistance in human cancers. *Crit. Rev. Oncol. Hematol.* 12, 243–253.
- (3) Gros, P., Neria, Y. B., Croop, J. M., and Housman, D. E. (1986) Isolation and expression of a complementary DNA that confers multidrug resistance. *Nature* 32, 728–731.
- (4) Ueda, K., Cardarelli, C., Gottesman, M. M., and Pastan, I. (1987) Expression of a full-length cDNA for the human MDR1 gene confers resistance to colchicine, doxorubicin, and vinblastine. *Proc. Natl. Acad. Sci. U.S.A.* 84, 3004–3008.
- (5) Higgins, C. F., and Gottesman, M. M. (1992) Is the multidrug transporter a flippase? *TIBS* 17, 18–21.
- (6) Lundescher, C., Thaler, J., Drach, D., Drach, J., Spitaler, M., Gattringer, C., Huber, H., and Hofmann, J. (1992) Detection of activity of P-glycoprotein in human tumor samples using rhodamine 123. *Br. J. Haematol.* 82, 161–168.
- (7) Nielsen, D., Maare, C., and Skovsgaard, T. (1994) Kinetics of daunorubicin transport in Erlich ascites tumor cells with different expression of P-glycoprotein. *Biochem. Pharmacol.* 47, 2125–2135.
- (8) Van-der-Blik, A. M., Van-der-Velde-Koerts, T., Ling, V., and Borst, P. (1986) Overexpression and amplification of five genes in a multidrug-resistant Chinese hamster ovary cell line. *Mol. Cell. Biol.* 6, 1671–1678.
- (9) Zacherl, J., Hamilton, G., Thalammer, T., Riegler, M., Cosentini, E. P., Ellinger, A., Bischof, G., Schweitzer, M., Telenky, B., and Koppena, T. (1994) Inhibition of P-glycoprotein-mediated vinblastine transport across HCT-8 intestinal carcinoma monolayers by verapamil, cyclosporine A and SDZ PSC 833 in dependence on extracellular pH. *Cancer Chemother. Pharmacol.* 34, 125–132.

- (10) Thierry, A. R., Jorgensen, T. J., Forst, D., Belli, J. A., Dritschillo, A., and Rahman, A. (1989) Modulation of multidrug resistance in Chinese hamster cells by liposome-encapsulated doxorubicin. *Cancer Commun.* 1, 311–316.
- (11) Seid, C. A., Fidler, I. J., Clyne, R. K., Earnest, L. E., and Fan, D. (1989) Overcoming murine tumor cell resistance to vinblastine by presentation of the drug in multilamellar liposomes consisting of phosphatidylcholine and phosphatidylserine. *Sel. Cancer Ther.* 7, 103–112.
- (12) Thierry, A. R., Dritschillo, A., and Rahman, A. (1992) Effect of liposomes on P-glycoprotein function in multidrug resistant cells. *Biochem. Biophys. Res. Commun.* 187, 1098–1105.
- (13) Rahman, A., Hussain, S. R., Siddiqui, J., Verma, M., Agresti, M., Center, M., Safa, A. R., and Glazer, R. I. (1992) Liposome-mediated modulation of multidrug resistance in human HL-60 leukemia cells. *J. Natl. Cancer Inst.* 84, 1909–1915.
- (14) Merlin, J. L., Marchal, S., Ramacci, C., Notter, D., and Vinegron, C. (1993) Antiproliferative activity of thermosensitive liposome-encapsulated doxorubicin combined with 43 degrees C hyperthermia in sensitive and multidrug-resistant MCF-7 cells. *Eur. J. Cancer* 29A, 2264–2268.
- (15) Thierry, A. R., Vige, D., Coughlin, S. S., Belli, J. A., Dritschillo, A., and Rahman, A. (1993) Modulation of doxorubicin resistance in multidrug-resistant cells by liposomes. *FASEB J.* 7, 572–579.
- (16) Coon, J. S., Knudson, W., Clodfelter, K., Lu, B., and Weinstein, R. S. (1991) Solutol HS 15, nontoxic polyoxyethylene esters of 12-hydroxystearic acid, reverses multiple drug resistance. *Cancer Res.* 51, 897–902.
- (17) Woodcock, D. M., Linsenmeyer, M. E., Chojnowski, G., Kriegler, A. B., Nink, V., Webster, L. K., and Sawyer, W. H. (1992) Reversal of multidrug resistance by surfactants. *Br. J. Cancer* 66, 62–68.
- (18) Chong, A. S., Markham, P. N., Gebel, H. M., Bines, S. D., and Coon, J. S. (1993) Diverse multidrug-resistance-modification agents inhibit cytolytic activity of natural killer cells. *Cancer Immunol. Immunother.* 36, 133–139.
- (19) Kabanov, A. V., Chekhonin, V. P., Alakhov, V. Yu., Batrakova, E. V., Lebedev, A. S., Melik-Nubarov, N. S., Arzhakov, S. A., Levashov, A. V., Morozov, G. V., Severin, E. S., and Kabanov, V. A. (1989) The neuroleptic activity of haloperidol increases after its solubilization in surfactant micelles. Micelles as microcontainers for drug targeting. *FEBS Lett.* 258, 343–345.
- (20) Kabanov, A. V., Batrakova, E. V., Melik-Nubarov, N. S., Fedoseev, N. A., Dorodnich, T. Yu., Alakhov, V. Yu., Chekhonin, V. P., Nazarova, I. S., and Kabanov, V. A. (1992) A new class of drug carriers: micelles of poly(oxyethylene)-poly(oxypropylene) block copolymers as microcontainers for drug targeting from blood in brain. *J. Controlled Release* 22, 141–158.
- (21) Page, M., and Alakhov, V. Yu. (1992) Elimination of P-gp-mediated multidrug resistance by solubilization in Pluronic micelles (Meeting Abstract). *Proc. Annu. Meet. Am. Assoc. Cancer Res.* 33, A3302.
- (22) Alakhov, V. Yu., Kabanov, A. V., Sveshnikov, P. G., and Severin, E. S. (1994) Composition of antineoplastic agents incorporated in micelles. PCT WO 94/08564.
- (23) Paradis, R., Noel, C., and Page, M. (1994) Use of Pluronic micelles to overcome multidrug resistance. *Int. J. Oncol.* 5, 1305–1308.
- (24) Alakhov, V. Yu., Batrakova, E. V., Klinsky, E. Yu., Moskaleva, E. Yu., and Kabanov, A. V. (1995) Poly(oxyethylene)-poly(oxypropylene) block copolymer micelles as a delivery vehicle for cytotoxic drugs. Reversion of multiple drug resistance to carcinoma cells. In *Abstracts of the 7th International Symposium on Recent Advances in Drug Delivery Systems*. Salt Lake City, pp 213–215.
- (25) Batrakova, E. V., Dorodnych, T. Yu., Klinskii, E. Yu., Klushnenkova, E. N., Shemchukova, O. B., Arjakov, S. A., Alakhov, V. Yu., and Kabanov, A. V. (1996) Pluronic block copolymers: activity against drug sensitive and resistant tumors. *Br. J. Cancer* (submitted for publication).
- (26) Shen, D. W., Fojo, A., Chin, J. E., Roninson, I. B., Richert, N., Pastan, I., and Gottesman, M. M. (1986) Human multidrug-resistant cell lines: increased *mdr1* expression can precede gene amplification. *Science* 232, 643–645.
- (27) Kabanov, A. V., Nazarova, I. R., Astafieva, I. V., Batrakova, E. V., Alakhov, V. Yu., Yaroslavov, A. A., and Kabanov, V. A. (1995) Micelle formation and solubilization of fluorescent probes in poly(oxyethylene-*b*-oxypropylene-*b*-oxyethylene) solutions. *Macromolecules* 28, 2303–2314.
- (28) Scudiero, D. A., Shoemaker, R. H., Paull, K. D., Monks, A., Tierney, S., Nofzinger, T. H., Currens, M. J., Seniff, D., and Boyd, M. R. (1988) Evaluation of a soluble tetrazolium/formazan assay for cell growth and drug sensitivity in culture using human and other tumor cell lines. *Cancer Res.* 48, 4827–4833.
- (29) Birnboim, H. C., and Jevcak, J. J. (1981) Fluorimetric method for rapid detection of DNA strand breaks in human peripheral white blood cells produced by low doses of radiation. *Cancer Res.* 41, 1889–1892.
- (30) Thierry, D., Rigaud, O., Duranton, I., Moustacchi, E., and Magdelenat, H. (1985) Quantitative measurement of DNA strand breaks and repair in γ -irradiated human leukocytes from normal and ataxia telangiectasia donors. *Radiat. Res.* 102, 347–358.
- (31) Weaver, J. L., Pine, P. S., Aszalos, A., Schoenlein, P. V., Currier, S. J., Padmanabhan, R., and Gottesman, M. (1991) Laser scanning confocal microscopy of daunorubicin, doxorubicin and Rhodamine 123 in multidrug-resistant cells. *Exp. Cell. Res.* 196, 323–329.
- (32) BASF Performance Chemicals, Specialty Products (1991) BASF Corp.
- (33) Schuurhuis, G. J., Broxterman, H. J., de Lange, J. H. M., Pinedo, H. M., van Heijningen, T. H. M., Kuiper, C. M., Scheffer, G. L., Scheper, R. J., van Kalken, C. K., Baak, J. P. A., and Lankelma, J. (1991) Early multidrug resistance, defined by changes in intracellular doxorubicin distribution, independent of P-glycoprotein. *Br. J. Cancer* 64, 857–861.
- (34) Giai, M., Biglia, N., and Sismondi, P. (1991) Chemoresistance in breast tumors. *Eur. J. Gynecol. Oncol.* 12, 359–373.
- (35) Fichtner, I., Stein, U., Hoffman, J., Winterfield, G., Pfeil, D., and Hentschel, M. (1994) Characterization of four drug-resistant P388 sublines: resistance/sensitivity in vivo, resistance and proliferation-markers, immunogenicity. *Anticancer Res.* 14, 1995–2003.
- (36) Van-der-Graaf, W. T., Mulder, N. H., Meijer, C., and de Vries, E. G. (1995) The role of methoxymorpholino anthracycline and cyanomorpholino anthracycline in a sensitive small-cell lung cancer cell line and its multidrug-resistant but P-glycoprotein-negative and cisplatin-resistant counterparts. *Cancer Chemother. Pharmacol.* 35, 345–348.
- (37) Lojewski, Z., and Loew, L. M. (1987) Insertion of amphiphilic molecules into membranes is catalyzed by a high molecular weight non-ionic surfactant. *Biochim. Biophys. Acta* 899, 104–112.
- (38) Slepnev, V. I., Kuznetsova, L. E., Gubin, A. N., Batrakova, E. V., Alakhov, V. Yu., and Kabanov, A. V. (1992) Micelles of poly(oxyethylene)-poly(oxypropylene) block copolymer (pluronic) as a tool for low-molecular compound delivery into a cell. Phosphorylation of intracellular proteins with micelle incorporated $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. *Biochem. Int.* 26, 587–595.
- (39) Kabanov, A. V., Slepnev, V. I., Kuznetsova, L. E., Batrakova, E. V., Alakhov, V. Yu., Melik-Nubarov, N. S., Sveshnikov, P. G., and Kabanov, V. A. (1992) Pluronic micelles as a tool for low-molecular compound vector delivery into a cell: effect of *Staphylococcus aureus* enterotoxin B on cell loading with micelle incorporated fluorescent dye. *Biochem. Int.* 26, 1035–1042.

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Synthesis of Cobalamin–Biotin Conjugates That Vary in the Position of Cobalamin Coupling. Evaluation of Cobalamin Derivative Binding to Transcobalamin II

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Six cobalamin–biotin conjugates have been prepared. The cobalamin–biotin conjugates were prepared to evaluate the effect that the location of attachment had on the binding with transcobalamin II (TCII), the cobalamin binding protein in plasma, and to evaluate their potential use for *in vitro* and *in vivo* applications. This study focused only on the effect of binding with TCII. To decrease the possibility of steric problems in binding of the cobalamin conjugates with TCII, and biotin's binding with streptavidin or avidin, moieties of 11–18 atoms in length were used as linkers. Four biotin conjugates were prepared which were attached to the corrin ring of the cobalamin molecule (on *b*-, *c*-, *d*-, and *e*-side chains). One conjugate was attached to the 5'-OH of the ribose moiety, and another conjugate was attached at the cobalt metal (in place of the cyanide moiety of cyanocobalamin). Competitive binding studies were conducted where various amounts of the cobalamin–biotin conjugates and their precursor cobalamin derivatives competed with [⁵⁷Co]cyanocobalamin for binding of recombinant human TCII (rhTCII). Evaluation of cobalamin derivatives which were conjugated at the 5'-OH of ribose or the cobalt metal center indicated that conjugation at either of these positions had little effect on binding with rhTCII. However, conjugates where the attachment was made on the corrin ring substituents had a large variation in binding with rhTCII. Conjugates on the *e*-propionamide side chain had little effect (relative affinity was equal to or decreased less than a factor of 3) on binding with rhTCII, conjugates of the *b*-isomer had decreased binding (relative affinity decreased less than a factor of 10), conjugates of the *d*-propionamide had further decreased binding (relative affinity decreased between 44 and 69 times), and conjugates on the *c*-acetamide group had poor binding to rhTCII (relative affinity decreased between 295 and 1160 times). The significance of the side chains on the corrin ring in providing specificity and high-affinity binding with rhTCII is discussed.

INTRODUCTION

Vitamin B₁₂, **1** (referred to as cyanocobalamin hereafter), is an essential micronutrient. Importantly, this vitamin must be obtained from external sources as it is not synthesized in mammalian cells (1, 2). In cells, cobalamin derivatives are involved in a number of enzymatic processes (3–6). Of particular note is the requirement of methylcobalamin for methionine synthase-catalyzed methyl group transfer from N⁵-methyltetrahydrofolate to homocysteine, producing tetrahydrofolate and methionine (7). Defects in absorption, transport, or metabolism of cobalamins will produce intracellular cobalamin deficiency (8–11), which leads to hematologic and neurologic abnormalities. Such abnormalities have been well-documented in pernicious anemia, in the congenital absence of cyanocobalamin binding proteins, and in inborn errors of cobalamin metabolism (12–14). These pathological conditions may be corrected by early detection and maintenance on pharmacological doses of cyanocobalamin.

The fact that cobalamin deficiency can cause decreased cellular proliferation, or stop cell growth altogether, makes it particularly attractive as a potential method for arresting the proliferation of rapidly dividing cells, such as those found in leukemias or lymphomas (15). Indeed, after earlier observation that prolonged nitrous oxide (N₂O) anesthesia caused severe (acute) bone marrow depression, clinicians reported the use of N₂O, to treat leukemia in 1959 (16) and 1963 (17). The mechanism of action was not known at the time therapy trials were undertaken, but it is now known that N₂O inactivates cobalamin by oxidation of cobalt from Co(I) to Co(II/III) (18–20). Further, it has been shown that exposure to N₂O leads to a rapid decrease in the level of methionine synthase activity (19). More recent therapeutic studies in rats have combined the effects of depletion of cobalamin with the commonly used antineoplastic agents, 5-fluorouracil (21), cycloleucine (22), and methotrexate (23, 24).

We have been investigating new methods of depletion of cellular cobalamin as part of a larger program designed to develop cellular growth blockers for the treatment of AIDS-related lymphoma. In that investigation, novel bioconjugates of cyanocobalamin are being prepared so that their properties as potential cellular growth blockers can be evaluated. The initial development of bioconjugates of cobalamin has focused on compounds which might affect the internalization and modulation of the cell surface receptor for cobalamin uptake. To be effective,

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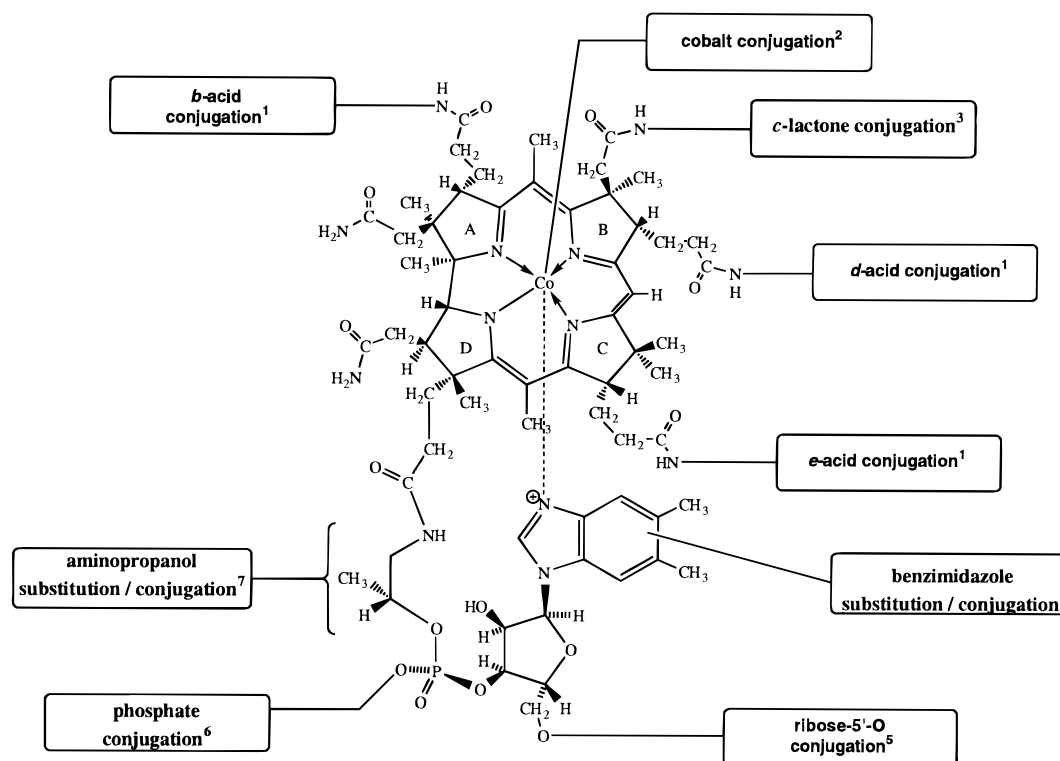


Figure 1. Possible sites for conjugation with the cobalamin molecule. Methods (or potential methods) of conjugation are as follows: ¹prepared from acid hydrolysis of propionamide side chains, followed by conjugation with amine-containing compounds; ²prepared by reductive decyanation, followed by reaction with alkyl halides; ³prepared by formation of *c*-lactone, followed by lactone opening with amine-containing compounds; ⁴potentially prepared by substitution of dimethylbenzimidazole with aminobenzimidazole, followed by reaction with carboxylate-containing molecules; ⁵prepared by reaction with succinic anhydride, followed by conjugation with amine-containing molecules; ⁶potentially prepared by alkylation of phosphate, followed by conjugation of reactive molecules (e.g. amines or carboxylates); ⁷potentially prepared by substitution of an appropriately substituted aminopropanol derivative, followed by conjugation of reactive molecules (e.g. amines or carboxylates). The designation of pyrroline rings with letters (A–D) is shown for reference in the Discussion.

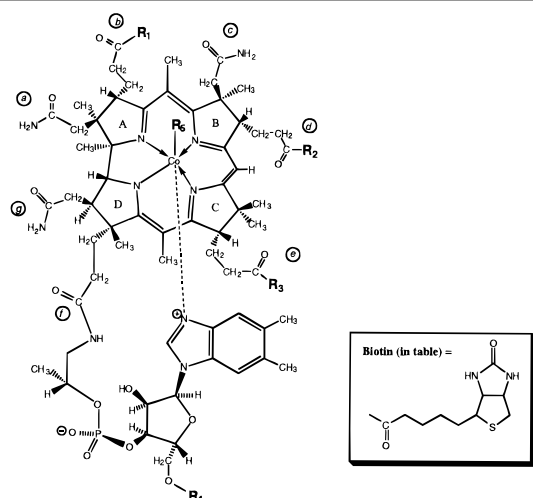
tive, it is essential that cobalamin derivatives be bound to the blood-borne carrier protein transcobalamin II (TCII¹) (25, 26) to obtain the requisite interaction with the cell surface receptor. To utilize this physiological pathway, it is important to ensure that the bioconjugates synthesized retain their high-affinity binding with TCII. The binding of cyanocobalamin to TCII is very strong (i.e. 10^{11} M^{-1}) (27), and it can be expected that many chemical modifications of the cobalamin molecule will result in decreased binding with TCII.

The cobalamin molecule (Figure 1) has several functional groups that can be modified for conjugation with other compounds (3, 4, 7, 28). A number of chemical modifications of cobalamin are described in the literature, but few have addressed the issue of how a modification affects its binding to TCII. The most readily derivatized position on cobalamin is that of alkylation on the Co metal center (29, 30). Another location that is relatively easily derivatized for conjugation is the 5'-hydroxyl on the ribose moiety (31, 32). Perhaps the most common site for conjugation has been that of coupling through carboxylates produced from acid hydrolysis of the corrin

ring propionamide side chains (33–40). Other positions that may be used for conjugation of molecules to cobalamin include the phosphate moiety (41), appropriately substituted (benz)imidazoles (42, 43), and derivatized aminopropanol moieties (44). In the investigation described here, conjugations involving four different locations on the corrin ring (side chains *b*-, *c*-, *d*-, and *e*-), conjugation with the 5'-hydroxyl of the ribose moiety, and conjugation with the Co metal were investigated.

Cobalamin–biotin conjugates were targeted and prepared in this investigation because these molecules had potential application in *in vitro* assays, and it was envisioned that further modification of the cobalamin–biotin conjugates would produce molecules of interest for our therapy studies involving cobalamin depletion. On the basis of the three-dimensional structure of cyanocobalamin, and its binding to proteins, it was apparent that a long spacer was needed between the cobalamin molecule and biotin. The length of the spacer was critical for binding of biotin to avidin and streptavidin as well, because it is known that biotin binds in deep pockets or grooves in these tetrameric molecules (45). The spacer chosen was diaminododecane. This linking molecule has been used to attach cobalamin derivatives to column matrices (34, 46, 47). As described above, six positional cyanocobalamin–biotin conjugates that differ in position of attachment were prepared (see Table 1 and Scheme 1). To assess the impact of conjugations at various positions of the cobalamin molecule on the binding with TCII, assays were conducted in which [⁵⁷Co]cyanocobalamin competed with cobalamin–biotin conjugates (8–10, 13, 15, and 18), or their precursor molecules (2–7, 11, 12, 14, 16, and 17), for binding with recombinant

¹ Abbreviations: Cbl, cobalamin; CN-Cbl, cyanocobalamin; DCC, dicyclohexylcarbodiimide; DMF, dimethylformamide; DMSO, dimethyl sulfoxide; EDC, 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide; 2HEDS, 2-hydroxyethyl sulfide; HOAc, acetic acid; HSA, human serum albumin; IF, intrinsic factor; LC, liquid chromatography; NaOAc, sodium acetate; 3NBA, 3-nitrobenzyl alcohol; NCS, *N*-chlorosuccinimide; NHS, *N*-hydroxysuccinimide; PBS, phosphate-buffered saline; rt, room temperature; Rxn, reaction; t-Boc, *tert*-butoxycarbonyl; TCII, transcobalamin II; rhTCII, recombinant human transcobalamin II; TFP, tetrafluorophenyl; TFP-OH, tetrafluorophenol.

Table 1. Chemical Structures of Cyanocobalamin, 1, and Cobalamin Derivatives 2–15^a

- 1 $R_1 = \text{NH}_2$; $R_2 = \text{NH}_2$; $R_3 = \text{NH}_2$; $R_4 = \text{H}$; $R_5 = \text{CN}$
- 2 $R_1 = \text{OH}$; $R_2 = \text{NH}_2$; $R_3 = \text{NH}_2$; $R_4 = \text{H}$; $R_5 = \text{CN}$
- 3 $R_1 = \text{NH}_2$; $R_2 = \text{OH}$; $R_3 = \text{NH}_2$; $R_4 = \text{H}$; $R_5 = \text{CN}$
- 4 $R_1 = \text{NH}_2$; $R_2 = \text{NH}_2$; $R_3 = \text{OH}$; $R_4 = \text{H}$; $R_5 = \text{CN}$
- 5 $R_1 = \text{NH}(\text{CH}_2)_{12}\text{NH}_2$; $R_2 = \text{NH}_2$; $R_3 = \text{NH}_2$; $R_4 = \text{H}$; $R_5 = \text{CN}$
- 6 $R_1 = \text{NH}_2$; $R_2 = \text{NH}(\text{CH}_2)_{12}\text{NH}_2$; $R_3 = \text{NH}_2$; $R_4 = \text{H}$; $R_5 = \text{CN}$
- 7 $R_1 = \text{NH}_2$; $R_2 = \text{NH}_2$; $R_3 = \text{NH}(\text{CH}_2)_{12}\text{NH}_2$; $R_4 = \text{H}$; $R_5 = \text{CN}$
- 8 $R_1 = \text{NH}(\text{CH}_2)_{12}\text{NH-Biotin}$; $R_2 = \text{NH}_2$; $R_3 = \text{NH}_2$; $R_4 = \text{H}$; $R_5 = \text{CN}$
- 9 $R_1 = \text{NH}_2$; $R_2 = \text{NH}(\text{CH}_2)_{12}\text{NH-Biotin}$; $R_3 = \text{NH}_2$; $R_4 = \text{H}$; $R_5 = \text{CN}$
- 10 $R_1 = \text{NH}_2$; $R_2 = \text{NH}_2$; $R_3 = \text{NH}(\text{CH}_2)_{12}\text{NH-Biotin}$; $R_4 = \text{H}$; $R_5 = \text{CN}$
- 11 $R_1 = \text{NH}_2$; $R_2 = \text{NH}_2$; $R_3 = \text{NH}_2$; $R_4 = \text{COCH}_2\text{CH}_2\text{COOH}$; $R_5 = \text{CN}$
- 12 $R_1 = \text{NH}_2$; $R_2 = \text{NH}_2$; $R_3 = \text{NH}_2$; $R_4 = \text{CO}(\text{CH}_2)_2\text{CONH}(\text{CH}_2)_{12}\text{NH}_2$; $R_5 = \text{CN}$
- 13 $R_1 = \text{NH}_2$; $R_2 = \text{NH}_2$; $R_3 = \text{NH}_2$; $R_4 = \text{CO}(\text{CH}_2)_2\text{CONH}(\text{CH}_2)_{12}\text{NH-Biotin}$; $R_5 = \text{CN}$
- 14 $R_1 = \text{NH}_2$; $R_2 = \text{NH}_2$; $R_3 = \text{NH}_2$; $R_4 = \text{H}$; $R_5 = \text{CH}_2\text{CH}_2\text{CH}_2\text{NH}_2$
- 15 $R_1 = \text{NH}_2$; $R_2 = \text{NH}_2$; $R_3 = \text{NH}_2$; $R_4 = \text{H}$; $R_5 = \text{CH}_2\text{CH}_2\text{CH}_2\text{NHCO}(\text{CH}_2)_5\text{NH-Biotin}$

^a Circled letters denote the corrin ring side chain nomenclature system.

human transcobalamin II (rhTCII). The synthesis of the six targeted cobalamin–biotin conjugates and the results obtained from competitive binding of cobalamin derivatives with rhTCII are reported herein.

EXPERIMENTAL PROCEDURES

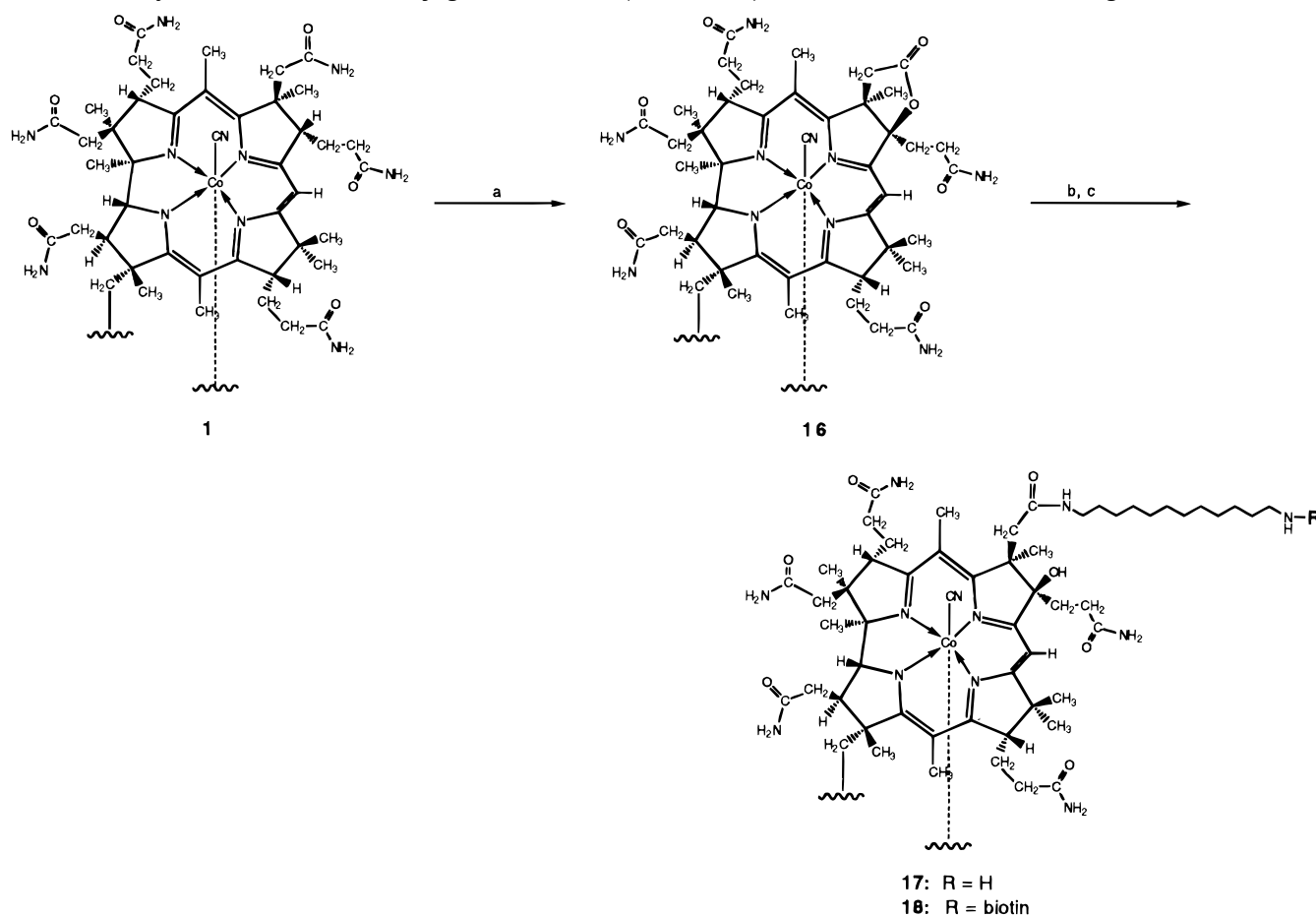
General. All chemicals purchased from commercial sources were of analytical grade or better and were used without further purification. Cyanocobalamin (vitamin B₁₂) and (*N*-hydroxysuccinimido)biotin were obtained from Sigma Chemicals (St. Louis, MO). *N*-Hydroxysuccinimide was purchased from Lancaster Synthesis Inc. (Windham, NH). All other reagents were obtained from Aldrich Chemical Co. (Milwaukee, WI) except where noted. Solvents for high-performance liquid chromatography (HPLC) analysis were obtained as HPLC grade and were filtered (0.2 μm) prior to use. Ion exchange chromatography was conducted with 2% cross-linked, 200–400 mesh, strongly basic anion Dowex 1-chloride (Aldrich Chemical Co.). Amberlite XAD-2 nonionic polymeric adsorbent and octadecyl functionalized silica gel for column chromatography were obtained from Aldrich Chemical Co. Bio-Sil NH₂ (aminopropyl-bonded silica) (40–63 μm) for column packing was purchased from Bio-Rad Laboratories (Hercules, CA). [⁵⁷Co]Cyanocobalamin was obtained from Amersham Corp. (Amersham, U.K.) at a specific activity of 210 $\mu\text{Ci}/\mu\text{g}$ (7.78 MBq/ μg). Phosphate-buffered saline (PBS) was prepared as a solution

containing 8.1 mM Na₂PO₄, 1.2 mM KH₂PO₄, and 138 mM NaCl (pH 7.4). Human serum albumin (HSA) was obtained from Miles, Inc. (Elkhart, IN). Counting of radioactive samples was conducted in a Beckman 300 γ counter.

Spectroscopic Data. ¹H NMR spectra were obtained on a Bruker AC-500 (500 MHz) instrument. The chemical shifts are expressed as parts per million (ppm) using tetramethylsilane as an internal standard ($\delta = 0.0$ ppm). IR data were obtained on a Perkin-Elmer 1420 infrared spectrophotometer. UV data were obtained on a Perkin-Elmer Lambda 2 UV/vis spectrophotometer or a Shimadzu UV 160U spectrophotometer. UV absorbances were obtained by dissolving a weighed sample (approximately 1.00 mg) into MeOH or H₂O in a 10 mL volumetric flask. A 1 mL aliquot of this solution was diluted to 2 mL in a volumetric flask. An aliquot of that solution was placed in a quartz cuvette, and the absorption was measured over the range of 200–1100 nm. Extinction coefficients (ϵ) were calculated from absorbances at the 360 nm peak using the Beer–Lambert equation. Mass spectral data were obtained on a VG 70SEQ mass spectrometer with a 11250J data system. Fast atom bombardment (FAB⁺) mass spectral data were obtained at 8 kV using a matrix of 3-nitrobenzyl alcohol (3NBA) for compounds which were soluble in CHCl₃ or CH₂Cl₂. Compounds soluble in MeOH were run with a matrix of MeOH and 2-hydroxyethyl disulfide (2HEDS). The cobalamin derivatives submitted for elemental analysis were dried under high vacuum; however, this procedure did not result in complete removal of water. Hydrated crystals are commonly obtained with cobalamin derivatives (48). Elemental analyses are reported with a calculated amount of water that resulted in the closest match of carbon present. For this reason, elemental analyses were only obtained on the final biotinylated derivatives.

Handling/Storage of Cobalamin Derivatives. In general, with the exception of the Co-conjugated derivatives of cobalamins, no special precautions were taken during reactions or workup. Preparative LC columns were covered with aluminum foil to protect the cobalamin from light during the time of elution (up to 8 d). The cobalamin derivatives were stored in aluminum foil-covered vials at refrigerator temperature (i.e. 4–7 °C). Under these storage conditions, most cobalamin derivatives were stable for several months. Cobalt-conjugated derivatives discolored within a few minutes when exposed to light. All reactions to prepare Co-conjugated derivatives were conducted in tightly closed aluminum foil-covered flasks. All workup of Co-conjugated derivatives was conducted in a dark room, with chromatography columns and other glassware being covered with aluminum foil. Standard laboratory safety practices were followed.

Analytical Chromatography. HPLC separations of compounds were conducted on a Hewlett-Packard quaternary 1050 gradient pumping system with a multiple wavelength UV detector (360 nm). Analyses of the HPLC data were conducted on Hewlett-Packard HPLC Chemstation software. All reactions were monitored by HPLC. Separations of the cyanocobalamin, 1, and cobalamin derivatives 2–18 were obtained on two different columns, an (aminopropyl)silica column and a C-18 reverse-phase column. Three different elution solvents were used with the C-18 column; thus, four different methods are described. In method 1, the majority of HPLC separations were conducted at a flow rate of 1 mL/min on a 5 μm , 4.6 $\times$ 250 mm mixed mode aminopropyl column (RAININ

Scheme 1. Synthetic Route for Conjugation at the *c*-(Acetamide) Side Chain of the Corrin Ring^a

^a (a) NCS, NaI, H₂O, HOAc, rt, 24 h; (b) diaminododecane, reflux, 24 h; (c) biotin-NHS, DMF, rt, 24 h.

microsorb-MV amino column) eluting with 58 μ M pyridine acetate (pH 4.4) in H₂O/THF (96:4) solution (49). Retention times for the cobalamins evaluated with this system were as follows: **1**, 2.7 min; **3**, 3.8 min; **2**, 4.4 min; **4**, 5.1 min; **5**, 2.2 min; **6**, 2.2 min; **7**, 2.2 min; **11**, 6.2 min; **12**, 2.2 min; **14**, 2.3 min; **16**, 2.7 min; and **17**, 2.2 min. In method 2, reverse-phase HPLC chromatography was carried out on a Hewlett-Packard LiChrospher 100 RP-18 (5 μ m, 125 $\times$ 4 mm) C-18 column using a gradient solvent system at a flow rate of 1 mL/min. Solvent A in the gradient was methanol. Solvent B was 1% HOAc in H₂O. Starting from 40% A, the initial solvent mixture was held for 2 min, and then the gradient was increased to 100% A over the next 10 min. Retention times under these conditions for biotin conjugates were as follows: **8**, 11.5 min; **9**, 11.5 min; **10**, 11.6 min; and **18**, 11.4 min. Method 3 was the same as method 2, with the exception of using H₂O as solvent B. The retention time of biotin conjugate **13** was 6.4 min under these conditions. Method 4 was the same as method 3, with the exception that the gradient was started at 20% A. The retention time of biotin conjugate **15** was 9.4 min under these conditions.

Identification of isomeric monocarboxylic acids² **2–4** was accomplished by evaluation of individual standards (alone and as mixtures) on HPLC using the method of Frenkel et al. (50). The exact conditions could not be duplicated due to differences in equipment. The conditions used were as follows. The HPLC equipment was the same as above. The column was a Hewlett-Packard

LiChrospher 100 RP-18 (5 μ m, 125 $\times$ 4 mm) C-18 column using a gradient solvent system at a flow rate of 1.8 mL/min. Solvent A in the gradient was MeOH containing 0.05 sodium acetate buffer (pH 4.0), and solvent B was 0.05 M sodium acetate in H₂O. A "concave" gradient was obtained by changing the slope of the gradient every 2 min over a 10 min period. The gradient started with 25% A, was held at 25% A for 2 min, was increased to 30% A over the next 2 min, was increased to 40% A over the next 2 min, was increased to 60% A over the next 2 min; and was increased to 90% A over the next 2 min (total of 10 min). The 90% A mixture was held for 5 min. Retention times under these conditions were as follows: **2** (b-), 6.8 min; **3** (d-), 7.1 min; and **4** (e-), 7.4 min (see Figure 3).

Preparative Liquid Chromatography. Preparative chromatographic separations were conducted on a system consisting of a Rainin Rabbit-plus peristaltic pump and a DYNAMAX (model UV-1) UV/vis absorbance detector. A Bio-Sil NH₂ (40–63 μ m) packed glass column (Alltech, 150 psi, 1000 mm $\times$ 25 mm) was used for separation of carboxylate-containing compounds, and a reverse-phase C-18 (BAKERBOND Octadecyl, 40 μ m) packed glass column (500 mm $\times$ 25 mm) was used for preparative purification of other cobalamin derivatives. Eluted compounds were collected in a Rainin DYNAMAX fraction collector, model FC-1, collecting 10.0 mL fractions.

Preparation of Cyanocobalamin Monocarboxylic Acids 2–4. Cyanocobalamin, **1** (3.7 mmol, 5 g), was dissolved in 500 mL of 0.1 N HCl and the mixture stirred at room temperature for 10–11 d under an argon atmosphere (flask covered with aluminum foil). The

² Cobalamin monocarboxylic acids **2–4** were numbered according to their order of elution from a reverse-phase (C-18) column.

solution was then neutralized with 6 N NaOH, and the cobalamins were desalted by extraction into phenol.

Phenol extraction was conducted by washing the collected aqueous fraction with 100 mL of 90% (w/w) phenol/H₂O and twice with 25 mL and once with 10 mL of phenol. The phenol extracts were combined, and to this solution were added 200 mL of H₂O, 480 mL of diethyl ether, and 160 mL of acetone. The aqueous layer was isolated and washed with 30 mL of ether to remove traces of phenol.

The aqueous cobalamin solution was applied to a Dowex 1 × 2 column (200 g, 60 × 4 cm) [prepared by washing with saturated sodium acetate until it was free from Cl[−] and then washing with 200 mL of water (acetate form), 200–400 mesh]. The column was eluted with water to remove unreacted cyanocobalamin and then eluted with 0.04 M NaOAc (pH 4.7). The first fraction of the second elution contained three monocarboxylic acids. This fraction was desalted by phenol extraction (as above). The aqueous solution of monocarboxylic acids was evaporated to dryness to yield 2.5 g (50%).

A 350 mg quantity of the mixture of three acids was then applied to 200 g of aminopropyl column packing (40–63 μm) in a glass column (1000 mm × 25 mm) and was eluted with 58 μM pyridine acetate (pH 4.4) in H₂O/THF (96:4); the eluate was collected with an automatic fraction collector. The first eluted acid was found to be *d*-monocarboxylic acid, **3**, the second eluted acid was *b*-monocarboxylic acid, **2**, and the third eluted acid was *e*-monocarboxylic acid, **4**. The separation process was repeated five times (1750 mg loaded onto the column) before isolation of the purified compounds to minimize loss due to workup. The collected fractions from the five separations were all checked by HPLC, and fractions containing pure samples were combined. Fractions containing mixtures of **2** and **4** were combined separately. The purified acid fractions were desalted by phenol extraction (as above). The solid obtained was recrystallized from aqueous acetone to yield the following: **3** (*d*-), 560 mg (16%); **2** (*b*-), 600 mg (17%); and **4** (*e*-), 200 mg (5.7%).³ An additional 300 mg (8.5%) was obtained as a mixture of **2** and **4**, which was saved for later separation, providing a total recovery from the column of approximately 95%.

d-Acid (**3**): mp 267–270 °C dec; ¹H NMR (MeOH-*d*₄) δ 0.43 (s, 3H), 1.00 (m, 2H), 1.18 (s, 3H), 1.24 (d, 3H), 1.36 (br s, 9H), 1.4 (s, 3H), 1.9 (d, 7H), 2.26 (d, 6H), 2.36 (d, 2H), 2.57 (s, 10H), 2.8 (m, 2H), 3.3 (m, 3H), 3.6 (m, 2H), 3.7 (d, 1H), 3.9 (d, 1H), 4.0 (m, 1H), 4.12 (d, 1H, C-19), 4.17 (s, 1H, C-3), 4.3 (m, 1H), 4.5 (m, 1H), 4.7 (m, 1H), 6.0 (s, 1H), 6.2 (s, 1H), 6.5 (s, 1H), 7.1 (s, 1H), 7.2 (s, 1H); MS (FAB⁺) *m/e* 1357 (M + 1); IR (KBr) 3400, 3200, 2950, 2060, 1660, 1570, 1490, 1060 cm^{−1}; UV (MeOH, λ) 360 (ε = 23 400).

b-acid (**2**): mp 245–250 °C dec; ¹H NMR (MeOH-*d*₄) δ 0.43 (s, 3H), 1.01 (m, 2H), 1.15 (s, 3H), 1.23 (d, 3H), 1.36 (br s, 9H), 1.4 (s, 3H), 1.83 (s, 4H), 1.93 (m, 6H), 2.22 (d, 6H), 2.35 (s, 3H), 2.5 (d, 13H), 2.9 (m, 1H), 3.2 (m, 1H), 3.4 (m, 1H), 3.6 (d, 1H), 3.7 (d, 1H), 3.9 (d, 1H), 4.0 (m, 2H), 4.1 (d, 1H), 4.2 (m, 2H), 4.6 (m, 1H), 6.0 (s, 1H), 6.3 (d, 1H), 6.5 (s, 1H), 7.0 (s, 1H), 7.2 (s, 1H); MS (FAB⁺) *m/e* 1357 (M + 1); IR (KBr) 3400, 3200, 2950, 2060, 1660, 1570, 1490, 1060 cm^{−1}; UV (MeOH, λ) 360 (ε = 21 800).

e-Acid (**4**): mp >300 °C; ¹H NMR (MeOH-*d*₄) δ 0.43 (s, 3H), 1.04 (m, 2H), 1.15 (s, 3H), 1.25 (d, 3H), 1.36 (br s, 9H), 1.4 (s, 3H), 1.85 (s, 4H), 2.01 (s, 6H), 2.23 (d, 8H), 2.38 (d, 3H), 2.53 (d, 13H), 2.6 (m, 5H), 2.9 (m, 1H), 3.3 (d, 1H), 3.4 (m, 1H), 3.6 (d, 1H), 3.7 (d, 1H), 3.9 (d, 1H), 4.0 (m, 2H), 4.1 (d, 1H), 4.3 (m, 2H), 6.0 (s, 1H), 6.3 (d, 1H), 6.5 (s, 1H), 7.1 (s, 1H), 7.2 (s, 1H); MS (FAB⁺) *m/e* 1357 (M + 1); IR (KBr) 3400, 3200, 2950, 2060, 1660, 1570, 1490, 1060 cm^{−1}; UV (MeOH, λ) 360 (ε = 22 100).

General Procedure for Reaction of 2, 3, or 4 with 1,12-Diaminododecane; Preparation of 5–7. Cyanocobalamin monocarboxylic acid (**2**, **3**, or **4**) (500 mg, 0.370 mmol) and *N*-hydroxysuccinimide (NHS) (170 mg, 1.48 mmol) were dissolved in 18.4 mL of DMF/H₂O (1:1), and 363 mg of NaCN was added. 1,12-Diaminododecane was dissolved in 18.4 mL of DMF/H₂O (1:1), and the pH was adjusted to 6 with 1 N HCl. The diaminododecane solution was then added in one portion to the cyanocobalamin solution. EDC (285 mg) was added, and the pH of the solution was readjusted to 5.5. The reaction mixture was then stirred overnight in the dark at room temperature. In five intervals of 6–14 h, 170 mg of NHS and 285 mg of EDC were added to the solution, readjusting the pH value to 5.5 each time. After a total reaction time of 4 d (monitored by HPLC), the solution was evaporated to dryness. The residue was digested with 100 mL of acetone, and the solvent was decanted. The solid residue was dissolved in 50 mL of H₂O and applied to an Amberlite XAD-2 column (200 g, 60 × 4 cm). The column was first washed with 1 L of H₂O, and then the desired product was eluted with 500 mL of methanol. The solution was evaporated to dryness, and the residue was dissolved in 25 mL of H₂O and was applied to a Dowex Cl[−] column (100 g, 60 × 2.5 cm, acetate form, 200–400 mesh). The product was eluted from the column with 250 mL of water, leaving any nonreacted acid bound to the column. This was followed by elution with 0.04 M sodium acetate buffer (pH 4.7). The fractions containing the final product were evaporated to dryness.

d-Isomer (**6**): yield 410 mg (82%); mp 172–174 °C dec; ¹H NMR (MeOH-*d*₄) δ 0.43 (s, 3H), 1.18 (s, 4H), 1.3 (m, 13H), 1.39 (m, 13H), 1.45 (s, 5H), 1.6 (m, 4H), 1.72 (m, 2H), 1.9 (s, 6H), 2.25 (d, 6H), 2.35 (m, 5H), 2.56 (m, 5H), 2.8–3.0 (m, 8H), 3.15 (m, 4H), 3.3 (m, 2H), 3.4 (m, 2H), 3.6 (m, 1H), 3.68 (m, 1H), 3.75 (m, 1H), 3.9 (d, 1H), 4.07 (m, 1H), 4.12 (d, 1H), 4.2 (br s, 1H), 4.3 (m, 1H), 4.47 (m, 1H), 4.7 (m, 1H), 6.0 (s, 1H), 6.2 (d, 1H), 6.5 (s, 1H, B4), 7.1 (s, 1H), 7.2 (s, 1H); MS (FAB⁺) *m/e* 1539 (M + 1); IR (KBr) 3400, 3200, 2950, 2060, 1660, 1570, 1490, 1060 cm^{−1}; UV (MeOH, λ) 360 (ε = 15 400).

b-Isomer (**5**): yield 430 mg (86%); mp 175–180 °C dec; ¹H NMR (MeOH-*d*₄) δ 0.43 (s, 3H), 1.17 (s, 4H), 1.22 (d, 4H), 1.29 (s, 24H), 1.36 (br s, 6H), 1.4 (s, 6H), 1.6 (m, 3H), 1.87 (s, 8H), 2.05 (m, 2H), 2.25 (s, 6H), 2.36 (m, 3H), 2.55 (d, 10H), 2.8 (s, 4H), 3.06 (t, 2H), 3.1 (m, 3H), 3.3 (s, 1H), 3.34 (m, 1H), 3.4 (m, 1H), 3.58 (m, 1H), 3.65 (m, 1H), 3.75 (d, 1H), 3.9 (d, 1H), 4.0 (m, 1H), 4.1 (d, 1H), 4.16 (m, 1H), 4.3 (m, 2H), 4.48 (m, 2H), 4.6 (m, 1H), 6.0 (s, 1H), 6.3 (d, 1H), 6.5 (s, 1H), 7.0 (s, 1H), 7.2 (s, 1H); MS (FAB⁺) *m/e* 1539 (M + 1); IR (KBr) 3400, 3200, 2950, 2060, 1660, 1570, 1490, 1060 cm^{−1}; UV (MeOH, λ) 360 (ε = 16 700).

e-Isomer (**7**): yield 400 mg (80%); mp 174–178 °C dec; ¹H NMR (MeOH-*d*₄) δ 0.43 (s, 3H), 1.07 (m, 3H), 1.2 (d, 4H), 1.27 (m, 15H), 1.35 (br s, 9H), 1.42 (s, 3H), 1.53 (m, 2H), 1.6 (m, 4H), 1.86 (s, 4H), 2.25 (d, 6H), 2.5 (d, 10H), 2.8 (s, 3H), 2.9 (m, 6H), 3.15 (m, 3H), 3.2 (m, 4H), 3.4 (m, 3H), 3.6 (d, 1H), 3.75 (d, 1H), 3.96 (d, 1H), 4.08 (m, 2H), 4.19 (m, 1H), 4.3 (m, 2H), 4.65 (m, 1H), 6.0 (s, 1H), 6.3 (d, 1H), 6.5 (s, 1H), 7.1 (s, 1H), 7.2 (s, 1H); MS (FAB⁺)

³ Yields are based on recovered material from the column being 50% of theoretical due to the yield of the mixture of isomers from the hydrolysis reaction. In a few separations, two (aminopropyl)silica preparative columns were used in series, and recovered yields of **2** and **4** were increased.

m/e 1539 (*M* + 1); IR (KBr) 3400, 3200, 2950, 2060, 1660, 1570, 1490, 1060 cm^{-1} ; UV (MeOH, λ) 360 (ϵ = 17 700).

General Procedure for Conjugation of Biotin with 5, 6, or 7; Preparation of 8–10. To a solution of **5**, **6**, or **7** (300 mg, 0.195 mmol) in 35 mL of DMF was added triethylamine (27 μL , 0.195 mmol). (*N*-Hydroxysuccinimido)biotin (100 mg, 0.295 mmol) was then added over a period of 10–15 min and evaporated to dryness. The solid residue was dissolved in 20 mL of H_2O and applied to a Dowex Cl^- column (75 g, 40×2 cm, acetate form, 200–400 mesh). The product was eluted using 250 mL of water. The aqueous solution was then evaporated to dryness, the residue was dissolved in 10 mL of methanol/water (7:3 v/v) mixture, and the solution was applied to a preparative reverse-phase C-18 column (500 mm $\times$ 25 mm) which was developed with the same solvent. The fractions containing the product (monitored by HPLC) were evaporated to dryness.

d-Isomer (**9**): yield 159 mg (53%); mp 210–212 $^\circ\text{C}$ dec; ^1H NMR (MeOH- d_4) δ 0.43 (s, 3H), 1.18 (s, 4H), 1.3 (m, 13H), 1.39 (m, 13H), 1.45 (s, 5H), 1.6 (m, 4H), 1.72 (m, 2H), 1.9 (s, 6H), 2.2 (d, 8H), 2.6 (d, 12H), 2.7 (m, 3H), 2.8–3.0 (m, 8H), 3.1 (m, 3H), 3.2 (m, 2H), 3.4 (s, 1H), 3.6 (m, 2H), 3.68 (d, 1H), 3.75 (m, 1H), 3.9 (d, 1H), 4.07 (m, 1H), 4.12 (d, 1H), 4.2 (s, 1H), 4.3 (m, 1H), 4.47 (m, 1H), 4.7 (m, 1H), 6.0 (s, 1H), 6.2 (d, 1H), 6.5 (s, 1H), 7.1 (s, 1H), 7.2 (s, 1H); MS (FAB $^+$) *m/e* 1764 (M^+); IR (KBr) 3400, 3200, 2950, 2060, 1660, 1570, 1490, 1060 cm^{-1} ; UV (MeOH, λ) 360 (ϵ = 23 700). Anal. Calcd for $\text{C}_{85}\text{H}_{127}\text{N}_{17}\text{O}_{16}\text{CoPS} \cdot 11\text{H}_2\text{O}$ (**9**): C, 51.98; H, 7.59; N, 12.13. Found: C, 51.91; H, 7.81; N, 12.31.

b-Isomer (**8**): yield 174 mg (58%); mp 222–224 $^\circ\text{C}$ dec; ^1H NMR (MeOH- d_4) δ 0.43 (s, 3H), 1.17 (s, 4H), 1.22 (d, 4H), 1.29 (s, 24H), 1.36 (br s, 6H), 1.4 (s, 6H), 1.6 (m, 4H), 1.72 (m, 2H), 1.87 (s, 4H), 2.17 (m, 3H), 2.25 (s, 6H), 2.36 (m, 3H), 2.55 (d, 10H), 2.64 (m, 2H), 2.8 (s, 4H), 2.97 (s, 4H), 2.17 (m, 3H), 3.3 (m, 1H), 3.4 (m, 1H), 3.58 (m, 1H), 3.65 (m, 1H), 3.75 (d, 1H), 3.9 (d, 1H), 4.0 (m, 1H), 4.1 (d, 1H), 4.16 (m, 1H), 4.3 (m, 2H), 4.48 (m, 2H), 4.6 (m, 1H), 6.0 (s, 1H), 6.3 (d, 1H), 6.5 (s, 1H), 7.0 (s, 1H), 7.2 (s, 1H); MS (FAB $^+$) *m/e* 1764 (M^+); IR (KBr) 3400, 3200, 2950, 2060, 1660, 1570, 1490, 1060 cm^{-1} ; UV (MeOH, λ) 360 (ϵ = 24 400). Anal. Calcd for $\text{C}_{85}\text{H}_{127}\text{N}_{17}\text{O}_{16}\text{CoPS} \cdot 9\text{H}_2\text{O}$ (**8**): C, 52.96; H, 7.53; N, 12.35. Found: C, 52.85; H, 7.55; N, 12.30.

e-Isomer (**10**): yield 165 mg (55%); mp 216–218 $^\circ\text{C}$ dec; ^1H NMR (MeOH- d_4) δ 0.43 (s, 3H), 1.16 (s, 3H), 1.2 (d, 4H), 1.28 (s, 15H), 1.35 (br s, 9H), 1.42 (s, 3H), 1.53 (m, 2H), 1.6 (m, 4H), 1.72 (m, 2H), 1.86 (s, 6H), 2.16 (m, 3H), 2.02 (m, 4H), 2.25 (d, 6H), 2.5 (d, 10H), 2.7 (d, 1H), 2.8 (m, 5H), 3.1 (m, 6H), 3.2 (m, 3H), 3.4 (m, 1H), 3.57 (m, 1H), 3.6 (d, 1H), 3.7 (d, 1H), 3.9 (d, 1H), 4.0 (m, 1H), 4.11 (d, 1H), 4.17 (m, 1H), 4.3 (m, 2H), 4.4 (m, 2H), 4.6 (m, 1H), 6.0 (s, 1H), 6.3 (d, 1H), 6.5 (s, 1H), 7.1 (s, 1H), 7.2 (s, 1H); MS (FAB $^+$) *m/e* 1764 (M^+); IR (KBr) 3400, 3200, 2950, 2060, 1660, 1570, 1490, 1060 cm^{-1} ; UV (MeOH, λ) 360 (ϵ = 29 800). Anal. Calcd for $\text{C}_{85}\text{H}_{127}\text{N}_{17}\text{O}_{16}\text{CoPS} \cdot 10\text{H}_2\text{O}$ (**10**): C, 52.46; H, 7.56; N, 12.24. Found: C, 52.27; H, 7.56; N, 12.34.

Preparation of Cyanocobalamin–Succinate (11**).** This compound was prepared in a manner similar to that previously reported (31). Cyanocobalamin (200 mg, 0.15 mmol) was dissolved in 40 mL of DMSO containing 8.0 g (80 mmol) of succinic anhydride and 6.4 mL of pyridine. After 14–16 h at room temperature, the excess succinic anhydride was destroyed by addition of 500 mL of water, keeping the pH of the reaction mixture at 6 with 10% KOH. KCN was then added at a final concentration of 0.01 M, and the pH of the solution was readjusted to 6 with 3 N HCl. After 1 h, the cobalamin components were

desalted by phenol extraction and applied to a Dowex Cl^- column (100 g, 60×2.5 cm, acetate form, 200–400 mesh). Cyanocobalamin was eluted with water. Monosuccinyl derivative **11** was eluted with 0.04 M aqueous NaOAc (pH 4.7) to yield 180 mg (85%): mp 208–210 $^\circ\text{C}$ dec; ^1H NMR (D_2O) δ 0.43 (s, 3H), 0.95 (m, 2H), 1.15 (s, 3H), 1.2 (d, 3H), 1.35 (d, 7H), 1.4 (s, 3H), 1.8 (s, 3H), 1.9 (s, 12H), 2.2 (d, 6H), 2.36 (d, 2H), 2.5 (d, 10H), 2.6–2.7 (m, 7H), 3.0 (m, 1H), 3.3 (d, 1H), 3.37 (m, 1H), 3.5 (d, 1H), 4.0 (d, 1H), 4.18 (m, 2H), 4.25 (m, 3H), 4.54 (d, 1H), 6.0 (s, 1H), 6.3 (d, 1H), 6.4 (s, 1H), 7.0 (s, 1H), 7.2 (s, 1H); MS (FAB $^+$) *m/e* 1455 (M^+); IR (KBr) 3400, 3200, 2950, 2060, 1660, 1570, 1490, 1060 cm^{-1} ; UV (MeOH, λ) 360 (ϵ = 26 000).

Conjugation of Diaminododecane with **11; Preparation of **12**.** Cyanocobalamin–ribose 5'-O-succinate, **11** (538 mg, 0.370 mmol), and *N*-hydroxysuccinimide (170 mg, 1.48 mmol) were dissolved in 18.4 mL of DMF/ H_2O (1:1), and 363 mg of NaCN was added. 1,12-Diaminododecane was dissolved in 18.4 mL of DMF/ H_2O (1:1), and the pH was adjusted to 6 with 1 N HCl. The diaminododecane solution was then added in one portion to the cyanocobalamin solution. EDC (285 mg) was added, the pH of the solution was readjusted to 5.5, and the reaction mixture was stirred overnight in the dark at room temperature. In five intervals of 6–14 h, 170 mg of NHS and 285 mg of EDC were added to the solution, readjusting the pH to 5.5 each time. After a total reaction time of 4 d, the solution was evaporated to dryness, the residue was digested with 100 mL of acetone, and the solvent was decanted. The solid residue was dissolved in 50 mL of H_2O and applied to an Amberlite XAD-2 column (200 g, 60×4 cm). The column was washed with 1 L of water, and the crude product was eluted with 500 mL of methanol. The solution was evaporated to dryness, and the residue was dissolved in 25 mL of water and was applied to a Dowex Cl^- column (100 g, 60×2.5 cm, acetate form, 200–400 mesh). The final product was eluted using 250 mL of water, thereby leaving nonreacted free acid bound to the column, which was later recovered by elution with 0.04 M sodium acetate buffer (pH 4.7). The fraction containing **12** was evaporated to dryness, yielding 425 mg (70%): mp 185–187 $^\circ\text{C}$ dec; ^1H NMR (MeOH- d_4) δ 0.43 (s, 3H), 1.15 (s, 3H), 1.2 (d, 3H), 1.3 (s, 27H), 1.4 (m, 3H), 1.55 (m, 6H), 1.85 (m, 12H), 2.2 (d, 6H), 2.3 (d, 6H), 2.5 (d, 10H), 2.8 (m, 10H), 3.0 (t, 3H), 3.1 (t, 3H), 3.2 (s, 6H), 3.3 (m, 4H), 3.58 (m, 2H), 3.6 (d, 1H), 4.1 (d, 1H), 4.2 (m, 2H), 4.3 (m, 1H), 4.4 (d, 1H), 6.0 (s, 1H), 6.2 (d, 1H), 6.5 (s, 1H), 7.1 (s, 1H), 7.2 (s, 1H); MS (FAB $^+$) *m/e* 1638 (M^+); IR (KBr) 3400, 3200, 2950, 2060, 1660, 1570, 1490, 1060 cm^{-1} ; UV (MeOH, λ) 360.

Conjugation of Biotin with **12; Preparation of **13**.** To a solution of **12** (300 mg, 0.183 mmol) in 35 mL of DMF was added triethylamine (25 μL , 0.183 mmol). (*N*-Hydroxysuccinimido)biotin (100 mg, 0.295 mmol) was then added over a period of 10–15 min, after which it was evaporated to dryness. The solid residue was dissolved in 20 mL of water the mixture adjusted to pH 10 with 1 N NaOH and applied to a Dowex Cl^- column (75 g, 40×2 cm, 200–400 mesh). The water fraction was discarded. The product was then eluted with 0.1 N aqueous NH_4OAc and was desalted by phenol extraction. The residue was dissolved in 10 mL of methanol/water (7:3 v/v), and the solution was applied to a C-18 reverse-phase column, which was developed with the same solvent. The fractions containing **13** (monitored by HPLC) were evaporated to dryness to yield 135 mg (45%): mp 198–205 $^\circ\text{C}$ dec; ^1H NMR (MeOH- d_4) δ 0.43 (s, 3H), 1.15 (s, 3H), 1.2 (d, 3H), 1.3 (s, 27H), 1.36 (m, 6H), 1.4 (m, 3H), 1.6 (m, 4H), 1.7 (m, 2H), 1.85 (m, 12H),

2.0 (d, 3H), 2.17 (m, 3H), 2.2 (d, 6H), 2.3 (d, 6H), 2.5 (d, 10H), 2.64 (m, 2H), 2.8 (m, 10H), 3.1 (m, 6H), 3.25 (m, 6H), 3.58 (m, 2H), 4.0 (m, 1H), 4.1 (m, 1H), 4.16 (m, 1H), 4.4 (m, 1H), 4.6 (s, 2H), 4.7 (m, 1H), 6.0 (s, 1H), 6.2 (d, 1H), 6.5 (s, 1H), 7.1 (s, 1H), 7.2 (s, 1H); MS (FAB⁺) *m/e* 1866 (M⁺); IR (KBr) 3400, 3200, 2950, 2060, 1660, 1570, 1490, 1060 cm⁻¹; UV (MeOH, λ) 360 (ϵ = 28 400).

Preparation of the Co–Aminopropyl Adduct (14).

This compound was prepared in a manner similar to that previously reported (51). To an aluminum foil-covered flask containing 500 mL of 10% aqueous ammonium chloride solution was added 1.0 g (0.70 mmol) of cyanocobalamin. After dissolution, zinc powder (20 g) was added. The resultant suspension was stirred vigorously under an argon atmosphere for 30 min. Then 0.50 g (3.85 mmol) of chloropropylamine was added to the brownish suspension, and it was stirred for an additional 15 min in the dark. The reaction mixture was desalted by phenol extraction. The isolated solid was recrystallized from acetone/water to yield 800 mg of **14** (80%): mp 232–236 °C dec; ¹H NMR (MeOH-*d*₄) δ 0.48 (s, 3H), 1.12 (s, 4H), 1.19 (d, 6H), 1.35 (s, 8H), 1.46 (s, 4H), 1.76 (s, 3H), 1.89 (s, 8H), 2.24 (s, 6H), 2.5 (d, 6H), 3.05 (m, 1H), 3.24 (d, 1H), 3.56 (d, 2H), 3.66 (t, 1H), 3.72 (dd, 1H), 3.86 (d, 1H), 4.1 (d, 2H), 4.2 (d, 1H), 4.33 (d, 2H), 4.7 (m, 1H), 6.18 (s, 1H), 7.15 (s, 1H); MS (FAB⁺) *m/e* 1387 (M⁺); IR (KBr) 3400, 3200, 2915, 1660, 1570, 1490, 1060 cm⁻¹.

Conjugation of Biotin–Aminocaproate TFP Ester with 14; Preparation of 15. (Aminopropyl)cobalamin, **14** (270 mg, 0.195 mmol), was dissolved in 15 mL of DMF under an argon atmosphere. Triethylamine (30 μ L, 0.292 mmol) was then added, followed by the TFP ester of biotin–aminocaproate, **19** (92 mg, 0.195 mmol). The reaction mixture was stirred in the dark for 24 h at room temperature (monitored by HPLC). The solvent was removed by distillation, and the residue was digested with acetone to yield 260 mg (77%) of **15**: mp 218–222 °C dec; ¹H NMR (MeOH-*d*₄) δ 0.55 (s, 3H), 1.08 (s, 4H), 1.19 (d, 6H), 1.35 (s, 8H), 1.49 (d, 4H), 1.78 (s, 2H), 1.82 (s, 6H), 2.15 (s, 4H), 2.18 (t, 4H), 2.25 (s, 6H), 2.5 (d, 11H), 2.9 (dd, 2H), 3.15 (d, 2H), 3.58 (t, 1H), 3.7 (m, 1H), 3.85 (m, 1H), 4.3 (m, 2H), 4.47–4.5 (dd, 1H), 4.7 (m, 1H), 6.2 (s, 1H), 7.17 (s, 1H); MS (FAB⁺) *m/e* 1727 (M⁺).

Synthesis of c-Lactone (16). Cyanocobalamin, **1** (2.0 g, 1.3 mmol), was dissolved in 600 mL of water, to which 30 mL of acetic acid was added. Sodium iodide (200 mg, 1.3 mmol) and *N*-chlorosuccinimide (360 mg, 2.64 mmol) were then added, and the reaction mixture was stirred at room temperature for 14–16 h, after which it was desalted by phenol extraction to yield 2.0 g (100%): mp 254–256 °C dec; ¹H NMR (MeOH-*d*₄) δ 0.49 (s, 3H), 1.16 (s, 3H), 1.25 (d, 3H), 1.28 (s, 1H), 1.39 (d, 6H), 1.49 (s, 2H), 1.59 (t, 1H), 1.83 (d, 2H), 1.92 (s, 2H), 1.98 (s, 1H), 2.04 (m, 1H), 2.14 (s, 1H), 2.27 (d, 6H), 2.6 (d, 9H), 2.8 (m, 3H), 3.2 (d, 1H), 3.3 (t, 1H), 3.67 (d, 1H), 3.76 (dd, 1H), 3.89 (dd, 1H), 4.0–4.2 (m, 2H), 4.3 (m, 1H), 4.48 (d, 1H), 4.67 (m, 1H), 5.00 (s, 1H), 6.28 (d, 1H), 6.5 (s, 1H), 7.16 (s, 1H), 7.30 (s, 1H); MS (FAB⁺) *m/e* 1353 (M⁺); IR (KBr) 3400, 3200, 2950, 2060, 1784, 1660, 1570, 1490, 1060 cm⁻¹; UV (H₂O, λ) 359 (ϵ = 26 300).

Conjugation of Diaminododecane with 16; Preparation of 17. A 40 g quantity of 1,12-diaminododecane was liquified by heating to 80 °C. Lactone **16** (1.0 g, 0.70 mmol) was then added, and the reaction mixture was stirred for 4 h under argon at 80 °C. After 4 h, a 500 mL quantity of preheated (to 80 °C) H₂O was added to the reaction mixture. This mixture was allowed to cool to room temperature, and 728 mg (14.9 mmol) of NaCN and 120 mL of 3 N HCl were added. The pH was adjusted to 6 with 1 N HCl, and a phenol extraction was

performed. The product was dissolved in 50 mL of H₂O and applied to an Amberlite XAD-2 column (175 g, 60 $\times$ 4 cm). The column was washed with 1 L of water, and then the product was eluted with 500 mL of methanol. The solution was evaporated to dryness, and the residue was dissolved in 25 mL of H₂O and was applied to a Dowex Cl⁻ column (100 g, 60 $\times$ 2.5 cm, acetate form, 200–400 mesh). The product was eluted with 250 mL of water and was evaporated to dryness. The solid was then suspended in acetone and filtered. The collected solid **17** was recrystallized from aqueous acetone to yield 320 mg (30%): mp >300 °C; ¹H NMR (MeOH) δ 0.44 (s, 3H), 1.2–1.3 (m, 24H), 1.45 (s, 4H), 1.6 (m, 6H), 1.88 (s, 9H), 2.2 (s, 7H), 2.46 (s, 5H), 2.6 (m, 4H), 2.8 (m, 4H), 3.3 (s, 2H), 3.6 (d, 1H), 3.7 (dd, 1H), 3.9 (d, 1H), 4.0 (m, 1H), 4.1 (m, 2H), 4.3 (m, 1H), 4.7 (m, 2H), 6.0 (s, 1H), 6.27 (s, 1H), 6.49 (s, 1H), 7.1 (s, 1H), 7.2 (s, 1H); MS (FAB⁺) *m/e* 1554 (M⁺); IR (KBr) 3400, 3200, 2950, 2060, 1660, 1570, 1490, 1060 cm⁻¹; UV (H₂O, λ) 361 (ϵ = 19 200).

Conjugation of Diaminododecane Adduct 17 with Biotin; Preparation of 18. Cobalamin derivative **17** (250 mg, 0.161 mmol) was dissolved in 30 mL of DMF. Triethylamine (40 μ L) and (*N*-hydroxysuccinimido)biotin (66 mg, 0.193 mmol) were added, and the reaction mixture was stirred at room temperature for 24 h. The DMF was removed by Kugelrohr distillation. The resultant solid was dissolved in 20 mL of methanol/H₂O (1:1) and applied to a preparative C-18 reverse-phase column (500 mm $\times$ 25 mm) which was conditioned with the same solvent. The fractions containing **18** (monitored by HPLC) were evaporated to dryness. The residue was suspended in acetone and filtered. The solid obtained was recrystallized from aqueous acetone to yield 100 mg (40%) of **18**: mp 234–236 °C dec; ¹H NMR (MeOH) δ 0.44 (s, 3H), 1.2–1.3 (m, 20H), 1.35 (d, 9H), 1.44 (s, 5H), 1.9 (s, 3H), 2.17 (s, 2H), 2.24 (s, 6H), 2.45 (s, 5H), 2.6 (m, 4H), 2.8–2.9 (m, 4H), 3.1–3.2 (m, 3H), 3.3 (m, 2H), 3.6 (d, 1H), 3.7 (d, 1H), 3.9 (d, 1H), 4.0 (m, 1H), 4.1 (m, 2H), 4.3 (m, 1H), 4.5 (m, 1H), 4.7 (m, 2H), 6.0 (s, 1H), 6.3 (s, 1H), 6.5 (s, 1H), 7.1 (s, 1H), 7.2 (s, 1H); IR (KBr) 3400, 3200, 2950, 2060, 1660, 1570, 1490, 1060 cm⁻¹. Anal. Calcd for C₈₅H₁₂₇N₁₇O₁₇CoPS·10H₂O (**18**): C, 52.04; H, 7.50; N, 12.14. Found: C, 52.12; H, 7.27; N, 11.87.

Synthesis of Biotin–Aminocaproate TFP Ester (19).⁴ *Reaction Step A; Synthesis of Biotin TFP Ester.* Biotin (3.0 g, 12.3 mmol) was dissolved in 60 mL of DMF (warmed at 70 °C) under an argon atmosphere. It was then allowed to cool to ambient temperature, and dicyclohexylcarbodiimide (DCC) (2.79 g, 13.5 mmol) was added, followed by tetrafluorophenol (TFP-OH) (4.08 g, 24.6 mmol). The reaction mixture was cooled to 0 °C and stirred for 30 min. It was brought back to ambient temperature and stirred for another 4–5 h. The reaction mixture was filtered, and the filtrate was evaporated to dryness. The precipitate was washed with 50 mL of acetonitrile and was filtered to yield 5.0 g (98%) of white solid: ¹H NMR (DMSO-*d*₆) δ 1.4 (m, 2H), 1.7 (m, 2H), 2.5 (t, 2H), 2.8 (t, 2H), 3.1 (m, 1H), 4.1 (m, 1H), 4.3 (m, 1H), 6.4 (d, 2H), 7.9 (m, 1H).

Reaction Step B; Synthesis of the Biotin–Caproate Adduct. 6-Aminocaproic acid (0.99 g, 7.5 mmol) was

⁴ The biotin–aminocaproic acid adduct can be obtained from commercial sources. This compound is referred to as LC-Biotin by Pierce (Rockford, IL) and Biotin-X by CALBIOCHEM (La Jolla, CA). The difference between Pierce's Immunopure NHS-LC-Biotin or CALBIOCHEM's Biotin-X-NHS and compound **19** is that those compounds have an NHS ester, whereas **19** has a TFP ester.

dissolved in 75 mL of H₂O. Triethylamine (0.5 mL) was added, followed by a solution of TFP ester of biotin (1.96 g, 5 mmol) in 300 mL of warm acetonitrile. The reaction mixture was stirred overnight at room temperature. It was then filtered, washed with 50 mL of H₂O, and dried under high vacuum to yield 0.870 g (47%). Additional product was obtained by evaporation of the filtrate to dryness. That residue was dissolved in 75 mL of boiling acetonitrile, filtered, and washed with hot acetonitrile. The solid was dried on high vacuum to yield an additional 0.6 g, for a total yield of 1.47 g (79%): ¹H NMR (DMSO-*d*₆) δ 1.2–1.6 (m, 8H), 2.0 (t, 2H); 2.2 (t, 2H), 2.5 (dd, 2H), 2.8 (dd, 2H), 3.1 (m, 3H), 4.1 (m, 1H), 4.3 (m, 1H), 6.4 (d, 2H), 7.7 (m, 1H).

Reaction Step C; Synthesis of the TFP Ester of the Biotin–Aminocaproate Adduct. The biotin–aminocaproate adduct (1.0 g, 2.68 mmol) was dissolved in 50 mL of DMSO. Triethylamine (0.4 mL, 2.9 mmol) was added, followed by the TFP ester of trifluoroacetic acid (1.05 g, 4.02 mmol) (52). The reaction mixture was stirred at room temperature for 15–20 min (monitored by HPLC) and was then evaporated to dryness. The residue was washed with ether and dichloromethane and then dried on high vacuum to yield 1.24 g (89%): mp 144–147 °C; ¹H NMR (DMSO-*d*₆) δ 1.2 (t, 2H), 1.3–1.7 (m, 5H), 2.1 (t, 2H), 2.6 (dd, 2H), 2.8 (m, 4H), 3.1 (m, 4H), 4.2 (m, 1H), 4.4 (m, 1H), 6.4 (d, 2H), 7.8 (t, 1H), 8.0 (m, 1H); IR (KBr) 3300, 2920, 1780, 1700, 1640, 1480, 1095 cm^{−1}; HRMS (*M* + *H*) calcd for C₂₂H₂₈N₃O₄F₄S 506.1737, found 506.1739.

Binding of Cobalamin Derivatives with rhTCII. Recombinant human transcobalamin II (rhTCII) was prepared as previously reported (53). Samples (2.0 mg) of cobalamin derivatives were dissolved in 100–200 μL of DMSO, diluted to 1.0 mL with H₂O, and stored at −20 °C until they were tested. Quantification of the cobalamin derivatives was accomplished by UV, on the basis of the measured extinction coefficients at 360 nm (data provided with individual compounds). Thus, a 20 μL aliquot was diluted to 1 mL with H₂O, and the absorbance at 360 nm was measured. An aliquot of the original solution was diluted to obtain a 1 μM solution, which was further diluted to 0.1 μM for use in the assay.

Recombinant human transcobalamin II (rhTCII) was partially purified on a cation exchange column as previously described (54). The rhTCII was diluted in phosphate-buffered saline (PBS) containing 0.025% HSA to bind approximately 10 pmol of cyanocobalamin, **1**, per milliliter. Aliquots of 100 μL of the rhTCII solution were added to tubes containing 0.01 pmol of [⁵⁷Co]**1** and 0.1–30 pmol of unmodified **1** or cobalamin derivatives **2–18**. The solution volume in each tube was adjusted to 1.0 mL with PBS. The samples were incubated at room temperature for 1 h. The protein-bound [⁵⁷Co]**1** was then separated from free [⁵⁷Co]**1** by batchwise adsorption to hemoglobin-coated charcoal (55). The amount of radioactivity in each of the fractions was determined in a γ counter. The inhibition of binding [⁵⁷Co]**1** in the presence of various amounts of a cobalamin derivative was calculated and graphed (Figure 4). The quantities of each derivative required to inhibit 50% of the [⁵⁷Co]**1** binding to rhTCII were determined, and the binding of the compounds relative to cyanocobalamin was calculated (Table 3).

RESULTS

A primary goal of this investigation was to evaluate the changes in cobalamin binding with TCII that occurred as a result of conjugation at different positions on the cobalamin molecule. A review of the potential

sites for conjugation (Figure 1) culminated in targeting of six different locations on the cobalamin molecule for conjugation. The targeted conjugates were chosen as their chemistry was perceived to be less difficult than that required for conjugation at other locations, and the resultant conjugates were thought to provide adequate information on which areas of the cobalamin were most affected by conjugation.

The targeted cobalamin–biotin conjugates were readily synthesized. Synthesis of biotin conjugates of cobalamin, in general, required three synthetic steps. The first step was production and activation of a site for conjugation on the cobalamin molecule. The second step was conjugation of the cobalamin–linker adduct with an activated carboxylate derivative (i.e. NHS ester) of biotin. Slightly different procedures were followed for production of the Co conjugate **15** and the *c*-lactone conjugate **18**. The structures of biotin conjugates, and their precursors, are provided in Table 2 and in Scheme 1. Discussion of the synthesis of each derivative is given in the following text.

Preparation of Corrin Ring *b*-, *d*-, and *e*-Carboxylate Conjugates. Conjugation at the *b*-, *d*-, and *e*-propionamide functionalities of the corrin ring (Figure 1) began with preparation of the corresponding three isomeric monocarboxylic acids. Studies of the hydrolysis of the *b*-, *d*-, and *e*-propionamides in cyanocobalamin to the corresponding monocarboxylates has been previously reported (56, 57). Importantly, the hydrolysis of the *b*-, *d*-, and *e*-propionamides can be accomplished preferentially over the hydrolysis of *a*-, *c*-, and *g*-acetamides, or the *f*-amide in the heterocyclic chain connecting the benzimidazole moiety. It was apparent from the literature that acid hydrolysis gave reasonable yields of the monocarboxylic acids, but it was unclear how the product compositions might be affected when acid strength and reaction temperature were varied. Thus, acid hydrolysis experiments were conducted with 1.0, 0.5, and 0.1 N HCl, at room temperature, 37 °C, and 50 °C, to optimize the yields of monocarboxylic acids. The reaction product compositions were followed by HPLC on an (aminopropyl)silica column, which provided good separation of the starting cyanocobalamin, the three isomeric monocarboxylates, and the di- and tricarboxylates (see Figure 2, panel A). The results of the acid hydrolysis optimization study are presented in Table 2. The reaction mixtures employing 1.0 or 0.5 N HCl contained more undesired products, many of which were unidentified. Hydrolyses at higher acid strengths resulted in having less cyanocobalamin remaining at an equivalent time than in the reactions employing 0.1 N HCl, but the yields of monocarboxylic acids were not improved. Likewise, elevation of the temperature to 37 or 50 °C gave similar results. Thus, an optimal yield of monocarboxylates, which had minimal di- and tricarboxylate derivatives and did not further degrade the carboxylates or nonhydrolyzed cyanocobalamin, was obtained at room temperature in 0.1 N HCl over a 10–11 d period (Table 2). Extending the hydrolysis time did not significantly improve the yields of monocarboxylates.

Hydrolysis reactions were routinely conducted on 5 g of cyanocobalamin. Following the hydrolysis, the unreacted cyanocobalamin was readily recovered from an ion exchange column as it was not retained when eluting with H₂O, whereas the carboxylate-containing products were retained. The three isomeric monocarboxylates were subsequently eluted as a mixture from the ion exchange column with dilute sodium acetate (pH 4.7) (see Figure 2, panel B). Preparative separation of the three isomeric monocarboxylic acids was not readily achieved

Table 2. HPLC Analysis of Product Mixture Compositions from Acid (HCl) Hydrolysis of Cyanocobalamin^a

reaction no. ^b (x N HCl)	time	CN–Cbl (%)	peak 1 (%)	peak 2 (%)	peak 3 (%)	peak 4 (%)	peak 5 (%)	peak 6 (%)
Reaction Temperature of 50 °C								
Rxn 1 ^c (1.0 N)	10 min	65.2	2.1	2.7	1.8	0.2		
	1 h	50.0	9.2	13.9	8.3	6.2		
Rxn 2 ^c (0.5 N)	10 min	97.4	0.4	0.7	0.4			
	30 min	81.9	3.5	6.4	3.7	0.5		
	1 h	68.1	6.3	11.0	6.4	1.5		
	2 h	45.5	9.3	16.8	9.3	7.2		
Rxn 3 ^c (0.1 N)	4 h	35.8	11.5	17.4	10.2	11.7		
	10 min	98.3	0.2	0.3				
	1 h	93.0	1.6	2.8	1.3			
	1.5 h	90.0	2.4	4.2	2.3			
	2 h	86.8	3.3	5.6	3.1			
	4.5 h	72.7	6.8	11.1	6.1	0.9		
	7 h	58.6	9.1	15.6	8.3	2.3	1.1	
Reaction Temperature of 37 °C								
Rxn 4 (0.5 N)	8 h	71.5	7.1	13.8	4.0			
	20 h ^b	27.3	8.6	18.8	8.6	10.4	10.9	4.9
Room-Temperature Reactions								
Rxn 5 (1.0 N)	1 h	92.6	0.6	2.1	1.4			
	2 h	81.9	1.6	5.4	3.4	0.4		
	5 h	68.1	2.9	9.2	5.8	1.2		
	1 d	38.4	11.3	16.9	9.4	9.0		
	4 d ^b	13.4	19.1	14.4	3.6	22.1		
Rxn 6 (0.5 N)	1 h	97.2	0.4	1.0	0.5			
	2 h	95.0	0.9	2.0	1.1			
	6 h	83.6	3.6	6.4	3.6	0.2		
	1 d	55.9	7.9	15.3	8.3	4.5		
	4 d ^b	20.0	7.1	22.0	12.0	16.4		
Rxn 7 (0.1 N)	1 h	98.0						
	3 h	97.1	0.5	0.8	0.3			
	20 h	90.1	2.6	4.1	1.8			
	2 d	79.1	5.2	7.9	4.0	0.3		
	5 d	60.5	8.4	16.8	8.0	2.1	1.8	1.3
	6 d	55.3	9.0	18.9	8.7	2.8	2.2	1.8
	7 d	50.2	9.6	21.1	9.3	3.6	4.7	
	10 d	49.4	10.3	20.2	9.8	4.2	1.7	3.7
Rxn 8 (0.1 N)	1 d	92.3	2.2	3.7	1.8			
	2 d	85.8	4.1	6.7	3.3			
	3 d	79.3	5.6	9.3	4.7	0.4	0.6	
	8 d	55.3	9.3	18.4	9.3	3.0	0.8	3.0
	8 d	55.3	9.3	18.4	9.3	3.0	0.8	3.0
	9 d	51.9	10.0	19.5	9.5	3.6	1.4	3.3
	10 d	49.4	10.3	20.2	9.8	4.2	1.7	3.7
	11 d	44.4	14.6	19.3	10.9	4.1	1.9	3.8
Rxn 9 (0.1 N)	1 d	92.4	1.9	3.2	1.6			
	2 d	85.4	3.9	6.4	3.3			
	3 d	80.4	5.2	8.8	4.5			
	4 d	71.5	8.1	11.4	6.2	0.8	0.7	
	7 d	58.2	10.8	15.9	8.6	2.1	1.0	1.9
	8 d	54.8	11.5	17.3	9.4	2.6	1.2	2.3
	9 d	50.6	12.3	18.0	10.1	3.0	1.4	2.7
	11 d	44.4	14.6	19.3	10.9	4.1	1.9	3.8

^a For example, see Figure 2, panel A. ^b Reaction number does not indicate order in which experiments were conducted. ^c Additional unidentified HPLC peaks present.

using procedures from the literature (34, 37). Since an (aminopropyl)silica HPLC column gave very good separations of the monocarboxylic acids (49), a low-pressure liquid chromatograph preparative column containing aminopropyl packing was prepared and evaluated. Initial separations were disappointing; however, slowing the flow rate to 0.15 mL/min (4 d for elution) improved the separation. This method provided pure *d*-carboxylate, **3**, but some fractions of *b*- and *e*-carboxylates (**2** and **4**, respectively) did not resolve completely. Base line separations of all three isomeric cyanocobalamin monocarboxylic acids were obtained by using a second aminopropyl column of the same size in series with the first. Although the separation took 8 d to elute the isomers, the isolated fractions were well-separated, and thus, the purified samples could be used directly for derivatization.

Assignment of *b*-, *d*-, and *e*-isomers to the isolated chromatographed compounds was difficult and cannot be considered unequivocal. Assignment could not be made from simple spectroscopic data. However, previous in-

vestigations have shown that NMR could be used to distinguish the isomers (58, 59). Assignments were still somewhat ambiguous until an elaborate set of NMR experiments were conducted to assign all of the ¹H and ¹³C resonances of two of the three isomers (*b*- and *e*-; *d*-was insoluble in adequate quantities for the experiment), and those assignments were used to correlate their connectivities in two-dimensional NMR spectroscopy with structures (60). That study unambiguously assigned structures to all three isomers (*d*-isomer by default). The key to our assignment of isomers was to determine which HPLC peaks correlated with the structures defined by NMR. To do this, we evaluated the mixture of monocarboxylic acid isomers **2–4** (Figure 3) under the HPLC conditions used in the NMR study (58). To be certain of the elution order, purified isomers collected from preparative LC were individually examined under the previous HPLC conditions as well. The order of elution on the C-18 column was found to be *b*-, *d*-, and *e*-, as previously described. The order of elution of the mono-

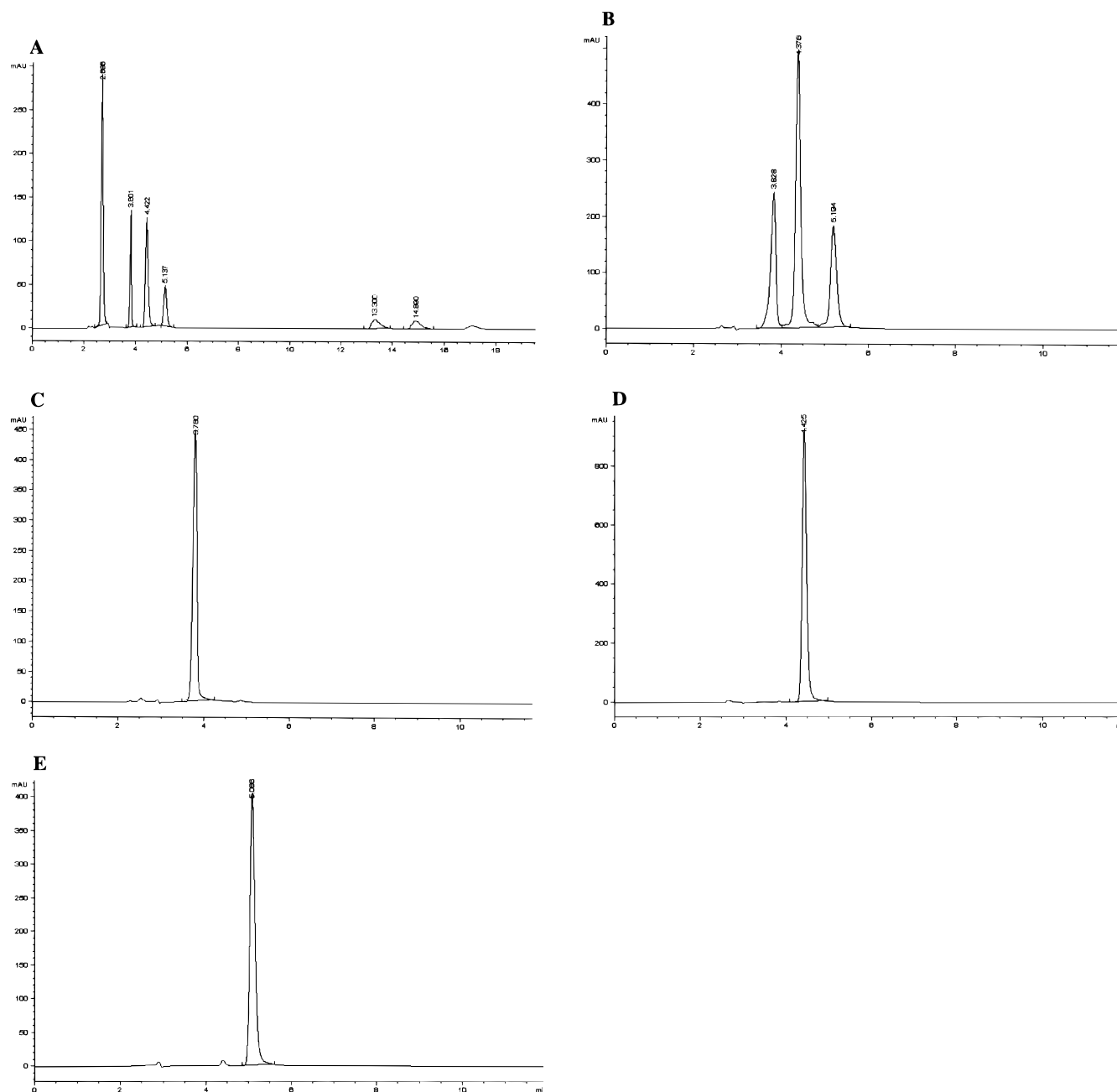


Figure 2. HPLC chromatograms showing hydrolysis of cyanocobalamin and isolation of its corrin ring side chain monocarboxylate products on an (aminopropyl)silica column. Chromatogram A shows the reaction mixture obtained after hydrolysis in 0.1 N HCl at room temperature for 11 days. The peak at 2.7 min is cyanocobalamin, peaks at 3.8, 4.4, and 5.1 min are monocarboxylates, and later peaks are those of di- and tricarboxylates. Chromatogram B shows the mixture of cobalamin monocarboxylates obtained after purification by ion chromatography. Chromatograms C–E show the purified carboxylates in the order of *d*-, *b*-, and *e*-, respectively (see discussion on structural assignments in the text). The HPLC conditions used for chromatograms A–E are listed in Materials and Methods. HPLC elutions were carried out for 30 min, but the chromatograms shown only display 12 or 20 min (chromatogram A) as there were no later-eluting compounds.

carboxylic acids on the (aminopropyl)silica column used in this investigation was *d*-, *b*-, and *e*-. Not surprisingly, the order of elution found on the (aminopropyl)silica column was the same as the previous investigators found on an anion exchange column, for which they initially assigned peaks I–III (58). An X-ray crystal structure of a cobalamin derivative of the *e*-monocarboxylic acid is being pursued to unequivocally assign that isomeric structure.

Coupling of the monocarboxylates with diaminododecane was first attempted using the water soluble carbodiimide, EDC, according to a published procedure (34). However, the major product obtained when the *b*-isomer, **2**, or *e*-isomer, **4**, was reacted did not have the correct mass (calculated, 1539 M + 1; found, 1512 *b*-isomer and

1513 *e*-isomer), and its ^1H NMR had unexpected shifts in the alkyl region.⁵ The structure of these cobalamin–diaminododecane adducts has not been elucidated, but the data appear to be consistent with loss of cyanide with subsequent coupling of the primary amine group with Co. Since it was suspected that the reaction involved loss of

⁵ The 500 MHz NMR spectrum had a triplet further upfield than any previously observed (1.06 ppm) corresponding to four protons. It also had a broad singlet at 1.29 ppm corresponding to 24 protons and a singlet at 2.39 ppm which were not seen in the spectrum for the diaminododecane adduct produced in the presence of cyanide ion. Interestingly, the unidentified diaminododecane adduct of **4** had nearly the same binding with rhTCII (50% decrease) as compared with its counterpart compound, the desired *e*-diaminododecane adduct **7**.

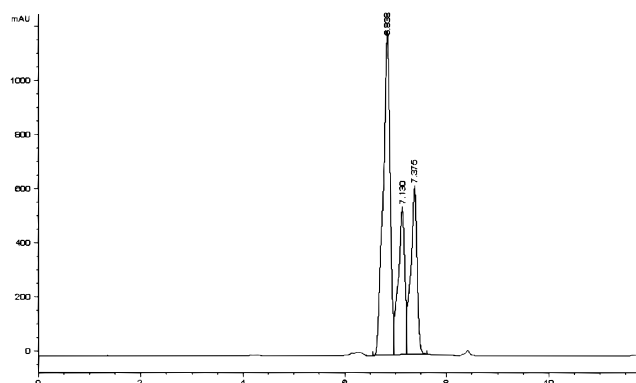


Figure 3. HPLC chromatogram of a mixture of isomeric cobalamin monocarboxylic acids **2–4** on a reverse-phase C-18 column. The order of elution is *b*- (**2**), *d*- (**3**), and *e*-monocarboxylates (**4**).

cyanide, it seemed that addition of more cyanide might give the desired product. Indeed, alteration of the reaction conditions by changing the reaction mixture to DMF/H₂O and adding NaCN and *N*-hydroxysuccinimide (**38**) provided the desired diaminododecane adducts, **5–7**. Unlike the previously obtained diaminododecane adducts, these conditions provided products that had the correct mass spectra and the expected ¹H NMR spectra. The conjugation to form conjugates, **8–10**, by reaction of **5**, **6**, or **7** with the NHS ester of biotin was facile.

Preparation of 5'-O-Ribose Conjugates **12 and **13**.** Other investigators have used succinylation of the 5'-O-ribose position to provide a carboxylate for conjugation (**31**, **46**). Although this produces an ester linkage, which may be cleaved *in vivo*, it was important to include conjugates at this position to evaluate how they affected binding with TCII. Conjugation to the 5'-hydroxyl group on the ribose ring was carried out initially by formation of the succinyl adduct **11** by reaction with succinic anhydride and pyridine in DMSO. As previously reported, the reaction was facile, providing an 85% yield after 14–16 h at room temperature. Conjugation of the succinic acid free carboxylate with diaminododecane was conducted using the conditions developed for its conjugation with the isomeric monocarboxylic acid. Thus, reaction of diaminododecane, EDC, NaCN, and NHS in DMF/H₂O provided a 70% yield of the desired adduct **12**. Conjugation of **12** with the NHS ester of biotin was more difficult than that of the monocarboxyl–diaminododecane adducts as the basic solution was incompatible with the succinate ester of **12**. Monitoring the reaction by HPLC indicated that a maximum yield could be obtained in 10–15 min under the conditions employed, after which time degradation decreased the amount of product present. Even with its instability to the reaction conditions, a 45% yield of **13** was obtained.

Preparation of Alkyl–Cobalt Conjugates **14 and **15**.** Many examples of cobalamin derivatives have been reported where the coupling has occurred on the cobalt atom (**29**, **30**). Although the products were expected to be light sensitive, making this position questionable for development of pharmaceuticals to be used as growth blockers, it seemed imperative that this position be evaluated in our studies as many other investigators have used derivatives that had been modified on the cobalt. The conjugations appear to be relatively facile as the reductive decyanation occurs very readily and the alkylation process is high yielding.

Our initial cobalt conjugation studies involved coupling of bromopropionic acid (**46**). We were interested in this product because it could potentially be derivatized in

Table 3. Binding of Cobalamin Derivatives with rhTCII Relative to CN-Cbl, **1**

cobalamin	50% binding inhibition ^a for [⁵⁷ Co]CN-Cbl	relative rhTCII ^b binding (%)
1	1	100
12	1	100
15	1.1	91
14	1.3	77
7	1.5	67
11	1.5	67
10	2	50
13	2.5	40
4	3	33
5	9	11
8	9.5	11
2	38	2.6
3	44	2.3
6	54	1.9
9	69	1.4
16	295	0.3
17	299	0.3
18	1160	0.09

^a The binding inhibition quantity is that amount (picomoles) of cobalamin required to obtain a 50% binding inhibition of 1 pmol of cyanocobalamin (containing trace [⁵⁷Co]cyanocobalamin) with rhTCII. ^b The relative rhTCII binding value (percent) is obtained by division 100 by the 50% binding inhibition quantity.

subsequent steps in a manner similar to that developed for the corrin ring monocarboxylates, **2–4**. Reaction of cyanocobalamin with Zn and bromopropionic acid appeared to yield the desired alkyl cobalt derivative by HPLC analysis. However, attempts to purify this compound proved unsuccessful due to its instability to light and column conditions. A diaminododecane adduct was formed upon reaction with the impure product, but it could not be readily purified either. Rather than developing conditions for preparing the propionate derivative, we targeted and prepared another Co adduct. In that reaction, chloropropylamine was reacted with reduced cyanocobalamin to yield the adduct **14** (**51**). The product obtained was a single entity by HPLC (>95%) which was used directly in the next step. Thus, crude **14** was reacted with the TFP ester of the aminocaproate adduct of biotin, **19**, to give the cobalamin–biotin conjugate **15**. Although sensitive to light, this compound appeared to be much less sensitive to degradation than the propionic acid derivative.

Preparation of c-Lactone Conjugates **17 and **18**.** Reaction of cyanocobalamin with NaI and NCS in 5% aqueous acetic acid results in the formation of a lactone ring from cyclization of the *c*-acetamide group, similar to previously reported reactions with I₂, Br₂, chloramine-T, and *N*-bromosuccinimide (**61–63**). This reaction is very efficient, yielding a nearly quantitative conversion within 16 h at room temperature. Although we were unaware of any previous reports on conjugation through ring opening of the lactone, it was reasoned that such a conversion might be achieved. In fact, reaction of the *c*-lactone, **16**, in a melt of neat diaminododecane at 80 °C for 4 h gave a 25% yield of the adduct **17**. Similar to the conjugation of diaminododecane with the corrin ring monocarboxylates, addition of excess cyanide ion was required to obtain the desired product. Higher yields of this adduct may be obtainable, but an optimization of the reaction was not conducted since the binding with rhTCII was found to be low (Table 3). Reaction conditions used to couple **17** with the NHS ester of biotin gave the desired biotin conjugate **18** in 40% yield.

TCII Binding Assays. Binding of the cobalamin derivatives to recombinant human transcobalamin II was conducted in nanomolar concentrations. The binding

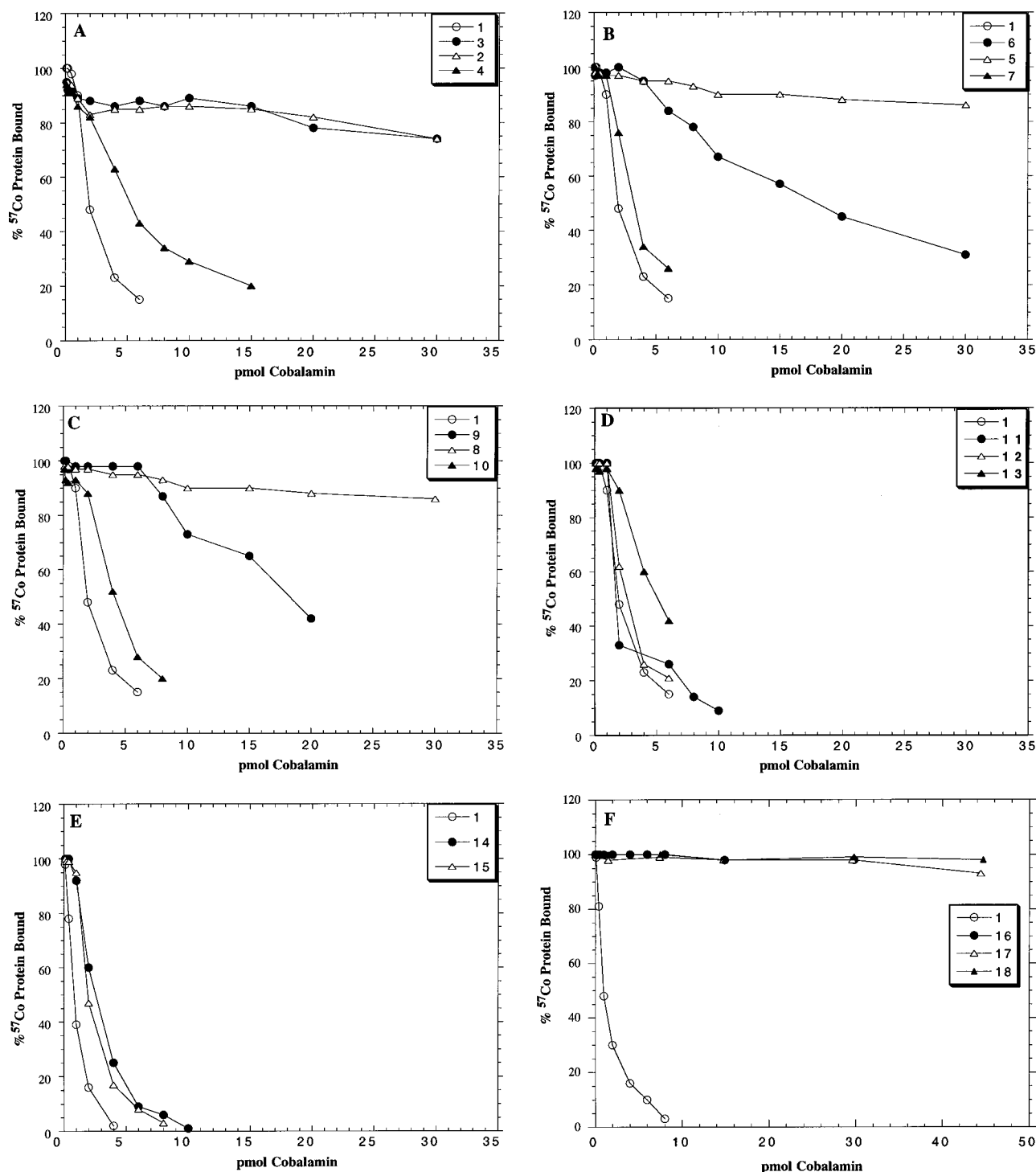


Figure 4. Competitive binding curves for [^{57}Co]cyanocobalamin, **1**, and cobalamin derivatives **2–18** with rhTCII. A quantity of rhTCII that would bind approximately 1 pmol of CN-Cbl was mixed with 0.01 pmol of [^{57}Co]cyanocobalamin and 0.01–30 pmol of unmodified **1** or cobalamin derivatives **2–18**. This mixture was diluted with PBS containing HSA and incubated at rt for 1 h. Free cobalamin was adsorbed onto a hemoglobin-coated charcoal column by batchwise mixing, and the amount of protein-bound radioactivity in the supernatant was measured in a γ counter. The amount of protein-bound ^{57}Co versus the amount of cobalamin derivative present is plotted in graphs shown as panels A–F. Panel A compares the competitive binding of the three monocarboxylates **2–4** with unmodified **1**. Panel B compares the competitive binding of the diaminododecane adducts **5–7** with **1**. Panel C compares the competitive binding of the biotin-containing cobalamin derivatives **8–10** with **1**. Panel D compares the binding of cobalamin-5'-*O*-ribose derivatives **11–13** with **1**. Panel E compares the binding of (aminopropyl)cobalamin derivatives **14** and **15** with **1**. Panel F compares the *c*-lactone derivative **16**, its diaminododecane adduct **17**, and biotinylated derivative **18** with **1**.

curves for the cobalamin derivatives are shown in panels A–F of Figure 4. The three cobalamin monocarboxylic acids, **2–4**, are shown with cyanocobalamin, **1**, in panel A. The binding of all carboxylic acid isomers with rhTCII is decreased relative to that of cyanocobalamin, but the decrease in *e*-isomer, **4**, binding was relatively small compared with that of *d*- and *b*-isomers, **3** and **2**,

respectively. Panel B of Figure 4 compares the binding of the diaminododecane adducts of the isomeric monocarboxylic acids, **5–7**, with cyanocobalamin. Interestingly, conversion of the monocarboxylic acids to substituted amides appeared to improve the binding of the *e*- and *b*-isomers. Indeed, the *e*-isomer, **7**, bound nearly as well as cyanocobalamin, and the *b*-isomer, **5**, had sig-

nificantly improved binding. Panel C of Figure 4 depicts the binding of biotinylated adducts **8–10** relative to cyanocobalamin. The binding results obtained parallel those for their precursors **5–7**. Panel D of Figure 4 depicts the rhTCII binding of the 5'-O-ribose adducts **11–13** relative to cyanocobalamin. Importantly, all of the derivatives appear to bind very competitively, with the biotin adduct **13** having only slightly decreased binding. With the presence of an ester linkage in the molecule, the question of its cleavage in the *in vitro* test must be considered. However, it seems unlikely that cleavage would occur in this assay.⁶ Panel E of Figure 4 compares the Co-conjugated derivatives **14** and **15** with cyanocobalamin. Very little decrease in binding as compared to that of cyanocobalamin was noted. Again, however, the potential for loss of the conjugated group from the cobalamin has to be considered. This too seems unlikely, as the loss of the alkyl moiety from cobalt should be accompanied by a shift in the λ_{max} of the UV absorbance, which was not observed. Panel F of Figure 4 compares c-lactone, **16**, its diaminododecane adduct **17**, and the biotinylated adduct **18** with cyanocobalamin.

DISCUSSION

We are investigating depletion of cellular cobalamins as a novel method of therapy for AIDS-related lymphoma. Prior studies have shown that inactivation of cobalamin by the use of the general anesthetic nitrous oxide (N₂O) had a therapeutic effect on patients with leukemia (16, 17). Unfortunately, this approach to therapy has severe limitations due to the length of time required for the patient to be in a N₂O-containing atmosphere, and some other apparent side effects. As an alternative method, we are attempting to deplete the cell of cobalamin by blocking its entry into cells. Since entry of cobalamin into cells at normal physiological concentrations of cobalamin (25, 64–66) is governed by receptor-mediated endocytosis, blockage of this receptor system could deplete the cells of cyanocobalamin. One potential method for blocking the entry of cobalamin into cells is to deplete the cell surface of receptor molecules. It seems possible that cobalamin derivatives could be internalized (67) and alter the endosomal/lysosomal recycling of the receptor and/or cobalamin derivative.

Cyanocobalamin and related cobalamin derivatives are of critical importance to growth and survival of mammalian cells. To compensate for the limited quantities of cobalamins [e.g. 3 mg in the human body with 5 μ g in diet/d (43)], there are specific protein systems that efficiently absorb, transport, and recycle these molecules (9, 25, 64, 65). In blood, cobalamins are bound to the transport protein TCII. TCII, which contains bound cobalamin (holoTCII), binds with a specific cell surface receptor. Once bound to the receptor, this complex is endocytosed into cells. The binding of cobalamin to TCII is a critical step in this process as these molecules do not bind with the cell surface receptor independently. Once in the cell, cobalamin derivatives are released through an as yet undefined process.

Our interest in blocking the cellular uptake of cyanocobalamin to effect a therapeutic response in rapidly proliferating cells makes it imperative that the cyanocobalamin derivatives prepared bind competitively with TCII. Thus, our first objective was to determine how the location of cobalamin conjugation affected binding with

TCII. After evaluating various positions that might be used for conjugation (Figure 1), we chose to conjugate with the corrin ring positions as acetamide and propionamide side groups designated *b*-, *c*-, *d*-, and *e*-; on the cobalt metal in place of the cyano group; and on the 5'-OH of the ribose moiety. No methods of conjugation with the phosphate group have been defined; therefore, we did not attempt to conjugate at that location. Conjugations which first required substitution of a different benzimidazole or propanolamine moiety were not chosen as large quantities of new cobalamin derivatives would have to be made (from microorganism cultures) before conjugation reactions were conducted. From earlier data in the literature, it appeared that conjugation of an appropriately substituted benzimidazole would have less effect on binding with TCII than a similar conjugation with a substituted derivative of the propanolamine. Thus, conjugation at the benzimidazole moiety was of interest but for the purpose of this study was not pursued.

Human TCII is a 45 kDa protein which binds very tightly with cyanocobalamin, hydroxycobalamin, methylcobalamin, and 5'-deoxyadenosylcobalamin (25, 64). The protein sequence of TCII is known, and its gene has been cloned (66–69), but its three-dimensional structure has not yet been elucidated. The structure of holoTCII (i.e. containing bound cyanocobalamin) is currently under investigation. However, since the native protein structure is not yet known, the exact location and environment of TCII binding has not been determined with certainty. This has made it difficult to evaluate the minimal length of a spacer molecule for separation of the cobalamin moiety from other conjugated molecules. It is known that extended length linkers are needed to facilitate binding with a cobalamin dependent enzymes (46, 47), so it might be concluded that this will also be necessary for TCII. Indeed, in the case of biotin, almost every atom in the molecule contributes to its extremely tight binding with avidin (70), and the need for a linker in derivatives with a minimum of six atoms has been well-documented (45). Since the two moieties were to be combined, it was surmised that a long chain linking molecule would have to be used. 1,12-Diaminododecane was chosen for the linker molecule as conjugations with this molecule had previously been reported (34, 46, 47). Considering the fact that 1,12-diaminododecane introduces a 14-atom spacer, we felt that this might be sufficient distance between the cobalamin and biotin moieties. Diaminododecane was also used as the linking moiety when the conjugation was made at the 5'-OH of the ribose, but an additional 4-atom spacer (succinate) was used to introduce a carboxylate for conjugation. Our initial plan was to also use the diaminododecane as a linker on the cobalt-conjugated biotin as well, but difficulties with the stability of Co-linked propionic acid led us to prepare another derivative. While it is likely that an adduct of mono-*t*-Boc-protected diaminododecane (or biotinylated diaminododecane) and 3-chloropropionic acid could be used to prepare the Co-conjugated derivative, the ease of preparing and working with Co-carbon-coupled aminopropane adduct made it the reaction sequence of choice. Coupling of the propylamine-cobalamin derivative with the adduct of aminocaproic acid and biotin to give **18** was found to be quite facile; however, that adduct's purification was found to be quite difficult due to its instability. The Co-conjugated cobalamin-biotin adduct had an 11-atom spacer, which was thought to be adequate for our evaluation. Data published recently by Russell-Jones et al. (39, 40) showed that increasing the spacer length beyond that of a diaminododecane gave increased binding when a cobalamin conjugate was evaluated. Studies

⁶ The cobalamin ester, **12**, was found to be relatively unstable even at 4 °C, undergoing up to 50% hydrolysis (to **1**) of the stored samples within 7–8 months. The biotin derivative **13** was also found to be somewhat unstable on storage.

planned which include coupling of the biotin moiety with streptavidin after binding with rhTCII will help in the identification of the length of the spacer arm needed for optimal binding.

The cobalamin derivatives synthesized in this study were examined for competitive binding with cyanocobalamin. The data obtained (Figure 4, panels A–F) indicate that conjugation (alkylation) on the cobalt metal site does not significantly decrease the binding to rhTCII. Other investigators had previously come to this conclusion for both TCII and IF (41, 62). The data also indicate that conjugation with the 5'-O-ribose moiety does not significantly decrease the binding with rhTCII. Conjugation with the corrin ring substituents, however, did show decreased binding in all locations. The corrin B-pyrroline ring with substituents on the *c*- and *d*-side chains had dramatically decreased binding, and conjugation with the pyrroline A ring on the *b*-propionamide appeared to decrease the binding to an intermediate degree. The *e*-monocarboxylate was found to bind to rhTCII nearly as well as cyanocobalamin.

The very low binding observed for the *c*-lactone is consistent with previous studies that have shown low binding with this compound. Also, the low bindings observed with the *c*-lactone adducts **17** and **18** are consistent with the low binding seen for the adducts of the *d*-monocarboxylic acids, which is in close proximity on the B-pyrroline ring (Table 1). Interestingly, Hippe et al. (27, 41) concluded that "changes in the B ring hampers binding of all three (transport) proteins" and that the A ring with its side chain may be involved in binding. In apparent contrast, Lien et al. reported that the hydrolyzed product of the *c*-lactone, the *c*-monocarboxylic acid, "forms a strong complex with intrinsic factor" (62). Their binding assay, however, was not a competitive assay, as they first incubated the hydrolyzed lactone over a 1 h period prior to adding the [⁵⁷Co]cyanocobalamin. The binding of the *c*-monoacid was strong enough to have a slow dissociation, but it is unclear how it might have competed with [⁵⁷Co]cyanocobalamin in a mixture of the two compounds. However, the same authors went on to conclude that the B-pyrroline ring of the corrin ring system is extremely important because of the relatively poor binding observed with the *c*-lactone and IF. It is readily apparent that the corrin B-pyrroline ring must be intimately involved in binding with TCII and that the A ring is also involved. The portion of the corrin ring that contains the C-pyrroline ring must have little or no involvement in the binding. Even though it may be important, it is not clear how the stereochemistry (above or below the corrin ring) of the substituents affects the binding. Some of these issues may not be resolved until a crystal structure of the holoTCII is obtained.

Our studies show that binding nearly equivalent to that of cyanocobalamin itself can be obtained when modifications are made on the Co metal, on the 5'-OH of the ribose, and on the *e*-propionamide side group of the corrin ring. For the purposes of preparing bioconjugates as pharmaceuticals to be used in treatment of human diseases, the Co-conjugated and ribose-conjugated derivatives prepared in this evaluation are probably unsuitable. The Co-conjugated biotin derivative **15** is quite susceptible to degradation by light, and intracellular enzymes are capable of efficiently cleaving this bond to substitute other moieties (71, 72). The ester-containing conjugate **13** is potentially susceptible *in vivo* to esterases. Therefore, the *e*-propionamide side chain is the location of choice for conjugation in our studies. It is unfortunate that this is the minor isomer, but it is likely

that this isomer will have fewer problems with stability. The other corrin ring isomers are also of interest, as derivatives with decreased binding can be used as negative controls in our studies, and may have other applications where decreased binding with transport proteins is desired.

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LITERATURE CITED

- (1) Milgrom, L. R. (1994) Vitamin B₁₂ the view from the summit. *Chem. Br.* 31, 923–927.
- (2) Battersby, A. R. (1994) How Nature Builds the Pigments of Life: The Conquest of Vitamin B₁₂. *Science* 264, 1551–1557.
- (3) Bonnet, R. (1963) The Chemistry of the Vitamin B₁₂ Group. *Chem. Rev.* 63, 573–605.
- (4) Smith, E. L. (1965) *Vitamin B₁₂*, John Wiley & Sons, New York.
- (5) Sebrell, W. H., and Harris, R. S., Eds. (1968) Vitamin B₁₂. *The Vitamins; Chemistry, Physiology, Pathology, Methods*, Chapter 4, pp 119–259, Academic Press, New York.
- (6) Kirschbaum, J. (1981) Cyanocobalamin. In *Analytical Profiles of Drug Substances* (K. Florey, Ed.) Vol. 10, pp 183–288, Academic Press, New York.
- (7) Hogenkamp, H. P. C. (1975) The Chemistry of Cobalamins and Related Compounds. In *Cobalamin, Biochemistry and Pathophysiology* (B. M. Babor, Ed.) Chapter 1, pp 21–73, John Wiley & Sons, New York.
- (8) Linnell, J. C. (1975) The Fate of Cobalamins *in vivo*. In *Cobalamin, Biochemistry and Pathophysiology* (B. M. Babor, Ed.) pp 287–333, John Wiley & Sons, New York.
- (9) Ellenbogen, L. (1975) Absorption and Transport of Cobalamin. In *Cobalamin, Biochemistry and Pathophysiology* (B. M. Babor, Ed.) pp 215–286, John Wiley & Sons, New York.
- (10) Mahoney, M. J., and Rosenberg, L. E. (1975) Inborn Errors of Cobalamin Metabolism. In *Cobalamin, Biochemistry and Pathophysiology* (B. M. Babor, Ed.) pp 369–402, John Wiley & Sons, New York.
- (11) Beck, W. S. (1975) Metabolic Features of Cobalamin Deficiency in Man. In *Cobalamin, Biochemistry and Pathophysiology* (B. M. Babor, Ed.) pp 403–450, John Wiley & Sons, New York.
- (12) Gimpert, E., Jakob, M., and Hitzig, W. H. (1975) Vitamin B₁₂ transport in blood. I. Congenital deficiency of transcobalamin II. *Blood* 45, 71–82.
- (13) Scott, C. R., Hakami, N., Teng, C. C., and Sagerson, R. N. (1972) Hereditary transcobalamin II deficiency: the role of transcobalamin II in vitamin B₁₂-mediated reactions. *J. Pediatr.* 81, 1106–1111.
- (14) Hitzig, W. H., Dohmann, U., Pluss, H. J., and Vischer, D. (1974) Hereditary transcobalamin II deficiency: clinical findings in a new family. *J. Pediatr.* 85, 622–628.
- (15) Faludy, J. E., and Linnell, J. C. (1990) Cobalamin metabolism in human leukaemia cell lines: a pilot study. In *Biomedicine and Physiology of Vitamin B₁₂* (J. C. Linnell and H. R. Bhatt, Eds.) pp 199–205, Children's Medical Charity, London.
- (16) Lassen, H. C. A., and Kristensen, H. S. (1959) Remission in chronic myeloid leukaemia following prolonged nitrous oxide inhalation. *Dan. Med. Bull.* 6, 252–254.
- (17) Eastwood, D. W., Green, C. D., Lambdin, M. A., and Gardner, R. (1963) Effect of nitrous oxide on the white-cell count in leukemia. *N. Engl. J. Med.* 268, 297–299.
- (18) Linnell, J. C., Quadros, E. V., Matthews, D. M., Jackson, B., and Hoffbrand, A. V. (1978) Nitrous Oxide and Megaloblastosis: Biochemical Mechanism. *Lancet*, 1372–1373.
- (19) Kondo, H., Osborne, M. L., Kolhouse, J. F., Binder, M. J., Podell, E. R., Utley, C. S., Abrams, R. S., and Allen, R. H. (1981) Nitrous oxide has multiple deleterious effects on cobalamin metabolism and causes decreases in activities of both mammalian cobalamin-dependent enzymes in rats. *J. Clin. Invest.* 67, 1270–1283.

- (20) Banerjee, R. V., and Matthews, R. G. (1990) Cobalamin-Dependent Methionine Synthase. *FASEB J.* 4, 1450–1459.
- (21) Kroes, A. C. M., Ermens, A. A. M., Lindemans, J., and Abels, J. (1986) Effects of 5-Fluorouracil Treatment of Rat Leukemia with Concomitant Inactivation of Cobalamin. *Anticancer Res.* 6, 737–742.
- (22) Kroes, A. C. M., Lindemans, J., and Abels, J. (1984) Synergistic growth inhibiting effect of nitrous oxide and cycloleucine in experimental rat leukaemia. *Br. J. Cancer* 50, 793–800.
- (23) Kroes, A. C. M., Lindemans, J., Schoester, M., and Abels, J. (1986) Enhanced therapeutic effect of methotrexate in experimental rat leukemia after inactivation of cobalamin (vitamin B₁₂) by nitrous oxide. *Cancer Chemother. Pharmacol.* 17, 114–120.
- (24) Ermens, A. A. M., Kroes, A. C. M., Lindemans, J., and Abels, J. (1990) Cobalamin inactivation as a target in cancer chemotherapy. In *Biomedicine and Physiology of Vitamin B₁₂* (J. C. Linnell and H. R. Bhatt, Eds.) pp 229–235, Children's Medical Charity, London.
- (25) Seetharam, B. (1994) Gastrointestinal Absorption and Transport of Cobalamin (Vitamin B₁₂). In *Physiology of the Gastrointestinal Tract* (L. R. Johnson, Ed.) pp 1997–2026, Raven Press, New York.
- (26) Hall, C. A. (1990) The role in Transcobalamin II in the Cellular Uptake of Cobalamin. In *Biomedicine and Physiology of Vitamin B₁₂* (J. C. Linnell and H. R. Bhatt, Eds.) pp 239–253, Children's Medical Charity, London.
- (27) Hippe, E., and Olesen, H. (1971) Nature of Vitamin B₁₂ Binding III. Thermodynamics of Binding to Human Intrinsic Factor and Transcobalamins. *Biochim. Biophys. Acta* 243, 83–89.
- (28) Golding, B. T. (1979) Vitamin B₁₂. In *Comprehensive Organic Chemistry: The Synthesis and Reactions of Organic Chemistry* (S. D. Barton and W. D. Ollis, Eds.) pp 549–584, Pergamon Press, New York.
- (29) Dolphin, D. (1971) Preparation of the Reduced Forms of Vitamin B₁₂ and of Some Analogs of the Vitamin B₁₂ Coenzyme Containing a Cobalt-Carbon Bond. *Methods Enzymol.* 18c, 34–54.
- (30) Hogenkamp, H. P. C. (1982) Reactions of Alkyl Ligands Coordinated to Cobalamins and Cobaloximes. In *B₁₂: Volume 1 - Chemistry* (Dolphin, D., Ed.) pp 295–323, John Wiley & Sons, New York.
- (31) Toraya, T., Ohashi, K., Ueno, H., and Fukui, S. (1975) Preparation, Properties and Biological Activities of Succinyl Derivatives of Vitamin B₁₂. *Bioinorg. Chem.* 4, 245–255.
- (32) Annunziato, M. E., Patel, U. S., Ranade, M., and Palumbo, P. S. (1993) *p*-Maleimidophenyl Isocyanate: A Novel Heterobifunctional Linker for Hydroxyl to Thiol Coupling. *Bioconjugate Chem.* 4, 212–218.
- (33) Morley, C. G. D., Blakley, R. L., and Hogenkamp, H. P. C. (1968) Analogs of Deoxyadenosylcobalamin with Alterations in a Side Chain of the Corrin Ring. *Biochemistry* 7, 1231–1239.
- (34) Yamada, R.-H., and Hogenkamp, H. P. C. (1972) The Synthesis of a 5'-Deoxyadenosylcobalamin-agarose Adsorbent and Its Utility in the Purification of Ribonucleotide Reductase. *J. Biol. Chem.* 247, 6266–6270.
- (35) Allen, R. H., and Majerus, P. W. (1972) Isolation of Vitamin B₁₂-Binding Proteins Using Affinity Chromatography. *J. Biol. Chem.* 247, 7695–7701.
- (36) Kenley, J. S., Leighton, M., and Bradbeer, C. (1978) Transport of Vitamin B₁₂ in *Escherichia coli*, Corrinoid Specificity of the Outer Membrane Receptor. *J. Biol. Chem.* 253, 1341–1346.
- (37) Toraya, T., Krodell, E., Mildvan, A. S., and Abeles, R. H. (1979) Role of Peripheral Side Chains of Vitamin B₁₂ Coenzymes in the Reaction Catalyzed by Dioldehydrase. *Biochemistry* 18, 417–426.
- (38) Huber, E., Dieckhoff, J., Klein, C., and Kürzinger, K. (1992) Cyanocobalamin Acid Hydrazides and Their Conjugates. U.S. Patent 5,171,679.
- (39) Russell-Jones, G. J., Westwood, S. W., Farnworth, P. G., Findlay, J. K., and Burger, H. G. (1995) Synthesis of LHRH Antagonists Suitable for Oral Administration via the Vitamin B₁₂ Uptake System. *Bioconjugate Chem.* 6, 34–42.
- (40) Russell-Jones, G. J. (1995) Vitamin B₁₂ Mediated Oral Delivery Systems for Granulocyte-Colony Stimulating Factor and Erythropoietin. *Bioconjugate Chem.* 6, 459–465.
- (41) Hippe, E., Haber, E., and Olesen, H. (1971) Nature of Vitamin B₁₂ Binding, II. Steric Orientation of Vitamin B₁₂ on Binding and Number of Combining Sites of Human Intrinsic Factor and the Transcobalamins. *Biochim. Biophys. Acta* 243, 75–82.
- (42) Kolhouse, J. F., and Allen, R. H. (1977) Absorption, Plasma Transport, and Cellular retention of Cobalamin Analogues in the Rabbit: Evidence for the existence of multiple mechanisms that prevent the absorption and tissue dissemination of naturally occurring cobalamin analogues. *J. Clin. Invest.* 60, 1381–1392.
- (43) Kondo, H., Kolhouse, J. F., and Allen, R. H. (1980) Presence of Cobalamin Analogues in Animal Tissue. *Proc. Natl. Acad. Sci. U.S.A.* 77, 817–821.
- (44) Heinrich, H. C., and Gabbe, E. E. (1965) Intestinale Resorbierbarkeit und Vitamin B₁₂-antagonistische Wirkungen eines hochwirksamen B₁₂-Antivitamins aus der Reihe der Alkanolamin-Analoga des Vitamin B₁₂ beim Menschen. *Klin. Wochenschr.* 43, 196–201.
- (45) Green, N. M., Konieczny, L., Toms, E. J., and Valentine, R. C. (1971) The Use of Bifunctional Biotinyl Compounds to Determine the Arrangement of Subunits in Avidin. *Biochem. J.* 125, 781–791.
- (46) Toraya, T., and Fukui, S. (1980) Immobilized Derivatives of Vitamin B₁₂ Coenzyme and Its Analogs. *Methods Enzymol.* 67, 57–66.
- (47) Toraya, T., and Fukui, S. (1980) The Synthesis of Several Immobilized Derivatives of Vitamin B₁₂ Coenzyme and Their use as Affinity Adsorbents for a Study of Interactions of Diol Dehydrase with the Coenzyme. *J. Biol. Chem.* 255, 3520–3525.
- (48) Hogenkamp, H. P. C., and Rush, J. E. (1967) Aquocobalamin. *Biochem. Prep.* 12, 121–124.
- (49) Lindemans, J. (1992) Cobalamins. In *Modern Chromatographic Analysis of Vitamins* (A. Leenheer, W. E. Lambert, and H. J. Nelis, Eds.) 2nd Ed., pp 469–513, Marcel Dekker, Inc., New York.
- (50) Frenkel, E. P., Kitchens, R. L., and Prough, R. (1979) High Performance Liquid Chromatographic Separations of Cobalamins. *J. Chromatogr.* 174, 393–400.
- (51) Jacobsen, D. W., Montejano, Y. D., and Huennekens, F. M. (1981) Rapid Purification of Cobalamin-Binding Proteins using Immobilized Aminopropylcobalamin. *Anal. Biochem.* 113, 164–171.
- (52) Gamper, H. B., Reed, M. W., Cox, T., Viroso, J. S., Adams, A. D., Gall, A. A., Scholler, J. K., and Meyer, R. B. (1993) Facile preparation of nuclease resistant 3' modified oligodeoxynucleotides. *Nucleic Acids Res.* 21, 145–150.
- (53) Quadros, E. V., Sai, P., and Rothenberg, S. P. (1993) Functional human transcobalamin II isoproteins are secreted by insect cells using the Baculovirus expression system. *Blood* 81, 1239–1245.
- (54) Quadros, E. V., Rothenberg, S. P., Pan, Y. Ch. E., and Stein, S. (1986) Purification and molecular characterization of human transcobalamin II. *J. Biol. Chem.* 261, 15455–15460.
- (55) Lau, K. S., Gottlieb, C., Wasserman, L. R., and Herbert, V. (1965) Measurement of serum vitamin B₁₂ level using radioisotope dilution and coated charcoal. *Blood* 26, 202–214.
- (56) Armitage, J. B., Cannon, J. R., Johnson, A. W., Parker, L. F. J., Smith, E. L., Stafford, W. H., and Todd, A. R. (1953) Chemistry of the Vitamin B₁₂ Group. Part III. The Course of Hydrolytic Degradations. *J. Chem. Soc.*, 3849–3864.
- (57) Bernhauer, V. K., Vogelmann, H., and Wagner, F. (1968) Gewinnung und Charakterisierung von Corrinoidcarbon-säure-Isomeren (Chemistry and biochemistry of corrinoids. XXVIII. Isolation and characterization of carboxylic acid isomers). *Hoppe-Seyler's Z. Physiol. Chem.* 349, 1281–1296.
- (58) Anton, D. L., Hogenkamp, H. P. C., Walker, T. E., and Matwiyoff, N. A. (1980) Carbon-13 Nuclear Magnetic Resonance Studies of the Monocarboxylic Acids of Cyanocobalamin. Assignments of the *b*-, *d*-, and *e*-Monocarboxylic Acids. *J. Am. Chem. Soc.* 102, 2215–2219.

- (59) Anton, D. L., Hogenkamp, H. P. C., Walker, T. E., and Matwiyoff, N. A. (1982) Carbon-13 Nuclear Magnetic Resonance Studies of Cyanocobalamin and Several of Its Analogues. *Biochemistry* 21, 2372–2378.
- (60) Pagano, T. G., and Marzilli, L. G. (1989) Vitamin B₁₂ Monocarboxylic Acids: Unambiguous Isomer Assignments by Modern Two-Dimensional NMR Spectroscopy. *Biochemistry* 28, 7213–7223.
- (61) Wagner, F. (1965) Reactions of the cyano and alkyl cobalamins. *Proc. R. Soc. London, Ser. A* 288, 344–347.
- (62) Lien, E. L., Ellenbogen, L., Law, P. Y., and Wood, J. M. (1974) Studies on the Mechanism of Cobalamin Binding to Hog Intrinsic Factor. *J. Biol. Chem.* 249, 890–894.
- (63) Bonnett, R. (1982) Reactions of the Corrin Macrocyclic. In *B₁₂: Volume 1 - Chemistry* (D. Dolphin, Ed.) pp 201–243, John Wiley & Sons, New York.
- (64) Allen, R. H. (1975) Human Vitamin B₁₂ Transport Proteins. *Prog. Hematol.* 9, 57–84.
- (65) Neale, G. (1990) B₁₂ Binding Proteins. *Gut* 31, 59–63.
- (66) Platica, O., Janeczko, R., Quadros, E. V., Regec, A., Romain, R., and Rothenberg, S. P. (1991) The cDNA Sequence and the Deduced Amino Acid Sequence of Human Transcobalamin II Show Homology with Rat Intrinsic Factor and Human Transcobalamin I. *J. Biol. Chem.* 266, 7860–7863.
- (67) Jacobsen, D. W., Amagasaki, T., and Green, R. (1990) Synthesis and recycling of the Transcobalamin II receptor. In *Biomedicine and Physiology of Vitamin B₁₂* (J. C. Linnell and H. R. Bhatt, Eds.) pp 293–306, Children's Medical Charity, London.
- (68) Li, N., Seetharam, S., and Seetharam, B. (1995) Genomic Structure of Human Transcobalamin II: Comparison to Human Intrinsic Factor and Transcobalamin I. *Biochem. Biophys. Res. Commun.* 208, 756–764.
- (69) Regec, A., Quadros, E. V., Platica, O., and Rothenberg, S. P. (1995) The Cloning and Characterization of the Human Transcobalamin II Gene. *Blood* 85, 2711–2719.
- (70) Green, N. M. (1975) Avidin. *Adv. Protein Chem.* 29, 85–133.
- (71) Quadros, E. V., Jackson, B., Hoffbrand, A. V., and Linnell, J. C. (1979) Interconversion of Cobalamins in Human Lymphocytes in Vitro and the Influence of Nitrous Oxide on Synthesis of Cobalamin Coenzymes. *Vitamin B₁₂* (B. Zagalak and W. Friedrich, Eds.) pp 1045–1054, Walter de Gruyter & Co., New York.
- (72) Quadros, E. V., and Jacobsen, D. W. (1995) The dynamics of cobalamin utilization in L-1210 mouse leukemia cells: a model of cellular cobalamin metabolism. *Biochim. Biophys. Acta* 1244, 395–403.

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Enhanced Binding and Inertness to Dehalogenation of α -Melanotropic Peptides Labeled Using *N*-Succinimidyl 3-Iodobenzoate

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Two peptides of potential utility for targeting melanoma cells, α -melanocyte-stimulating hormone (α -MSH) and its more potent analogue [Nle⁴,D-Phe⁷]- α -MSH, were radioiodinated in 45–65% yield using *N*-succinimidyl 3-[¹²⁵I]iodobenzoate (SIB). To determine whether this labeling method resulted in improved *in vitro* and *in vivo* characteristics, these peptides also were labeled with ¹³¹I by direct iodination with the iodogen method. For α -MSH, the rapid tissue clearance of both radionuclides in mice was consistent with rapid degradation of the peptide; however, significantly lower levels of ¹²⁵I were observed in thyroid and stomach, reflecting a greater inertness to deiodination. More extensive comparisons were performed with [Nle⁴,D-Phe⁷]- α -MSH. The *in vitro* binding of [Nle⁴,D-Phe⁷,Lys¹¹-(¹²⁵I)IBA]- α -MSH (prepared using SIB) to the murine B-16 melanoma cell line, $34.1 \pm 4.7\%$, was more than twice as high as that for [Tyr²(¹³¹I),Nle⁴,D-Phe⁷]- α -MSH ($15.0 \pm 0.1\%$), and its K_D was more than 10-fold lower than that for conventionally labeled peptide (10 ± 5 versus 140 ± 14 pM). The normal tissue clearance of [Nle⁴,D-Phe⁷,Lys¹¹-(¹²⁵I)IBA]- α -MSH in mice was faster than that of [Tyr²(¹³¹I),Nle⁴,D-Phe⁷]- α -MSH. The 19–40-fold lower activity concentrations of [Nle⁴,D-Phe⁷,Lys¹¹-(¹²⁵I)IBA]- α -MSH in tissues accumulating free iodide (thyroid and stomach) suggest a greater inertness of this peptide to deiodination. The primary urinary catabolite of [Nle⁴,D-Phe⁷,Lys¹¹-(¹²⁵I)IBA]- α -MSH was the lysine conjugate of iodobenzoic acid, whereas radioiodide was the chief catabolite generated from [Tyr²(¹³¹I),Nle⁴,D-Phe⁷]- α -MSH. We conclude that further evaluation of [Nle⁴,D-Phe⁷,Lys¹¹-(¹²⁵I)IBA]- α -MSH for targeting α -MSH receptors is warranted and that SIB may be a useful method for the radioiodination of peptides.

INTRODUCTION

Radiolabeled peptides, including those radioiodinated directly on constituent tyrosine residues, have been valuable tools for *in vitro* evaluation of receptor status and other biochemical processes. Recent investigations have extended this approach to determine whether peptides could be exploited for the selective delivery of radionuclides to specific cell populations. Examples of this strategy include the evaluation of chemotactic peptides for imaging infection (Babich et al., 1993a,b) and octreotide analogues (Bakker et al., 1991; Krenning et al., 1992) for the delineation of somatostatin receptor positive tumors. *In vivo* applications such as these provide additional constraints for peptide labeling; the labeling method should yield a stable bond between the radionuclide and the peptide and result in the generation of labeled catabolites which are excreted rapidly from normal tissues.

Iodine radionuclides are attractive for *in vivo* applications (Larson and Carrasquillo, 1988) because their wide range of decay properties is well-suited for single photon emission tomography (¹²⁵I), positron emission tomographic imaging (¹²⁴I), β -particle therapy (¹³¹I), and Auger/conversion electron therapy (¹²³I and ¹²⁵I). Unfortunately, direct radioiodination of tyrosine residues on peptides via electrophilic substitution often yields a

molecule which is susceptible to deiodination *in vivo* (Bakker et al., 1990, 1991). In addition, the oxidants used in the labeling reaction can alter amino acids such as methionine and, as a result, sometimes have a deleterious effect on the affinity of labeled peptide binding to receptor (Eberle et al., 1991).

Previous work from our laboratory (Zalutsky and Narula, 1987, 1988; Zalutsky et al., 1989a; Schuster et al., 1991) and other groups (Wilbur et al., 1989; Badger et al., 1990) has demonstrated that use of *N*-succinimidyl 3- or 4-iodobenzoate (SIB) for labeling proteins significantly reduced thyroid uptake (an *in vivo* indicator of dehalogenation) in mice compared with proteins labeled by direct iodination methods. In some cases, monoclonal antibodies (MAbs) labeled using SIB also exhibited better *in vitro* binding characteristics, higher accumulation in human tumor xenografts, and enhanced therapeutic efficacy (Zalutsky and Narula, 1988; Zalutsky et al., 1989a; Schuster et al., 1991).

The present study was undertaken to determine the potential utility of SIB for the radioiodination of peptides. These investigations were performed with the melanotropic peptide α -melanocyte-stimulating hormone (α -MSH) (Eberle, 1988) and its more stable analogue [Nle⁴,D-Phe⁷]- α -MSH (Sawyer et al., 1980). The amino acid sequences of these peptides are shown in Figure 1. Because of the presence of α -MSH receptors on human melanoma cell lines (Seigrist et al., 1989; Solca et al., 1989) and biopsy tissue (Ghanem et al., 1989; Tatro et al., 1990), α -MSH and its analogues are of interest for the development of specific diagnostic and therapeutic approaches for melanoma. Also relevant to the current study are the difficulties noted in the literature in production of a radioiodinated α -MSH analogue with

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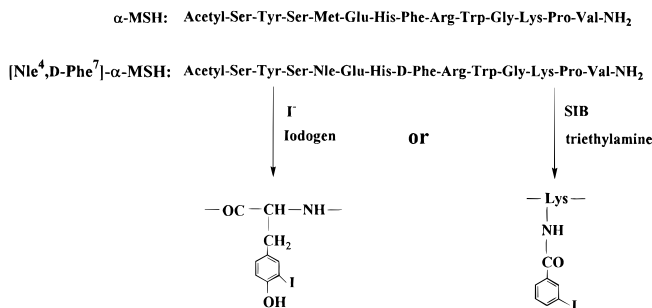


Figure 1. Amino acid sequence for α -MSH and its [Nle⁴,D-Phe⁷]- α -MSH analogue. Radioiodination is assumed to occur at Tyr² with the iodogen method and at Lys¹¹ with SIB reagent.

suitable properties for routine use in receptor assays (Heward et al., 1979; Eberle et al., 1991).

In the present study, α -MSH and [Nle⁴,D-Phe⁷]- α -MSH were radioiodinated using SIB and the iodogen method. The labeled peptides were evaluated with regard to binding to B-16 murine melanoma cells, tissue distribution in normal mice, and urinary catabolites associated with the two labeling methods. Our results suggest that the SIB reagent offers distinct advantages for the radioiodination of these peptides.

EXPERIMENTAL PROCEDURES

Materials. Sodium [¹³¹I]iodide (1200 Ci/mmol) and sodium [¹²⁵I]iodide (no-carrier-added; 2200 Ci/mmol calculated specific activity) in pH 7–11 NaOH were obtained from DuPont-New England Nuclear. Iodogen was purchased from Pierce Chemical Co. The peptides α -MSH and [Nle⁴,D-Phe⁷]- α -MSH were obtained from Bachem California (Torrence, CA). The lysine and glycine conjugates of 3-iodobenzoic acid (IBA) were synthesized using previously described procedures (Garg et al., 1995). All other chemicals were purchased from Aldrich Chemical Co.

Radioiodination of Peptides Using Iodogen. A slight variation of the original iodogen method was used for the radioiodination of α -MSH (Fraker and Speck, 1978). A solution of α -MSH (100 μ g, 5 mg/mL in phosphate buffer, pH 7.4) was added to sodium [¹³¹I]iodide in a glass vial coated with 10 μ g of iodogen. After a 3 min incubation at room temperature, the radioiodinated peptide was isolated by performing high-performance liquid chromatography (HPLC) using a reverse-phase column (Alltech adsorbosphere C-18 10 μ m) eluted at a flow rate of 1.2 mL/min using a 5 to 60% linear gradient of solvent A [70% acetonitrile in water and 0.1% trifluoroacetic acid (TFA)] in solvent B (0.1% TFA in water) over 45 min. Using this system, α -MSH eluted at 35.0 min and the radioiodinated peptide, [Tyr²(¹³¹I)]- α -MSH, eluted at 38.2 min. [Nle⁴,D-Phe⁷]- α -MSH was radioiodinated in 75–85% radiochemical yield following the method described above and was isolated using the same reverse-phase HPLC system. Under these conditions, [Nle⁴,D-Phe⁷]- α -MSH eluted at 36.6 min and the radioiodinated peptide [Tyr²(¹³¹I),Nle⁴,D-Phe⁷]- α -MSH eluted at 39.4 min.

Synthesis of [Nle⁴,D-Phe⁷,Lys¹¹-IBA]- α -MSH. This compound was synthesized to serve as a reference standard in the HPLC catabolite analysis studies and to aid in the confirmation of the lysine residue as the site of IBA conjugation. SIB was synthesized by reaction of 3-iodobenzoic acid with *N*-hydroxysuccinimide and dicyclohexylcarbodiimide as described in a prior publication (Garg et al., 1995). To SIB (0.19 mg) dissolved in 15 μ L of dimethylformamide (DMF) were added 2 μ L of triethylamine and [Nle⁴,D-Phe⁷]- α -MSH (0.8 mg in 160 μ L of

DMF). The reaction mixture was incubated at room temperature and was monitored for the disappearance of SIB by thin layer chromatography on a silica plate with hexane/ethyl acetate/acetic acid (30/70/0.12) as the solvent. The desired product was isolated from the reaction mixture by HPLC with a reverse-phase C18 column (Alltech adsorbosphere C-18 10 μ m) eluted at a flow rate of 1.2 mL/min with a 5 to 60% linear gradient of solvent A [acetonitrile/water (70/30) containing 0.1% TFA] in solvent B (0.1% TFA in water) over 45 min. Retention times for IBA, SIB, and [Nle⁴,D-Phe⁷,Lys¹¹-IBA]- α -MSH were 38.1, 43.6, and 48.5 min, respectively. Mass spectra were obtained on a Finigan MAT 700 TSQ machine (Oneida Research Services, Whitesboro, NY) to confirm the molecular weight for the desired peak: MS *m/z* (electron spray, direct infuse) 1894 (16%, M⁺), 1878 (47%, M⁺ – 17).

Radioiodination of Peptides Using SIB. The radioiodination agent [¹²⁵I]SIB was prepared according to a previously reported method (Garg et al., 1989). Briefly, 10 μ L of 5% acetic acid, 20 μ L of *tert*-butylhydroperoxide, and 5 μ mol of *N*-succinimidyl 3-(tri-*n*-butylstannyl)-benzoate were added to 100–500 μ Ci of sodium [¹²⁵I]iodide. After a 10 min reaction at room temperature, [¹²⁵I]SIB was isolated by HPLC using a silica column (Alltech adsorbosphere-10 10 μ m, 250 $\times$ 4.6 mm) eluted with ethyl acetate/hexane/AcOH (30/70/0.12). After solvent evaporation, α -MSH (100 μ g, 5 mg/mL in DMF) and triethylamine (2 μ L) were added to the [¹²⁵I]SIB residue and the reaction mixture was incubated for 15–90 min at room temperature or at 37 $^{\circ}$ C. After incubation, [Lys¹¹-(¹²⁵I)IBA]- α -MSH was isolated in 45–65% radiochemical yield using the same reverse-phase HPLC system described for the iodogen procedure. Unlabeled α -MSH eluted at 35.0 min, and [Lys¹¹-(¹²⁵I)IBA]- α -MSH eluted at 46.2 min. Using the same procedure, [Nle⁴,D-Phe⁷,Lys¹¹-(¹²⁵I)IBA]- α -MSH was labeled in 45–61% radiochemical yield. The retention time for [Nle⁴,D-Phe⁷,Lys¹¹-(¹²⁵I)IBA]- α -MSH was 48.5 min, identical to that seen for the cold compound.

In Vitro Binding of Radioiodinated Peptides to B16-F1 Melanoma Cells. The binding of the two radioiodinated [Nle⁴,D-Phe⁷]- α -MSH analogues to the murine melanoma line B16-F1 (ATCC, Rockville, MD) was determined *in vitro*. Cells were grown in 150 cm² tissue culture flasks at 37 $^{\circ}$ C in a humidified atmosphere of 95% air and 5% CO₂ in modified essential media (MEM) with Earle's salts supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 1% MEM nonessential amino acids, 1.5% MEM vitamin solution, 50 units/mL penicillin, and 50 μ g/mL streptomycin.

Competitive binding experiments were performed using assay conditions described by Siegrist et al. (1989). Briefly, the binding media consisted of MEM supplemented with 25 mM HEPES, 0.2% bovine serum albumin, and 1.0 mM 1,10-phenanthroline. Cells (2 $\times$ 10⁶ per tube) were incubated in Eppendorf tubes with approximately 15 000 cpm of [Tyr²(¹³¹I),Nle⁴,D-Phe⁷]- α -MSH or [Nle⁴,D-Phe⁷,Lys¹¹-(¹²⁵I)IBA]- α -MSH. Binding in the presence of 10^{–6} M peptide was considered to be nonspecific. Incubations were performed in triplicate in the presence or absence of varying concentrations (10^{–6} to 10^{–13} M) of [Nle⁴,D-Phe⁷]- α -MSH. After a 3 h incubation at 15 $^{\circ}$ C, media was removed and the cells were washed two times with pH 7.4 phosphate-buffered saline. The cell pellets along with input standards were counted for ¹²⁵I and ¹³¹I activity using an automated γ counter. Binding data were analyzed using the RADLIG computer program developed by G. A. McPherson (Biosoft, Cambridge, U.K.).

Biodistribution Studies. Two paired-label experiments were performed in male Balb/c mice weighing 20–25 g. In the first, animals were injected intravenously with 4 μ Ci each of [Tyr²(¹³¹I)]- α -MSH and [Lys¹¹-(¹²⁵I)-IBA]- α -MSH; in the second, animals received 4 μ Ci each of [Tyr²(¹³¹I),Nle⁴,D-Phe⁷]- α -MSH and [Nle⁴,D-Phe⁷,Lys¹¹-(¹²⁵I)IBA]- α -MSH. The radiochemical purity of all preparations used in the biodistribution studies, determined by HPLC, was greater than 98%. Groups of five animals were killed at 0.5, 1, 2, and 4 h postinjection. Tissues of interest were removed, washed with saline, and counted for radioactivity in an automated γ counter. A correction was applied for the presence of ¹³¹I in the ¹²⁵I counting window. After comparison of the tissue activities to injection standards of appropriate count rate, the results were expressed as percent injected dose per gram of tissue (%ID/g) or percent injected dose per organ (%ID).

Urine samples were obtained from mice killed 1 h after injection of [Tyr²(¹³¹I),Nle⁴,D-Phe⁷]- α -MSH and [Nle⁴,D-Phe⁷,Lys¹¹-(¹²⁵I)IBA]- α -MSH, combined, and passed through a 0.45 μ m filter. The HPLC system consisted of a reverse-phase column (Alltech adsorbosphere C-18 10 μ m, 250 $\times$ 4.6 mm) eluted with MeOH/H₂O/AcOH (45/55/0.2) at a flow rate of 1 mL/min. Iodotyrosine and IBA, as well as the IBA conjugates of lysine and glycine, also were analyzed on this system to aid in the identification of these potential catabolites.

RESULTS

Initially, purification of labeled peptides was attempted by Sephadex LH-20 chromatography as described by Eberle and Hübscher (1979). However, when an aliquot from the purified product was analyzed by HPLC, the presence of unlabeled peptide was observed on the UV trace. Reverse-phase HPLC was then adopted as the purification method and permitted good separation of all radioiodinated peptides from unlabeled peptide; no cold peak was observed on the UV trace along with the radioiodinated product, making these essentially no-carrier-added preparations. Using iodogen, yields for the radioiodination of α -MSH and [Nle⁴,D-Phe⁷]- α -MSH generally were greater than 75% with an estimated specific activity of about 1200 Ci/mmol. The efficiency for the coupling of [¹²⁵I]SIB to α -MSH and [Nle⁴,D-Phe⁷]- α -MSH was similar and ranged between 45 and 61% for 15 min to 2 h reaction periods. Although there appeared to be a trend for higher conjugation efficiencies at longer reaction times, these differences were not statistically significant. Running the reaction at 37 °C did not improve labeling yield significantly. A specific activity greater than 1500 Ci/mmol was calculated for peptides labeled with [¹²⁵I]SIB on the basis of the UV detection limit of our HPLC system.

In vitro binding of radioiodinated [Nle⁴,D-Phe⁷]- α -MSH analogues was investigated using the B16-F1 murine melanoma line. Specific binding of [Nle⁴,D-Phe⁷,Lys¹¹-(¹²⁵I)IBA]- α -MSH, 34.1 $\pm$ 4.7%, was more than twice as high as that for [Tyr²(¹³¹I),Nle⁴,D-Phe⁷]- α -MSH (15.0 $\pm$ 1.0%). The specific/nonspecific binding ratio for [Nle⁴,D-Phe⁷,Lys¹¹-(¹²⁵I)IBA]- α -MSH and [Tyr²(¹³¹I),Nle⁴,D-Phe⁷]- α -MSH was 26/1 and 5/1, respectively. The apparent equilibrium dissociation constants were calculated for these labeled ligands using [Nle⁴,D-Phe⁷]- α -MSH as a competitor (Figure 2). The K_D for [Tyr²(¹³¹I),Nle⁴,D-Phe⁷]- α -MSH was 140 $\pm$ 14 pM compared with a K_D of 10 $\pm$ 5 pM for [Nle⁴,D-Phe⁷,Lys¹¹-(¹²⁵I)IBA]- α -MSH.

A tissue distribution study was performed with [Tyr²(¹³¹I)]- α -MSH and [Lys¹¹-(¹²⁵I)IBA]- α -MSH to determine whether SIB improved peptide stability. As summarized in Table 1, the tissue clearance of radioiodine was quite

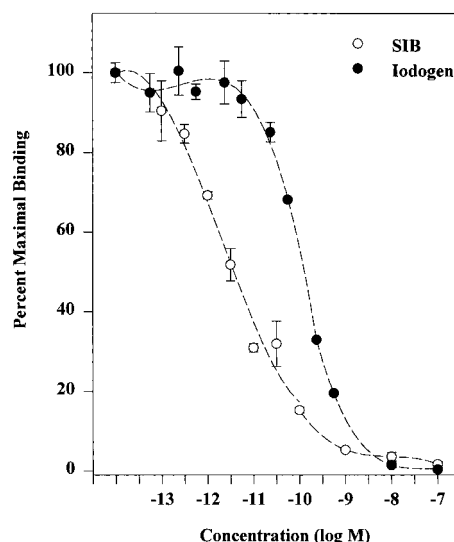


Figure 2. Competitive binding of radioiodinated [Nle⁴,D-Phe⁷]- α -MSH derivatives to the murine B16-F1 melanoma cell line. Binding of [Tyr²(¹³¹I),Nle⁴,D-Phe⁷]- α -MSH and [Nle⁴,D-Phe⁷,Lys¹¹-(¹²⁵I)IBA]- α -MSH measured in the presence of varying concentrations of [Nle⁴,D-Phe⁷]- α -MSH.

Table 1. Paired-Label Tissue Distribution of Radioiodine in Normal Mice Following Injection of [Tyr²(¹³¹I)]- α -MSH and [Lys¹¹-(¹²⁵I)IBA]- α -MSH

tissue	percent injected dose per gram of tissue ^a			
	1 h		4 h	
	Lys ¹¹ -(IBA)	Tyr ² (I)	Lys ¹¹ -(IBA)	Tyr ² (I)
liver	0.15 $\pm$ 0.06	1.44 $\pm$ 0.66	0.02 $\pm$ 0.01	0.11 $\pm$ 0.05
spleen	0.04 $\pm$ 0.02	1.41 $\pm$ 0.46	0.02 $\pm$ 0.01	0.09 $\pm$ 0.04
lungs	0.18 $\pm$ 0.13	2.29 $\pm$ 0.76	0.04 $\pm$ 0.01	0.21 $\pm$ 0.08
heart	0.07 $\pm$ 0.04	1.29 $\pm$ 0.56	0.03 $\pm$ 0.02	0.08 $\pm$ 0.04
kidney	0.33 $\pm$ 0.14	3.30 $\pm$ 0.77	0.09 $\pm$ 0.03	0.79 $\pm$ 0.07
small intestine	0.34 $\pm$ 0.15	2.14 $\pm$ 1.17	0.02 $\pm$ 0.01	0.16 $\pm$ 0.03
large intestine	0.29 $\pm$ 0.15	1.19 $\pm$ 0.53	0.13 $\pm$ 0.04	0.23 $\pm$ 0.04
muscle	0.10 $\pm$ 0.04	0.61 $\pm$ 0.31	0.03 $\pm$ 0.02	0.04 $\pm$ 0.02
blood	0.14 $\pm$ 0.09	3.01 $\pm$ 1.16	0.05 $\pm$ 0.02	0.17 $\pm$ 0.08
brain	0.02 $\pm$ 0.01	0.20 $\pm$ 0.08	0.01 $\pm$ 0.00	0.01 $\pm$ 0.00

^a Mean $\pm$ standard deviation for five animals.

rapid for both labeling methods. At 1 h after injection, the ¹³¹I percent injected dose per gram of tissue activity concentrations were significantly higher ($P < 0.01$) than those of ¹²⁵I. Differences were particularly apparent in the thyroid and stomach, two tissues known to accumulate radioiodide (Figure 3). For example, thyroid uptake for [Tyr²(¹³¹I)]- α -MSH 1 h after injection was 3.26 $\pm$ 0.70 %ID compared with 0.02 $\pm$ 0.01 %ID for [Lys¹¹-(¹²⁵I)IBA]- α -MSH; similar differences were seen in stomach (1 h; iodogen, 4.50 $\pm$ 0.90 %ID; SIB, 0.06 $\pm$ 0.02 %ID).

A more extensive paired-label experiment compared the tissue distributions of radioiodine following the injection of [Tyr²(¹³¹I),Nle⁴,D-Phe⁷]- α -MSH and [Nle⁴,D-Phe⁷,Lys¹¹-(¹²⁵I)IBA]- α -MSH. The tissue distribution of [Tyr²(¹³¹I),Nle⁴,D-Phe⁷]- α -MSH (Table 2) was similar to that observed for [Tyr²(¹³¹I)]- α -MSH (Table 1), while retention of activity in most normal tissues was higher for [Nle⁴,D-Phe⁷,Lys¹¹-(¹²⁵I)IBA]- α -MSH (Table 2) compared with that of [Lys¹¹-(¹²⁵I)IBA]- α -MSH (Table 1). When the biodistribution data for [Nle⁴,D-Phe⁷]- α -MSH labeled using SIB and iodogen were compared (Table 2), no clear trend was seen at 0.5 h with regard to normal tissue retention. As time progressed, increased tissue activity concentrations generally were observed for [Tyr²(¹³¹I),Nle⁴,D-Phe⁷]- α -MSH. Again, 26–40 times lower

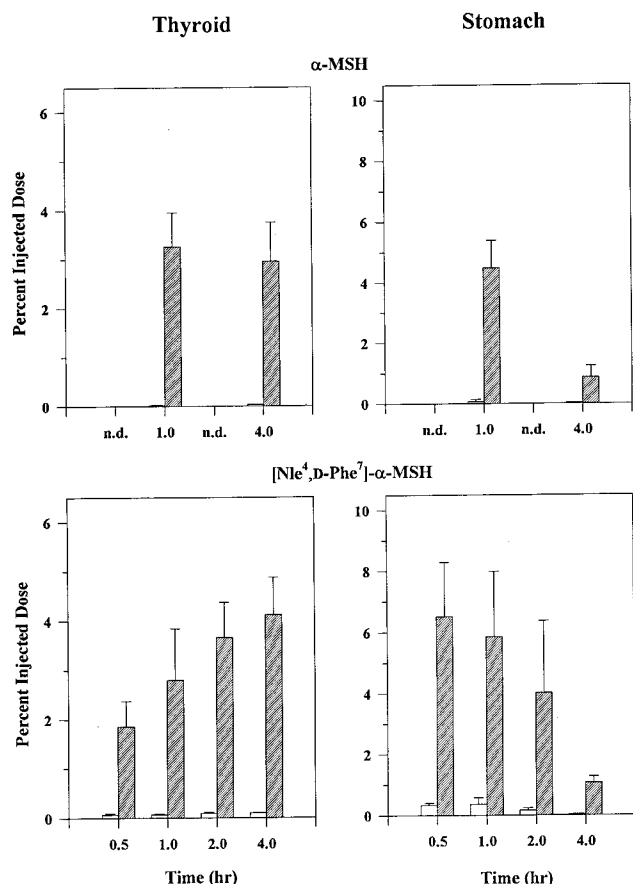


Figure 3. Percent of injected dose of radioiodine localized in the thyroid and stomach following the injection of radioiodinated α -MSH and [Nle⁴,D-Phe⁷]- α -MSH derivatives labeled using the iodogen (cross-hatched bars) and SIB methods (open bars).

thyroid uptake and 19–25 times lower stomach accumulation were observed for [Nle⁴,D-Phe⁷,Lys-(¹²⁵I)-IBA]- α -MSH.

HPLC analyses were performed on the 1 h urine samples obtained from mice injected with the radioiodinated [Nle⁴,D-Phe⁷]- α -MSH analogues. Using the peptide HPLC purification column, there was no evidence for excretion of intact [Tyr²(¹³¹I),Nle⁴,D-Phe⁷]- α -MSH, while about 10% of ¹²⁵I was excreted as intact [Nle⁴,D-Phe⁷,Lys¹¹-(¹²⁵I)IBA]- α -MSH. The reverse-phase HPLC system was used for the analysis of lower molecular weight species. As shown in Figure 4, with [Tyr²(¹³¹I),Nle⁴,D-Phe⁷]- α -MSH, >95% of the ¹³¹I activity eluted with a retention time corresponding to radioiodide, while with [Nle⁴,D-Phe⁷,Lys¹¹-(¹²⁵I)IBA]- α -MSH, the principal labeled species had an elution time corresponding to the IBA-lysine conjugate. In addition, radioiodide (9%), IBA (3%), and the IBA-glycine conjugate (8%) also were observed.

DISCUSSION

A wealth of data is available concerning the *in vitro* binding specificity and mechanisms of interaction of radioiodinated peptides to tumor-associated receptors. Extension of promising peptides to *in vivo* applications using iodine radionuclides is attractive because it minimizes the need for performing extensive validation studies with chemically dissimilar radiolabels. An additional feature of peptide radioiodination is that methodology often can be adapted for use with other radiohalogens such as 2 h ¹⁸F and 7.2 h ²¹¹At, which are of interest for positron emission tomographic imaging and α -particle radiotherapy, respectively. For example, SIB analogues

have been used to label MABs with ¹⁸F (Vaidyanathan et al., 1992) and ²¹¹At (Zalutsky et al., 1989b).

Because receptors for α -MSH have been identified on the surface of human melanoma cells and tissue biopsies (Seigrist et al., 1989; Solca et al., 1989; Ghanem et al., 1989; Tatro et al., 1990), these peptides are of practical interest for tumor targeting. Moreover, the less than optimal *in vitro* and *in vivo* properties of its radioiodinated analogues make α -MSH a useful paradigm for the development of better peptide radioiodination strategies. Radioiodination of α -MSH using electrophilic iodination has been investigated using a number of procedures (Heward et al., 1979; Eberle and Hübscher, 1979; Ghanem et al., 1982; Eberle, 1988). Unfortunately, the methionine residue at position 4 is easily oxidized so these labeling methods yield a labeled peptide with substantially reduced receptor affinity. In addition, the tissue distribution in mice of α -MSH labeled with ¹²⁵I using chloramine-T reflects significant degradation and/or deiodination of the labeled peptide *in vivo* (Ghanem et al., 1991). Alternative radioiodination methods and/or analogues of α -MSH are clearly needed to provide a more suitable labeled melanotropic peptide for *in vitro* and *in vivo* applications.

With regard to radioiodination methodology, SIB has been demonstrated to decrease MAB deiodination and increase immunoreactivity compared with MAB labeled using iodogen or chloramine-T (Zalutsky and Narula, 1988; Zalutsky et al., 1989a; Wilbur et al., 1989). An advantage of the SIB method is that it avoids exposure of the biomolecule to oxidizing or reducing agents. In addition, the iodination site which is created is structurally dissimilar to iodotyrosine, a compound which is susceptible to the action of multiple endogenous deiodinases.

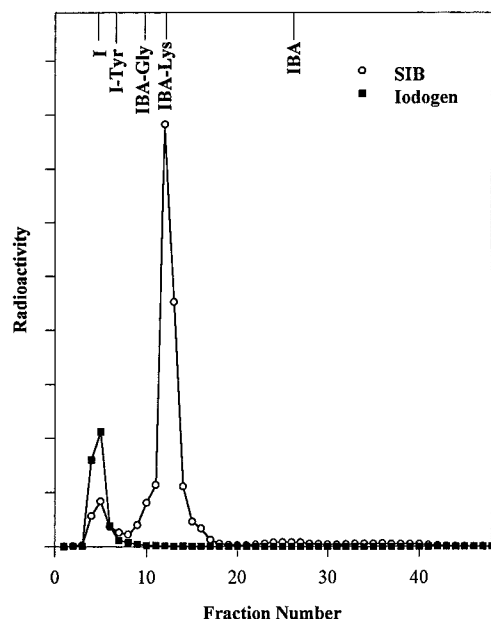
The procedure developed for peptide radioiodination with SIB was modified slightly from that used for MABs. With MABs, the coupling reaction was performed in pH 8.5 borate buffer in order to minimize protein denaturation even though higher pH results in greater conjugation yields (Zalutsky and Narula, 1987). In the current study, peptides were dissolved in DMF in the presence of triethylamine, conditions which should increase coupling yield due to the availability of a greater proportion of lysine ϵ -amino groups for reaction. In addition, the use of nonaqueous media should minimize competitive hydrolysis of the SIB active ester. The conjugation yields for SIB to these peptides (45–61%) were comparable to those obtained with MABs at similar protein concentrations and reaction times (Zalutsky and Narula, 1988; Zalutsky et al., 1989). Increasing peptide concentration should permit higher α -MSH coupling efficiencies since we have used this strategy to label a chemotactic peptide with the analogous *N*-succinimidyl 4-[¹⁸F]fluorobenzoate in greater than 80% yield (Vaidyanathan et al., 1995).

In vitro studies have documented that α -MSH can be degraded under physiological conditions and in the presence of melanoma cell membranes (Deschodt-Lankman et al., 1990), a characteristic which probably prohibits the use of α -MSH for *in vivo* applications. Nonetheless, we performed a paired-label tissue distribution study to confirm this behavior and to determine whether deiodination was a contributory factor. In a previous study with an ¹²³I-labeled somatostatin analogue, high thyroid uptake was noted, indicating that extensive dehalogenation had occurred (Bakker et al., 1990).

An important parameter for the evaluation of the potential utility of a radiolabeling method is the extent to which it minimizes the retention of radioactivity in normal tissues. Previously, we have demonstrated that,

Table 2. Paired-Label Tissue Distribution of Radioiodine in Normal Mice Following Injection of [Tyr²(¹³¹I),Nle⁴,D-Phe⁷]- α -MSH and [Nle⁴,D-Phe⁷,Lys¹¹-(¹²⁵I)IBA]- α -MSH

tissue	percent injected dose per gram of tissue ^a							
	0.5 h		1 h		2 h		4 h	
	Lys ¹¹ -(IBA)	Tyr ² (I)	Lys ¹¹ -(IBA)	Tyr ² (I)	Lys ¹¹ -(IBA)	Tyr ² (I)	Lys ¹¹ -(IBA)	Tyr ² (I)
liver	2.56 $\pm$ 0.84	1.25 $\pm$ 0.36	1.11 $\pm$ 0.25	1.04 $\pm$ 0.41	0.47 $\pm$ 0.26	0.37 $\pm$ 0.05	0.22 $\pm$ 0.03	0.28 $\pm$ 0.08
spleen	0.50 $\pm$ 0.14	1.39 $\pm$ 0.32	0.32 $\pm$ 0.03	1.05 $\pm$ 0.30	0.20 $\pm$ 0.06	0.49 $\pm$ 0.14	0.17 $\pm$ 0.04	0.35 $\pm$ 0.10
lung	1.40 $\pm$ 0.56	2.48 $\pm$ 0.80	0.63 $\pm$ 0.28	1.99 $\pm$ 0.83	0.46 $\pm$ 0.21	0.75 $\pm$ 0.12	0.39 $\pm$ 0.09	0.57 $\pm$ 0.10
heart	0.36 $\pm$ 0.10	1.46 $\pm$ 0.37	0.13 $\pm$ 0.01	1.03 $\pm$ 0.25	0.06 $\pm$ 0.02	0.47 $\pm$ 0.20	0.03 $\pm$ 0.01	0.36 $\pm$ 0.10
kidney	5.18 $\pm$ 1.42	2.99 $\pm$ 0.64	1.77 $\pm$ 0.37	2.01 $\pm$ 0.52	0.80 $\pm$ 0.16	0.91 $\pm$ 0.08	0.27 $\pm$ 0.07	0.65 $\pm$ 0.11
small intestine	5.43 $\pm$ 1.43	2.34 $\pm$ 0.35	4.23 $\pm$ 1.29	1.79 $\pm$ 0.59	0.55 $\pm$ 0.14	0.63 $\pm$ 0.15	0.16 $\pm$ 0.05	0.54 $\pm$ 0.19
large intestine	0.24 $\pm$ 0.06	1.10 $\pm$ 0.26	1.94 $\pm$ 0.84	1.02 $\pm$ 0.24	1.96 $\pm$ 0.57	0.65 $\pm$ 0.18	0.63 $\pm$ 0.32	0.52 $\pm$ 0.20
muscle	0.21 $\pm$ 0.08	2.36 $\pm$ 0.43	0.09 $\pm$ 0.03	1.88 $\pm$ 0.28	0.03 $\pm$ 0.04	1.24 $\pm$ 0.19	0.03 $\pm$ 0.01	0.76 $\pm$ 0.08
blood	0.58 $\pm$ 0.17	2.68 $\pm$ 0.68	0.18 $\pm$ 0.05	2.22 $\pm$ 0.79	0.07 $\pm$ 0.03	0.66 $\pm$ 0.13	0.02 $\pm$ 0.01	0.60 $\pm$ 0.15
brain	0.05 $\pm$ 0.02	0.20 $\pm$ 0.06	0.03 $\pm$ 0.02	0.16 $\pm$ 0.02	0.03 $\pm$ 0.02	0.46 $\pm$ 0.38	0.01 $\pm$ 0.01	0.10 $\pm$ 0.08

^a Mean $\pm$ standard deviation for five animals.**Figure 4.** Reverse-phase HPLC chromatogram of a urine sample obtained from normal mice 1 h after coinjection of [Tyr²(¹³¹I),Nle⁴,D-Phe⁷]- α -MSH and [Nle⁴,D-Phe⁷,Lys¹¹-(¹²⁵I)IBA]- α -MSH.

compared with the Iodogen method, utilization of SIB for the radioiodination of an intact MAb (Zalutsky et al., 1989a) and a F(ab')₂ fragment (Zalutsky and Narula, 1988) resulted in significant reduction in activity levels in most normal tissues. Paired-label tissue distribution experiments were performed to determine whether SIB labeling would confer a similar advantage for the labeling of α -MSH and its more metabolically inert [Nle⁴,D-Phe⁷]- α -MSH analogue.

The clearance of radioiodine activity from normal tissues following the injection of both [Tyr²(¹³¹I)- α -MSH and [Lys¹¹-(¹²⁵I)IBA]- α -MSH was quite rapid and qualitatively similar to the results reported previously for α -MSH labeled with chloramine-T (Ghanem et al., 1991). The lower levels of radioiodine in the thyroid and stomach for peptide labeled using SIB suggest that this labeling method reduced deiodination significantly. Activity levels in other normal tissues from [Tyr²(¹³¹I)- α -MSH and [Lys¹¹-(¹²⁵I)IBA]- α -MSH were quite similar to those measured previously for iodide and IBA, respectively (Zalutsky and Narula, 1988). Thus, even though SIB appeared to reduce peptide deiodination, rapid degradation of labeled α -MSH was the predominant determinant, rendering this peptide of minimal interest for *in vivo* applications.

For this reason, subsequent studies focused on the evaluation of radioiodinated [Nle⁴,D-Phe⁷]- α -MSH. In this α -MSH analogue, norleucine is substituted for methionine at position 4 to avoid susceptibility to oxidative degradation and a D-phenylalanine is substituted for its L-isomer to increase resistance to proteolytic degradation (Sawyer et al., 1980). The affinity of [Nle⁴,D-Phe⁷]- α -MSH for the α -MSH receptor has been reported to be 3–5 times higher than that of α -MSH itself (Siegrist et al., 1989; Chhajlani and Wilkberg, 1992). Using the murine B16 melanoma cell line and [Nle⁴,D-Phe⁷]- α -MSH as a competitor, a K_D of 0.32 ± 0.13 nM for [Tyr²(¹²⁵I),Nle⁴,D-Phe⁷]- α -MSH has been measured (Siegrist et al., 1988).

The binding characteristics determined in the current study for [Tyr²(¹³¹I),Nle⁴,D-Phe⁷]- α -MSH compare favorably with those reported in the literature for this tracer. Using [Nle⁴,D-Phe⁷]- α -MSH as a competitor, a $K_D = 0.140 \pm 0.014$ nM was determined for [Tyr²(¹³¹I),Nle⁴,D-Phe⁷]- α -MSH binding to B16-F1 murine melanoma cells. The maximum specific binding in the absence of carrier and the nonspecific binding percentage measured in the current study also were slightly more favorable than those determined previously for [Tyr²(¹³¹I),Nle⁴,D-Phe⁷]- α -MSH prepared using chloramine-T (Siegrist and Eberle, 1993; Eberle et al., 1991; Tatro and Reichlin, 1987; Eberle et al., 1993). These results are consistent with those obtained with other proteins and peptides which show that radioiodination using iodogen can provide greater retention of biological activity than that using chloramine-T (Fraker and Speck, 1978).

The apparent K_D measured for [Nle⁴,D-Phe⁷,Lys¹¹-(¹²⁵I)IBA]- α -MSH was 10 ± 5 pM, a value lower than that reported previously for radioiodinated α -MSH analogues. In addition, the maximal specific binding of this tracer to B16 melanoma cells was high, $34.1 \pm 4.7\%$, with a low degree of nonspecific binding. The latter property is important because addition of IBA increased peptide lipophilicity as reflected by its longer retention time on reverse-phase HPLC. The excellent *in vitro* binding properties of [Nle⁴,D-Phe⁷,Lys¹¹-(¹²⁵I)IBA]- α -MSH to melanoma cells may in part reflect a greater inertness of this compound to dehalogenation. This is consistent with the observation that after only a 10 min incubation *in vitro* the fraction of intact [Tyr²(¹²⁵I),Nle⁴,D-Phe⁷]- α -MSH remaining had declined to $92.0 \pm 1.0\%$ (Tatro and Reichlin, 1987). Another possibility is the greater proximity of Lys¹¹ compared with Tyr² to the amino acid residues considered to be involved in receptor binding (Eberle, 1988); as proteolytic breakdown proceeds, a label on Lys¹¹ might be more likely to remain associated with a peptide fragment retaining receptor binding capabilities.

Because of their potential for the diagnosis and treatment of melanomas, the two radioiodinated [Nle⁴,D-Phe⁷]-

α -MSH derivatives were compared in a paired-label tissue distribution experiment. [$\text{Nle}^4, \text{D-Phe}^7, \text{Lys}^{11-(^{125}\text{I})}$]-IBA]- α -MSH exhibited significantly lower activity levels compared with [$\text{Tyr}^2(^{131}\text{I})$]- α -MSH in thyroid and stomach, two tissues known to accumulate free radioiodide. The biodistribution pattern for [$\text{Tyr}^2(^{131}\text{I})$]- α -MSH was similar to that seen for [$\text{Tyr}^2(^{125}\text{I})$]- α -MSH, suggesting that the enhanced stability of the [$\text{Nle}^4, \text{D-Phe}^7$]- α -MSH structure could not be exploited due to the predominating role of deiodination in determining the catabolism of the label. Indeed, more than 95% of the radioactivity excreted in the urine was identified as radioiodide with no evidence for monoiodotyrosine. Although absolute percent injected dose per gram of tissue values were not included, Tatro and Reichlin (1987) reported extensive deiodination of [$\text{Tyr}^2(^{125}\text{I})$]- α -MSH 10 min after injection.

It thus appears that, to be able to exploit the potential advantages of [$\text{Nle}^4, \text{D-Phe}^7$]- α -MSH for *in vivo* applications, it would be preferable to utilize a labeling method which reduces deiodination. Minimization of retention of radioactivity in normal tissues is another consideration in the selection of a method for labeling peptides since the low molecular weight of these carrier molecules should result in low normal tissue uptake levels. This has not always been the case with ^{111}In -labeled α -MSH analogues where significant activity levels have been observed in liver and kidneys (Bard et al., 1990; Bagutti et al., 1994). In the current study, rapid normal tissue clearance was seen for [$\text{Nle}^4, \text{D-Phe}^7, \text{Lys}^{11-(^{125}\text{I})}$]-IBA]- α -MSH, with liver and kidney retaining less than 0.3% ID/g at 4 h.

An investigation of the labeled species present in the urine after administration of [$\text{Nle}^4, \text{D-Phe}^7, \text{Lys}^{11-(^{125}\text{I})}$]-IBA]- α -MSH revealed that Lys-IBA was the primary labeled catabolite. This observation suggests that labeling had occurred at Lys¹¹ on the peptide. Lys-IBA also was determined to be the major catabolite for a MAb F(ab')₂ fragment labeled using SIB (Garg et al., 1995), and Lys-drug and Lys-chelate conjugates have been reported as major catabolites of lysine-modified proteins (Franssen et al., 1992; Duncan and Welch, 1993).

Gly-IBA is an additional catabolite which has been observed with MAb fragments labeled using SIB (Garg et al., 1995), and a small amount of Gly-IBA also was seen in the urine when the peptide was labeled by this method. We have previously reported that IBA reacts *in vivo* with endogenous glycine to form Gly-IBA conjugates (Garg et al., 1989b), an observation consistent with the well-known ability of glycine to form conjugates with benzoic acids (Gatley and Scheratt, 1976). Thus, the observation of low levels of Gly-IBA in the urine of mice injected with [$\text{Nle}^4, \text{D-Phe}^7, \text{Lys}^{11-(^{125}\text{I})}$]-IBA]- α -MSH most likely reflects the cleavage of IBA from the labeled peptide itself or from lower-molecular weight IBA-conjugate intermediate catabolites.

In summary, [$\text{Nle}^4, \text{D-Phe}^7, \text{Lys}^{11-(^{125}\text{I})}$]-IBA]- α -MSH appears to be a promising reagent for α -MSH receptors because of its high affinity and low nonspecific binding and for *in vivo* applications because of its inertness to deiodination. When labeled with ^{123}I , this tracer might be valuable for the localization of melanoma metastases. Experiments to evaluate the pharmacokinetics of [$\text{Nle}^4, \text{D-Phe}^7, \text{Lys}^{11-(^{125}\text{I})}$]-IBA]- α -MSH in a murine melanoma model are planned. In addition, the investigation of the applicability of the SIB reagent for the radioiodination of other peptides appears to be warranted.

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LITERATURE CITED

- Babich, J. W., Solomon, H., Pike, M. C., Kroon, D., Graham, W., Abrams, M. J., Tompkins, R. G., Rubin, R. H., and Fischman, A. J. (1993a) $^{99\text{m}}\text{Tc}$ -labeled hydrazino nicotinamide derivatized chemotactic peptides for imaging focal sites of bacterial infection. *J. Nucl. Med.* 34, 1964–1974.
- Babich, J. W., Graham, W., Barrow, S. A., Dragotakes, S. C., Tompkins, R. G., Rubin, R. H., and Fischman, A. J. (1993b) Technetium-99m-labeled chemotactic peptides: comparison with indium-111-labeled white blood cells for localizing acute bacterial infections in rabbits. *J. Nucl. Med.* 34, 2176–2181.
- Badger, C. C., Wilbur, D. S., Hadley, S. W., Fritzberg, A. R., and Bernstein, I. D. (1990) Biodistribution of *p*-iodobenzoyl (PIP) labeled antibodies in a murine lymphoma model. *Nucl. Med. Biol.* 17, 381–387.
- Bagutti, C., Stolz, B., Albert, R., Bruns, C., Pless, J., and Eberle, A. N. (1994) [^{111}In]-DTPA-labeled analogues of α -melanocyte stimulating hormone for melanoma targeting: receptor binding *in vitro* and *in vivo*. *Int. J. Cancer* 58, 749–755.
- Bakker, W. H., Krenning, E. P., Breeman, W. A., Koper, J. W., Kooij, P. P., Reubi, J.-C., Klijn, J. G., Visser, T. J., Docter, R., and Lamberts, S. W. (1990) Receptor scintigraphy with a radioiodinated somatostatin analogue: radiolabeling, purification, biologic activity, and *in vivo* application in animals. *J. Nucl. Med.* 31, 1501–1509.
- Bakker, W. H., Krenning, E. P., Breeman, W. A., Kooij, P. P., Reubi, J.-C., Koper, J. W., de Jong, M., Laméris, J. S., Visser, T. J., and Lamberts, S. W. (1991) *In vivo* use of a radioiodinated somatostatin analogue: dynamics, metabolism, and binding to somatostatin receptor-positive tumors in man. *J. Nucl. Med.* 32, 1184–1189.
- Bard, D. R., Knight, C. G., and Page-Thomas, D. P. (1990) A chelating derivative of α -melanocyte stimulating hormone as a potential imaging agent for malignant melanoma. *Br. J. Cancer* 62, 919–922.
- Chhajlani, V., and Wikberg, J. E. S. (1992) Molecular cloning and expression of the human melanocyte stimulating hormone receptor cDNA. *FEBS Lett.* 309, 417–420.
- Deschodt-Lanckman, M., Vanneste, Y., Loir, B., Michel, A., Libert, A., Ghanem, G., and Lejeune, F. (1990) Degradation of α -melanocyte stimulating hormone (α -MSH) by Calla/endopeptidase 24.11 expressed by human melanoma cells in culture. *Int. J. Cancer* 46, 1124–1130.
- Eberle, A. N. (1988) *The Melanotropins: Chemistry, Physiology and Mechanisms of Action*, pp 1–409, Karger, Basel.
- Eberle, A. N., and Hübscher, W. (1979) α -Melanotropin labelled at its tyrosine² residue: synthesis and biological activities of 3'-iodotyrosine²-3'- ^{125}I -iodotyrosine²-3',5'-diiodotyrosine² and (3',5'-3H₂)-tyrosine²- α -melanotropin, and of related peptides. *Helv. Chim. Acta* 62, 2460–2483.
- Eberle, A. N., Verin, V. J., Solca, F., Siegrist, W., Kuenlin, C., Bagutti, C., Stutz, S., and Girard, J. (1991) Biologically active monoiodinated α -MSH derivatives for receptor binding studies using human melanoma lines. *J. Recept. Res.* 11, 311–322.
- Eberle, A. N., Siegrist, W., Bagutti, C., Chluba-DeTapia, J., Solca, F., Wikberg, J. E. S., and Chhajlani, V. (1993) Receptors for melanocyte-stimulating hormone on melanoma cells. *Ann. N. Y. Acad. Sci.* 680, 320–341.
- Fracker, P. J., and Speck, J. C. (1978) Protein and cell membrane iodinations with a sparingly soluble chloramide 1,3,4,6-tetrachloro-3 α -6 α -diphenylglycouril. *Biochem. Biophys. Res. Commun.* 80, 849–857.
- Garg, P. K., Archer, G. E., Jr., Bigner, D. D., and Zalutsky, M. R. (1989a) Synthesis of radioiodinated *N*-succinimidyl iodobenzoate: optimization for use in antibody labeling. *Appl. Radiat. Isot.* 40, 485–490.
- Garg, P. K., Slade, S. K., Harrison, C. L., and Zalutsky, M. R. (1989b) Labeling proteins using aryl iodide acylation agents:

- influence of *meta* vs *para* substitution on *in vivo* stability. *Nucl. Med. Biol.* 7, 669–673.
- Garg, P. K., Alston, K. L., and Zalutsky, M. R. (1995) Catabolism of radioiodinated murine monoclonal antibody F(ab')₂ fragment labeled using *N*-succinimidyl 3-iodobenzoate and iodogen methods. *Bioconjugate Chem.* 6, 493–501.
- Gatley, J. S., and Sherratt, H. S. A. (1977) The synthesis of hippurate from benzoate and glycine by rat liver mitochondria. *Biochem. J.* 166, 39–47.
- Ghanem, G., Legros, F., Lejeune, F., and Fröling, J. (1982) Comparison and evaluation of different methods for α -MSH labeling. *J. Immunol. Methods* 54, 223–232.
- Ghanem, G., Verstegen, J., Libert, A., Arnould, R., and Lejeune, F. (1989) Alpha-melanocyte-stimulating hormone immunoreactivity in human melanoma metastases extracts. *Pigm. Cell Res.* 2, 519–523.
- Ghanem, G., Libert, A., Arnould, L. R., Vercammen, A., and Lejeune, F. (1991) Human melanoma targeting with α -MSH-melphalan conjugate. *Melanoma Res.* 1, 105–114.
- Heward, C. B., Yang, Y. C. S., Ormberg, J. F., Hadley, M. E., and Hruby, V. J. (1979) Effects of chloramine T on the biological activity of melanotropin. *Hoppe-Seyler's Z. Physiol. Chem.* 360, 1851–1859.
- Krenning, E. P., Bakker, W. H., Kooij, P. P. M., Breeman, W. A. P., Oei, H. Y., de Jong, M., Reubi, J. C., Visser, T. J., Bruns, C., Kwekkeboom, D. J., Reijts, A. E. M., van Hagen, P. M., Koper, J. W., and Lamberts, S. W. J. (1992) Somatostatin receptor scintigraphy with indium-111-DTPA-D-Phe-1-octreotide in man: metabolism, dosimetry and comparison with iodine-123-tyr-3-octreotide. *J. Nucl. Med.* 33, 652–658.
- Larson, S. M., and Carrasquillo, J. A. (1988) Advantages of radioiodine over radioindium labeled monoclonal antibodies for imaging solid tumors. *Nucl. Med. Biol.* 15, 231–234.
- Sawyer, T. K., Sanfilippo, P. J., Hruby, V. J., Engel, M. H., Heward, C. B., Burnett, J. B., and Hadley, M. E. (1980) 4-Norleucine, 7-D-phenylalanine- α -melanocyte-stimulating hormone: a highly potent α -melanotropin with ultralong biological activity. *Proc. Natl. Acad. Sci. U.S.A.* 77, 5754–5758.
- Schuster, J. M., Garg, P. K., Bigner, D. D., and Zalutsky, M. R. (1991) Improved therapeutic efficacy of a monoclonal antibody radioiodinated using *N*-succinimidyl-3-(tri-*n*-butylstannyl)-benzoate. *Cancer Res.* 51, 4164–4169.
- Siegrist, W., and Eberle, A. N. (1993) Homologous regulation of the MSH receptor in melanoma cells. *J. Recept. Res.* 13, 263–281.
- Siegrist, W., Oestreicher, M., Stutz, S., Girard, J., and Eberle, A. N. (1988) Radioreceptor assay for α -MSH using mouse B16 melanoma cells. *J. Recept. Res.* 8, 323–343.
- Siegrist, W., Solca, F., Stutz, S., Giuffrè, L., Carrel, S., Girard, J., and Eberle, A. N. (1989) Characterization of receptors for α -melanocyte-stimulating hormone on human melanoma cells. *Cancer Res.* 49, 6352–6358.
- solca, F., Siegrist, W., Drozd, R., Girard, J., and Eberle, A. N. (1989) The receptor for α -melanotropin of mouse and human melanoma cells. *J. Biol. Chem.* 264, 14277–14281.
- Tatro, J. B., and Reichlin, S. (1987) Specific receptors for α -melanocyte-stimulating hormone are widely distributed in tissues of rodents. *Endocrinology* 121, 1900–1907.
- Tatro, J. B., Atkins, M., Mier, J. W., Hardarson, S., Wolfe, H., Smith, T., Entwistle, M. L., and Reichlin, S. (1990) Melanotropin receptors demonstrated *in situ* in human melanoma. *J. Clin. Invest.* 85, 1825–1832.
- Vaidyanathan, G., and Zalutsky, M. R. (1992) Labeling of proteins with fluorine-18 using *N*-succinimidyl 4-[¹⁸F]fluorobenzoate. *Nucl. Med. Biol.* 19, 275–281.
- Vaidyanathan, G., and Zalutsky, M. R. (1995) Fluorine-18-labeled chemotactic peptides: a potential approach for the PET imaging of bacterial infection. *Nucl. Med. Biol.* (in press).
- Wilbur, D. S., Hadley, S. W., Hylarides, M. D., Abrams, P. G., Beaumier, P. A., Morgan, A. C., Reno, J. M., and Fritzberg, A. R. (1989) Development of a stable radioiodinating reagent to label monoclonal antibodies for radiotherapy of cancer. *J. Nucl. Med.* 30, 216–226.
- Zalutsky, M. R., and Narula, A. S. (1987) A method for the radiohalogenation of proteins resulting in decreased thyroid uptake of radioiodine. *Appl. Radiat. Isot.* 38, 1051–1055.
- Zalutsky, M. R., and Narula, A. S. (1988) Radiohalogenation of a monoclonal antibody using an *N*-succinimidyl 3-(tri-*n*-butylstannyl)benzoate intermediate. *Cancer Res.* 48, 1446–1450.
- Zalutsky, M. R., Noska, M. A., Colapinto, E. V., Garg, P. K., and Bigner, D. D. (1989a) Enhanced tumor localization and *in vivo* stability of a monoclonal antibody radioiodinated using *N*-succinimidyl-3-(tri-*n*-butylstannyl)benzoate. *Cancer Res.* 49, 5543–5549.
- Zalutsky, M. R., Garg, P. K., Friedman, H. S., and Bigner, D. D. (1989b) Labeling monoclonal antibodies and F(ab')₂ fragments with the alpha particle emitting nuclide astatine-211: Preservation of immunoreactivity and *in vivo* localizing capacity. *Proc. Natl. Acad. Sci. U.S.A.* 86, 7149–7153.

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Novel Non-Nucleosidic Building Blocks for the Preparation of Multilabeled Oligonucleotides

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Synthesis of new non-nucleosidic phosphoramidites **1** and **2** derived from achiral precursors containing two reporter groups or amino functions is described. Among them, only phosphoramidites **1a–c** allow multiple derivatization of synthetic oligonucleotides. The usefulness of building blocks **1a–c** is demonstrated by introduction of several amino functions or fluorescent dansyl reporters at the 3'- and 5'-termini of synthetic oligonucleotides and their phosphorothioate analogues. It is demonstrated that multilabeled non-nucleosidic tether has no or only minor influence on the hybridization properties of the oligonucleotides.

INTRODUCTION

Oligodeoxynucleotides bearing either reporter groups, bioaffinity groups, or additional functional groups have proven to be potent tools in molecular biology, finding applications in hybridization diagnostics, sequencing, chemical tailoring, and molecular recognition studies of nucleic acids (1). Related structural modifications have also been exploited to increase the cellular uptake of oligonucleotides and to improve their stability toward nucleases. The methods elaborated to introduce various conjugate groups into synthetic oligonucleotides fall into two categories. The first alternative is derivatization of nucleotide units at either the base, sugar, or phosphodiester moiety (1). All these modifications change, sometimes even dramatically, the ionic, tautomeric, and/or steric properties of the oligonucleotide. In particular, introduction of several conjugate groups within the sense sequence that is engaged in the hybrid formation may cause problems. The second alternative is to extend the sense sequence with a non-nucleosidic tail designed to bear the desired functionalities. Obviously, this approach is the method of choice for multilabeling.

The non-nucleosidic tails used for multilabeling of oligonucleotides include both branched and linear structures. Branched dendrimeric structures have, for example, been assembled on the 5'-terminus of the oligonucleotide by using bis(4,4'-dimethoxytrityl)alkanetriol phosphoramidite as a building block (2–4). Each condensation step thus doubles the number of 5'-terminal hydroxyl functions. At the end of the chain assembly, all the terminal hydroxy functions are reacted with a phosphoramidite building block derived from the desired conjugate group, such as a biotinyl (2–4), dinitrophenyl (3), or protected aminoalkyl (3, 4) group. Polyamino oligonucleotides may finally be labeled in solution, *e.g.*, with fluorescein, digoxigenin, or biotin (3, 4).

Linear tails have, in turn, been prepared by using non-nucleosidic phosphoramidites derived from substituted diols that carry either a protected amino function or a reporter group (4–11). Among them, derivatives of 3-amino-1,2-propanediol (5–9) and glycerol (9–11) have been used most extensively. The 1,2-diol system has been used to construct the non-nucleosidic backbone, the

remaining functionality (either amino or the third hydroxy group) being tethered to the conjugate group. Biotinyl (5, 10), pyrenyl (6, 7), dansyl (DNS) (7), carborane (8), dinitrophenyl (9), phosphotyrosinyl (10), or fluoresceinyl (11) residues have been introduced in this manner. It is also possible to keep the remaining functionality appropriately protected during the chain assembly and label it postsynthetically after deprotection (5). The 4,4'-dimethoxytrityl (DMTr) group has, however, been observed to retard sterically the coupling reaction of the vicinal phosphoramidite function (6, 10, 11). Moreover, the presence of two chemically different hydroxy groups (primary and secondary) results in phosphate migration at the terminal moiety (12).

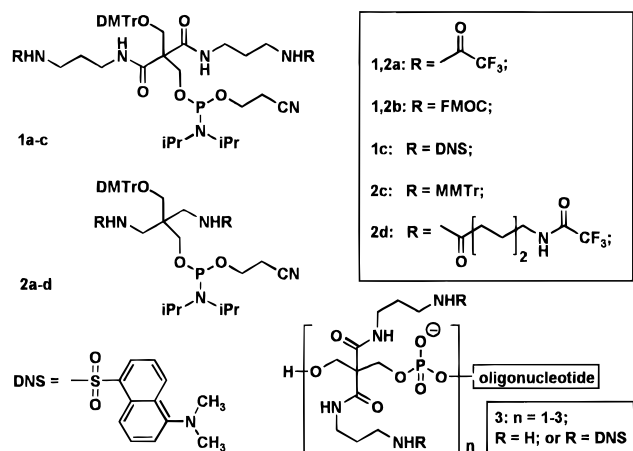
In order to improve the efficiency of synthesis, as well as to mimic the stereochemical properties of the natural polynucleotide backbone, building blocks derived from 1,3-diols have been used instead of their 1,2-diol counterparts. The compounds employed include 2-amino-1,3-butanediol (13), 2-(4-aminobutyl)-1,3-propanediol (14), 2-methyl-2-[2-(benzyloxycarbonyl)ethyl]-1,3-propanediol (15), and 1-[(6-aminohexanoyl)amino]-2,4-propanediol (4). More recently, phosphoramidites from longer α,ω -diols have also been used for the same purpose (8, 16, 17).

The non-nucleosidic building blocks discussed above suffer from two common shortcomings. Except for 5-(aminomethyl)-1,3-benzenedimethanol-based phosphoramidite (17), they are derived from chiral compounds giving upon chain assembly a family of diastereomeric oligonucleotide conjugates. In few cases, a pure enantiomer has been employed as a starting material (8, 13, 16). Furthermore, they allow introduction of only one functionality per one building block added. Both of these drawbacks have been avoided in one case. The phosphoramidite of achiral *N,N'*-bis(2-hydroxyethyl)-*N,N'*-bis(2,4-dinitrophenyl)-1,2-ethanediamine has been used to prepare oligonucleotide conjugates containing up to 10 labels (18). The reporter groups can hardly be introduced postsynthetically, since the secondary amino functions are less reactive than primary ones. With fluorescent reporter groups, attachment *via* longer linkers would also be desirable to reduce intermolecular quenching.

These data clearly indicate that, regardless of the nature of the reporter (functional) group, the structure of the linker tethering the conjugate group to the oligonucleotide is of major importance for the properties of the entire conjugate. It seems to be likely that a non-

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Scheme 1. Structures of Non-Nucleosidic Phosphoramidites 1 and 2 and Multilabeled Oligonucleotides 3

nucleosidic building block optimal for preparation of multilabeled oligonucleotides should meet the following requirements. (i) The parent structure should be 1,3-diol. (ii) This structure should contain neither chiral nor prochiral centers. (iii) It should bear at least two functional groups for labeling, situated sufficiently far from each other and the groups engaged in the oligomer backbone. As an attempt to fulfill these requirements, we now report on preparation of several novel phosphoramidites, **1** and **2**, that are derived from achiral precursors and that possess two primary amino groups (either protected or labeled with reporter groups). Among them, building blocks **1a–c** bearing two *N*-(3-aminopropyl)carboxamido linkers were found to meet all the requirements of oligonucleotide synthesis, deprotection, and labeling (Scheme 1). Their usefulness is demonstrated by preparation of oligonucleotide conjugates (**3**) and their phosphorothioate analogues that bear up to four primary amino functions or up to six fluorescent DNS labels at either the 3'- or 5'-terminus. Melting point measurements showed that the bulky non-nucleosidic tether only slightly retards hybridization of the oligonucleotide moiety (15-mer) with a longer DNA target (27-mer).

EXPERIMENTAL PROCEDURES

General. Diethyl 2,2-bis(hydroxyethyl)malonate was purchased from Aldrich. The reagents for oligonucleotide synthesis were from Cruachem. Adsorption column chromatography was performed on silica gel 60 (Merck). NMR spectra were recorded on a JEOL GX-400 spectrometer operating at 399.8 and 161.9 MHz for ^1H and ^{31}P , respectively. Either CDCl_3 or $\text{DMSO}-d_6$ was used as the solvent, with either tetramethylsilane (TMS) as an internal (^1H) or H_3PO_4 as an external (^{31}P) standard.

Melting Experiments. A λ 2 UV/vis spectrophotometer equipped with a PTP-6 temperature programmer, comprising an electronic control unit and a Peltier cell holder (Perkin-Elmer), was used. The temperature was increased at a rate of $1^\circ\text{C}/\text{min}$. The melting curves were recorded at 260 nm in a buffer containing 20 mM Tris-HCl and 0.1 M NaCl (pH 7.0).

High-Performance Liquid Chromatography (HPLC) Techniques. The oligonucleotides were analyzed and isolated by reversed phase (RP) chromatography [column, Nucleosil 300-5C18, 4.0×250 mm, Macherey-Nagel; buffer A, 0.05 M NH_4OAc ; buffer B, 0.05 M NH_4OAc in 65% MeCN; flow rate, 1.0 mL min^{-1} ; a linear gradient from 5 to 60% B in 30 min was applied for

dimethoxytrityl-protected hexathymidylates and for **31** and **32**; otherwise (**28–30** and **33–41**) from 30 to 100% B in 35 min and then 100% B for 5 min].

Oligodeoxyribonucleotide Synthesis. The protected oligonucleotides were assembled on an Applied Biosystems 392 DNA synthesizer in 0.2 and $1.0 \mu\text{mol}$ scales using either commercial solid supports or **27** (**19**) and phosphoramidite chemistry. Phosphoramidites **1a–c** and **2a–d** were used as 0.1 M solutions in dry MeCN, with unaltered coupling time. In the synthetic protocol for the attachment of non-nucleosidic units derived from **1a–c**, the capping subroutine was excluded, and the detritylation step was carried out by using two subsequent “#14 (acid solution) to column” steps (2×60 s), separated by a trityl flush step (3 s). In other cases, the recommended protocols were employed. The oligonucleotides bearing 5'-terminal non-nucleosidic moieties were subjected to the final deprotection in DMT-on mode. The support-bound material was deprotected with concentrated aqueous NH_3 (2 h at room temperature, then 7 h at 55°C), and products were isolated by RP HPLC. The 5'-DMTr group was cleaved with 80% aqueous AcOH for 20 min at ambient temperature, followed by analysis on both RP and ion exchange columns, RP isolation, and final desalting of the target oligonucleotides.

Labeling of the Amino-Derivatized Oligonucleotides in Solution. DNS-Cl [solution in dimethylformamide (DMF), 10 mg/mL, 120 μL] was added to the oligonucleotides **17**, **31**, or **32** (triethylammonium form, desalted, ca. 4 OD) dissolved in 0.1 M $\text{NaHCO}_3/\text{Na}_2\text{CO}_3$ buffer (pH 10.3, 80 μL). The mixture was incubated in the dark for 3 h, the second portion of the DNS-Cl solution (50 μL) was added, and the reaction mixture was incubated for another 3 h. The reaction mixture was treated with 0.05 M aqueous NH_4OAc (800 μL), and the excess of label was extracted with ethyl acetate ($2 \times 200 \mu\text{L}$). The organic layer was discarded, and the labeled oligonucleotides **19**, **28**, and **29** were isolated from aqueous phase by RP HPLC. The fractions were evaporated *in vacuo* and detritylated with 80% aqueous AcOH (3 mL) for 20 min. After the removal of AcOH by evaporation, the material was separated by RP HPLC, and the fractions collected were finally desalted to give the DNS-labeled oligonucleotides **20**, **33**, or **34**.

Labeling of the Amino-Derivatized Oligonucleotides on the Solid Support. The oligonucleotide-derivatized solid supports **21** and **23** (4 mg, ca. $0.1 \mu\text{mol}$) were placed in a synthetic column. For **21**, the standard detritylation step was carried out. For **23**, the column was treated with a mixture of DMF/piperidine (1.5 mL, 9/1 v/v) for 10 min by a two-syringe method (20). In both cases, the deprotection was completed by extensive washing with MeCN. The solid supports **24** obtained by both routes were reacted with dabsyl chloride in *N,N*-diisopropylamine/pyridine (500 μL , 1/20/80 w/v/v) for 12 h in the dark. The liquid phases were withdrawn, and the solid supports, **25**, were washed with dioxane (5×2 mL) and treated with concentrated ammonia for 2 h. The solutions obtained were evaporated, and the residues were separated by RP HPLC to give **26** in 85 and 60% yields, respectively.

2-Ethoxy-5,5-bis(ethoxycarbonyl)-1,3-dioxane (6). Concentrated H_2SO_4 (0.2 mL) was added to a solution of diethyl 2,2-bis(hydroxymethyl)malonate, **4** (**21**) (33.0 g, 150 mmol), and triethyl orthoformate (33.4 g, 225 mmol) in dry tetrahydrofuran (THF) (50 mL). The reaction mixture was left overnight and then slowly poured into a magnetically stirred ice-cold solution of NaHCO_3 (5% aqueous, 300 mL). The product was extracted with

diethyl ether, washed with saturated aqueous NaCl, and dried over Na₂SO₄. The solvent was evaporated, and the residue was distilled *in vacuo* to give **6** (34.8 g, 84%): bp 135–137 °C (1 Torr); *n*_D²⁰ 1.4416; ¹H NMR (CDCl₃) δ 5.36 (s, 1H, C²-H), 4.49 (d, 2H, *J*_{AB} = 11.2 Hz, C⁴-H^e, C⁶-H^e), 4.26 (2H, q, *J*³ = 7.0 Hz, CH₂OC^aO), 4.24 (d, 2H, *J*_{AB} = 11.2 Hz, C⁴-H^a, C⁶-H^a), 4.23 (2H, q, *J*³ = 7.0 Hz, CH₂OC^aO), 3.64 (2H, q, *J*³ = 7.0 Hz, CH₂OC²), 1.28 (t, *J*³ = 7.0 Hz), 1.27 (t, *J*³ = 7.0 Hz), 1.25 (t, *J*³ = 7.0 Hz, CH₃-CH₂OC², 2 × CH₃CH₂OCO); IR ν 1731 cm⁻¹ (ν_{C=O}); TLC *R*_f 0.3 (CH₂Cl₂), 0.8 (49/1 CH₂Cl₂/MeOH). Anal. (C₁₂H₂₀O₇) C, H.

2-Ethoxy-5,5-bis[N-[3-(trifluoroacetamido)propyl]carboxamido]-1,3-dioxane (8a). Compound **6** (10.0 g, 36.2 mmol) was added to a mixture of 1,3-propanediamine (11.1 g, 150 mmol) and THF (50 mL) and stirred overnight at ambient temperature. The colorless solution obtained was evaporated *in vacuo*, and the residue was coevaporated with water (3 × 100 mL) and *p*-xylene (5 × 100 mL) until no 1,3-propanediamine could be detected by TLC (1/1/1/5 concentrated aqueous ammonia/H₂O/MeOH/THF). The viscous residue was dried on an oil pump at 50 °C to give 12 g (100%) of crude 2-ethoxy-5,5-bis[N-(3-aminopropyl)carboxamido]-1,3-dioxane (**7**): ¹H NMR (D₂O) δ 5.48 (s, 1H, C²-H), 4.53 (d, 2H, *J*_{AB} = 12.4 Hz, C⁴-H^e, C⁶-H^e), 4.23 (d, 2H, *J*_{AB} = 12.4 Hz, C⁴-H^a, C⁶-H^a), 3.74 (q, 2H, *J*³ = 7.0 Hz, CH₂O), 3.30 (t, 2H, *J*³ = 6.8 Hz, CH₂NHC^aO), 3.25 (t, 2H, *J*³ = 6.8 Hz, CH₂NHC^aO), 2.61 (t, 2H, *J*³ = 7.0 Hz, C^aH₂NH₂), 2.58 (t, 2H, *J*³ = 7.3 Hz, C^eH₂NH₂), 1.65 (m, 2H, C-C^aH₂-C), 1.62 (m, 2H, C-C^eH₂-C), 1.21 (t, 3H, *J*³ = 7.0 Hz, CH₃). The content of **7** in the crude sample was more than 95%, and it was hence used without further purification for the preparation of **8a–c**. A mixture of crude **7** (4.75 g, 14.3 mmol), methyl trifluoroacetate (4.20 g, 32.8 mmol), triethylamine (4.05 g, 40 mmol), and dry pyridine (50 mL) was kept for 5 h at room temperature. The reaction mixture was evaporated to an oil, dissolved in CH₂Cl₂ (200 mL), washed with aqueous NaHCO₃ (5%, 2 × 50 mL), and dried over Na₂SO₄. The solvent was evaporated *in vacuo*, and the residue was recrystallized twice from benzene/ethyl acetate to give **8a** (5.76 g, 77%). An analytical sample was recrystallized once to give pure **8a**: mp 152.5–153 °C; ¹H NMR (CDCl₃) δ 7.79 (2H, t, NH), 7.66 (2H, t, NH), 5.39 (1H, s, C²-H), 4.50 (2H, d, *J*_{AB} = 12.2 Hz, C⁴-H^e, C⁶-H^e), 4.19 (2H, d, *J*_{AB} = 12.2 Hz, C⁴-H^a, C⁶-H^a), 3.68 (2H, q, *J*³ = 7.3 Hz, CH₂O), 3.38 (8H, m, CH₂NHCO), 1.78 (4H, m, C-CH₂-C), 1.28 (3H, t, *J*³ = 7.3 Hz, CH₃); TLC *R*_f 0.51 (9/1 CH₂Cl₂/MeOH), 0.40 (19/1 CH₂Cl₂/MeOH). Anal. (C₁₈H₂₆F₆N₄O₇) C, H, N.

2-Ethoxy-5,5-bis[N-[3-[(9-fluorenylmethoxycarbonyl)aminol]propyl]carboxamido]-1,3-dioxane (8b). Fmoc-Cl (4.75 g, 16.5 mmol) was added in small portions to a stirred solution of crude **7** (2.5 g, 7.5 mmol) and NaHCO₃ (2.5 g, 30 mmol) in 40% aqueous dioxane (100 mL) on ice bath. The stirring was continued for 2 h and then for 4 h at room temperature. The suspension was evaporated to one-third of the initial volume and diluted with water (100 mL). The precipitate was extracted with ethyl acetate (3 × 100 mL), and the extracts were washed with water (3 × 50 mL) and dried over Na₂SO₄. After evaporation to dryness, the pure **8b** (4.3 g, 74%) was isolated by recrystallization (hexane/benzene). Two additional recrystallizations from the same mixture gave an analytical sample: mp 95–97 °C; ¹H NMR (CDCl₃) δ 7.75 (4H, m, aromatic), 7.58 (4H, m, aromatic), 7.39 (4H, m, aromatic), 7.30 (4H, m, aromatic), 5.38 (1H, s, C²-H), 4.53 (2H, d, *J*_{AB} = 13.4 Hz, C⁴-H^e, C⁶-H^e), 4.39 (4H, d, *J*³ = 6.8 Hz, CHCH₂O-C=O in Fmoc), 4.21 (2H, d, *J*_{AB} =

13.4 Hz, C⁴-H^a, C⁶-H^a), 4.19 (2H, t, *J*³ = 6.8 Hz, CHCH₂O-C=O in Fmoc), 3.65 (2H, q, *J*³ = 7.1 Hz, CH₃CH₂O), 3.32 (4H, m, CH₂NHCO), 3.21 (4H, m, CH₂NH-Fmoc), 1.67 (4H, m, C-CH₂-C), 1.26 (3H, t, *J*³ = 7.1 Hz, CH₃-CH₂O); TLC *R*_f 0.70 (9/1 CH₂Cl₂/MeOH), 0.45 (19/1 CH₂Cl₂/MeOH). Anal. (C₄₄H₄₈N₄O₉) C, H, N.

2-Ethoxy-5,5-bis[N-[3-[5-(dimethylamino)-1-naphthalenesulfonamido]propyl]carboxamido]-1,3-dioxane (8c). DNS-Cl (1350 mg, 5.0 mmol) was added portionwise to a solution of crude **7** (660 mg, 2.0 mmol) in dry pyridine (25 mL) during 2 h. The mixture was left overnight at room temperature and the reaction quenched by addition of MeOH (0.5 mL). The reaction mixture was evaporated to an oil, dissolved in CH₂Cl₂ (200 mL), washed with aqueous NaHCO₃ (5%, 2 × 50 mL), and dried over Na₂SO₄. The solvent was evaporated *in vacuo*, and the pure compound was isolated on a silica gel column, eluting with a gradient of MeOH (0 to 7%) in CH₂Cl₂ to give **8c** (1295 mg, 81%): ¹H NMR (CDCl₃) δ 8.51 (2H, dd, *J* = 0.5 and 8.5 Hz), 8.33 (2H, dd, *J* = 3.9 and 8.5 Hz), 8.22 (2H, dt, *J* = 1.2 and 7.3 Hz), 7.58–7.50 (4H, m), 7.16 (2H, dt, *J* = <1 and 7.6 Hz (aromatic)), 7.36 (2H, s, NH), 5.77 (2H, t, NH), 5.24 (1H, s, C²-H), 4.28 (2H, d, *J*_{AB} = 12.0 Hz, C⁴-H^e, C⁶-H^e), 3.93 (2H, d, *J*_{AB} = 12.0 Hz, C⁴-H^a, C⁶-H^a), 3.61 (2H, q, *J*³ = 7.1 Hz, CH₂O), 3.28 (4H, m, CH₂NHCO), 2.93–2.86 (4H, m, CH₂NHSO₂), 2.87 (12H, s, N(CH₃)₂), 1.64–1.56 (4H, m, C-CH₂-C), 1.22 (3H, t, *J*³ = 7.3 Hz, CH₃); TLC *R*_f 0.52 (9/1 CH₂Cl₂/MeOH), 0.68 (19/1 CH₂Cl₂/MeOH). Anal. (C₃₈H₅₀N₆O₉S₂) C, H, N, S.

N,N'-Bis[3-(trifluoroacetamido)propyl]-2,2-bis(hydroxymethyl)malonodiamide (9a). Compound **8a** (2.20 g, 4.2 mmol) was dissolved in 80% aqueous AcOH (50 mL) and left for 2 h at room temperature. The solution was evaporated to an oil and coevaporated with water (3 × 25 mL). At this step, the reaction mixture consisted of **9a** and its monoformate. The deprotection was completed by treating the material with MeOH/Et₃N (90/10, 50 mL) for 20 min. *The use of alkalis or primary or secondary amines causes degradation of 9a.* The solution was evaporated, and the residue was coevaporated with toluene (2 × 50 mL). Chromatography on a short silica gel column eluted with a gradient of MeOH (3 to 20%) in CH₂Cl₂ gave **9a** (1.75 g, 89%): mp 101–102 °C (benzene/MeOH); ¹H NMR (DMSO-*d*₆/CDCl₃) δ 9.30 (2H, t, NH), 7.76 (2H, t, NH), 4.86 (2H, t, *J*³ = 4.9 Hz, OH), 3.81 (4H, d, *J*³ = 4.9 Hz, CH₂O), 3.18 (4H, m), 3.11 (4H, m, CH₂NHCO), 1.62 (4H, tt, 2 × *J*³ = 6.6 Hz, C-CH₂-C); TLC *R*_f 0.32 (9/1 CH₂Cl₂/MeOH), 0.12 (19/1 CH₂Cl₂/MeOH). Anal. (C₁₅H₂₂F₆N₄O₆) C, H, N.

N,N'-Bis[3-[(9-fluorenylmethoxycarbonyl)aminol]propyl]-2,2-bis(hydroxymethyl)malonodiamide (9b) was obtained from **8b** (932 mg, 1.2 mmol) by a procedure analogous to that described for **9a**. After the removal of MeOH and Et₃N, the product was dissolved in CH₂Cl₂, washed with saturated aqueous NaCl, and dried over Na₂SO₄. The solvent was evaporated *in vacuo*, and the pure compound was isolated on a silica gel column, eluting with a gradient of MeOH (0 to 12%) in CH₂Cl₂ to give **9b** (700 mg, 81%) as a colorless foam: ¹H NMR (CDCl₃) δ 7.77–7.73 (4H, m, aromatic), 7.73–7.70 (2H, br m, NH), 7.62–7.56 (4H, m, aromatic), 7.41–7.35 (4H, m, aromatic), 7.32–7.27 (4H, m, aromatic), 5.27 (2H, br, m, NH), 4.38 (4H, d, *J*³ = 6.3 Hz, CHCH₂O-C=O in Fmoc), 4.18 (2H, t, *J*³ = 6.3 Hz, CHCH₂O-C=O in Fmoc), 3.99 (2H, br t, OH), 3.91 (4H, d, *J*³ = 5.5 Hz, CH₂OH), 3.32 (4H, m, CH₂NHCO), 3.20 (4H, m, CH₂NH-Fmoc), 1.68 (4H, m, C-CH₂-C); TLC *R*_f 0.50 (9/1 CH₂Cl₂/MeOH), 0.35 (19/1 CH₂Cl₂/MeOH). Anal. (C₄₁H₄₄N₄O₈) C, H, N.

***N,N'*-Bis[3-[5-(dimethylamino)-1-naphthalene-sulfonamidol]propyl]-2,2-bis(hydroxymethyl)malonodiamide (9c)** was obtained from **8c** (800 mg, 1.0 mmol) by a procedure analogous to that described for **9a**. Chromatography on a short silica gel column eluted with a gradient of MeOH (0 to 12%) in CH₂Cl₂ gave **9c** (667 mg, 90%) as a green foam: ¹H NMR (CDCl₃) δ 8.52 (2H, dd, *J* = 0.7 and 8.5 Hz), 8.31 (2H, dd, *J* = 0.6 and 8.6 Hz), 8.22 (2H, dd, *J* = 1.2 and 7.3 Hz), 7.57–7.49 (4H, m), 7.17 (2H, d, *J* = 7.5 Hz (aromatic)), 7.59 (2H, m, NH), 5.97 (2H, t, NH), 3.74 (4H, s, CH₂OH), 3.22 (4H, m, CH₂NHCO), 2.88 (12 H, s, N(CH₃)₂), 2.81 (4H, m, CH₂NHSO₂), 1.50 (4H, m, C-CH₂-C); UV (MeCN) λ_{max} = 260 (ε 26 500), 340 nm (ε = 9700); TLC *R*_f 0.54 (9/1 CH₂Cl₂/MeOH), 0.36 (19/1 CH₂Cl₂/MeOH). Anal. (C₃₅H₄₆N₆O₈S₂) C, H, N, S.

General Procedure for the Selective Dimethoxytritylation of Diols: 3-[(4,4'-dimethoxytrityl)oxy]-2,2-bis(azidomethyl)propanol (12). A solution of 4,4'-dimethoxytrityl chloride (880 mg, 2.60 mmol) in dry dioxane (40 mL) was added dropwise to 2,2-bis(azidomethyl)-1,3-propanediol, **11** (22) (700 mg, 3.75 mmol), in dry pyridine (15 mL). The reaction mixture was left overnight at ambient temperature and the reaction stopped by addition of methanol (0.5 mL). Upon neutralization with NaHCO₃ (5% in water, 10 mL), the mixture was evaporated *in vacuo* to an oil, dissolved in CH₂Cl₂ (100 mL), washed with aqueous saturated NaCl (2 × 50 mL), and dried over Na₂SO₄. The solvent was removed *in vacuo*, and the residue was separated on a silica gel column (30 × 100 mm), eluting with a CH₂Cl₂/hexane mixture (1/3). Fractions containing pure product were pooled, evaporated, and dried on an oil pump to give **12** (1040 mg, 83% on DMTr-Cl) as a colorless oil: ¹H NMR (CDCl₃) δ 7.42–7.20 (9H, m, aromatic), 6.88–6.80 (4H, m, aromatic), 3.80 (6H, s, CH₃O), 3.53 (2H, d, *J*³ = 6.3 Hz, CH₂-OH), 3.43 (2H, s), 3.42 (2H, s, 2 × CH₂N₃), 3.08 (2H, s, C-O-CH₂), 1.79 (1 H, t, *J*³ = 6.3 Hz, HO-CH₂); IR ν 3510 (O-H), 2102 cm⁻¹ (N₃); TLC *R*_f 0.2 (100/1 CHCl₃/EtOH). Anal. (C₂₆H₂₈N₆O₄) C, H, N. Further elution of the column with a mixture of MeOH and CH₂Cl₂ (3/97) gave about 10% of the starting material, **11**. ***N,N'*-Bis[3-(trifluoroacetamido)propyl]-2-[(4,4'-dimethoxytrityl)oxy]methyl-2-(hydroxymethyl)malonodiamide (10a)** was prepared analogously from **9a** (1400 mg, 3.0 mmol) and DMTr-Cl (1020 mg, 3.0 mmol). Isolation on a silica gel column, eluting with a gradient of MeOH (0 to 10%) in CH₂Cl₂, gave first pure **10a** (1660 mg, 72%) along with unreacted **9a** (20%): ¹H NMR (CDCl₃) δ 7.58 (2H, t, *J*³ = 6 Hz, NH), 7.52 (2H, t, *J*³ = 6.2 Hz, NH), 7.38–7.13 (9H, m, aromatic), 6.84–6.80 (4H, m, aromatic), 4.13 (2H, d, *J*³ = 6.0 Hz, CH₂-OH), 3.79 (6H, s, OCH₃), 3.59 (1H, t, *J*³ = 6.0 Hz, OH), 3.50 (2H, s, CH₂O), 3.39–3.22 (8H, m, CH₂NHCO), 1.67 (4H, m, C-CH₂-C); TLC *R*_f 0.42 (19/1 CH₂Cl₂/MeOH), 0.15 (49/1 CH₂Cl₂/MeOH). Anal. (C₃₆H₄₀F₆N₄O₈) C, H, N.

***N,N'*-Bis[3-[(9-fluorenylmethoxycarbonyl)amino]propyl]-2-[(4,4'-dimethoxytrityl)oxy]methyl-2-(hydroxymethyl)malonodiamide (10b)** was prepared analogously from **9b** (577 mg, 0.8 mmol) and DMTr-Cl (271 mg, 0.8 mmol). Isolation by using a gradient of MeOH (0 to 8%) in CH₂Cl₂ gave first pure **10b** (614 mg, 75%) as a white foam along with unreacted **9b** (18%). ¹H NMR (CDCl₃) δ 7.75–7.73 (4H, m, aromatic), 7.58–7.56 (4H, m, aromatic), 7.53–7.46 (2H, br m, NH), 7.40–7.35 (6H, m, aromatic), 7.29–7.16 (11H, m, aromatic), 6.83–6.80 (4H, m, aromatic), 5.29 (2H, br m, NH), 4.36 (4H, d, *J*³ = 6.8 Hz, CHCH₂O-C=O), 4.18 (2H, t, *J*³ = 6.8 Hz, CHCH₂O-C=O), 4.08 (4H, d, *J*³ = 6.8 Hz, CH₂OH), 3.75 (6H, s, OCH₃), 3.50 (2H, s, CH₂ODMTr), 3.29 (2H, m),

3.26 (2H, m, 2 × CH₂NHCO), 3.14 (4H, m, CH₂NHCOO), 1.68–1.57 (4H, m, C-CH₂-C); IR ν 3500–3250 (O-H, N-H), 1698 (ν_{C=O}, amid I), 1539 cm⁻¹ (N-H, amid II); TLC *R*_f 0.50 (19/1 CH₂Cl₂/MeOH), 0.2 (49/1 CH₂Cl₂/MeOH). Anal. (C₆₂H₆₂N₄O₁₀) C, H, N.

***N,N'*-Bis[3-[5-(dimethylamino)-1-naphthalene-sulfonamidol]propyl]-2-[(4,4'-dimethoxytrityl)oxy]methyl-2-(hydroxymethyl)malonodiamide (10c)** was prepared analogously from **9c** (525 mg, 0.7 mmol) and DMTr-Cl (237 mg, 0.7 mmol). Isolation by using a gradient of MeOH (0 to 9%) in CH₂Cl₂ gave first pure **10b** (600 mg, 82%) along with unreacted **9b** (12%): ¹H NMR (CDCl₃) δ 8.49 (2H, d, *J* = 8.5 Hz, DNS), 8.28 (2H, d, *J* = 8.5 Hz, DNS), 8.19 (2H, dd, *J* = 1.0 and 7.3 Hz, DNS), 7.49–7.44 (4H, m, DNS), 7.32 (2H, m), 7.26–7.11 (9H, m, DMTr and NH), 7.12 (2H, d, *J* = 7.6 Hz, DNS), 6.83–6.80 (4H, m, DMTr), 5.94 (2H, t, NH), 3.98 (2H, d, *J*³ = 6.0 Hz, CH₂OH), 3.79 (6H, s, OCH₃), 3.51 (1H, t, *J*³ = 6.0 Hz, OH), 3.39 (2H, s, CH₂ODMTr), 3.07 (4H, m, CH₂NHCO), 2.87 (12H, s, N(CH₃)₂), 2.71 (4H, m, CH₂NHSO₂), 1.33 (4H, m, C-CH₂-C); TLC *R*_f 0.60 (19/1 CH₂Cl₂/MeOH), 0.25 (49/1 CH₂Cl₂/MeOH). Anal. (C₅₆H₆₄N₆O₁₀S₂) C, H, N, S.

3-[(4,4'-Dimethoxytrityl)oxy]-2,2-bis[(trifluoroacetamido)methyl]propanol (14a). (a) *Reduction with 1,3-Propanedithiol/NaBH₄.* Compound **12** (966 mg, 2.0 mmol), 1,3-propanedithiol (20 μL, 0.2 mmol), and Et₃N (0.6 mL, 4.0 mmol) were dissolved in *i*-PrOH (20 mL). NaBH₄ (380 mg, 10 mmol) was added under vigorous stirring in several portions, and the mixture was stirred overnight at room temperature. The solvent was evaporated, and the residue was dissolved in CH₂Cl₂ (150 mL), washed with 10% NaOH in saturated aqueous NaCl (25 mL) and saturated aqueous NaCl (5 × 25 mL), and dried over Na₂SO₄. The solvent was evaporated, and the resulting oil was separated on a silica gel column, eluting with a gradient of MeOH (2 to 20%) in CH₂Cl₂/Et₃N (95/5). The combined fractions were evaporated to dryness, and the resulting oil was redissolved in CH₂Cl₂/hexane (7/3, 25 mL). The turbid solution was filtered through a paper filter and evaporated to give 3-[(4,4'-dimethoxytrityl)oxy]-2,2-bis(aminomethyl)propanol (**13**) (688 mg, 80%) as a colorless foam which was used without further purification: ¹H NMR (CDCl₃) δ 7.42–7.20 (9H, m, aromatic), 6.85–6.80 (4H, m, aromatic), 3.79 (6H, s, CH₃O), 3.71 (2H, s, CH₂-OH), 3.04 (2H, s, C-O-CH₂), 2.91 (2H, d, *J*² = 10.0 Hz, 2 × CH^AH^BNH₂), 2.88 (2H, d, *J*² = 10.0 Hz, 2 × CH^AH^BNH₂), 2.47 (4H, br s, NH₂), 1.27 (1H, s, HO); TLC *R*_f 0.6 (85/10/5 CH₂Cl₂/MeOH/Et₃N).

(b) *Reduction with Ph₃P.* Ph₃P (2070 mg, 7.9 mmol) was added to a solution of **12** (1820 mg, 3.8 mmol) in pyridine (15 mL) for 30 min. The mixture was stirred for 3 h, treated with concentrated aqueous ammonia (3 mL), and left overnight. After evaporation *in vacuo* and coevaporation with toluene (3 × 20 mL), the resulting gum was separated on a silica gel column to give **13** (605 mg, 37%) which was identical to that synthesized by reduction with 1,3-propanedithiol/NaBH₄ (TLC and ¹H NMR).

A solution of **13** (646 mg, 1.5 mmol) and methyl trifluoroacetate (0.45 mL, 4.5 mmol) in dry pyridine (5 mL) was kept overnight at ambient temperature. Aqueous NaHCO₃ (5%, 3 mL) was added, and the mixture was evaporated *in vacuo* to one-third of the initial volume. Water (10 mL) was added to the residue, and the product was extracted with CH₂Cl₂ (2 × 50 mL), washed with saturated aqueous NaCl, and dried over Na₂SO₄. The solvent was evaporated, and the product was isolated by silica gel column chromatography, eluting with a gradient of MeOH in CH₂Cl₂ (0 to 4%) to obtain 820 mg (87%) of

14a as a white foam: ^1H NMR (CDCl_3) δ 7.43–7.20 (9H, m, (aromatic), 6.90 (2H, br m, NH), 6.88–6.84 (4H, m, aromatic), 3.80 (6H, s, CH_3O), 3.47 (2H, br s, $\text{CH}_2\text{-OH}$), 3.43 (2H, dd, $J^2 = 14.2$ Hz, $J^3 = 7.8$ Hz, $2 \times \text{CH}^{\text{A}}\text{H}^{\text{B}}\text{NH}$), 3.08 (2H, s, C-O- CH_2), 2.97 (2H, dd, $J^2 = 14.2$ Hz, $J^3 = 5.9$ Hz, $2 \times \text{CH}^{\text{A}}\text{H}^{\text{B}}\text{NH}$), 1.58 (1H, br, s, HO); IR ν 3600–3300 (O-H, N-H), 1714 ($\nu_{\text{C=O}}$, amid I), 1556 (N-H, amid II), 1216 and 1177 cm^{-1} ($\nu_{\text{C-F}}$); TLC R_f 0.7 (19/1 $\text{CH}_2\text{Cl}_2/\text{MeOH}$). Anal. ($\text{C}_{30}\text{H}_{30}\text{F}_6\text{N}_2\text{O}_6$) C, H, N.

3-[(4,4'-Dimethoxytrityl)oxy]-2,2-bis[[N-(9-fluorenylmethoxycarbonyl)amino]methyl]propanol (14b) was prepared from crude **13** (300 mg, 0.7 mmol) and Fmoc-Cl (520 mg, 2.0 mmol) by a procedure analogous to that for **8b**. Upon isolation by silica gel chromatography (gradient from 0 to 10% MeOH in CH_2Cl_2), the pure **14b** (350 mg, 57%) was obtained as a colorless foam. ^1H NMR (CDCl_3) δ 7.8–7.7, 7.6–7.15 (25H, m, aromatic), 6.9–6.8 (4H, m, aromatic), 5.45 (1H, m, OH), 5.06 (2H, br m, NH), 4.46 (2H, d, $J^3 = 6.3$ Hz, CH_2OH), 4.38 (2H, dd, $J^3 = 7.3$ Hz, $J^2 = 10.5$ Hz, $\text{CHCH}^{\text{A}}\text{H}^{\text{B}}\text{O-C=O}$), 4.34 (2H, dd, $J^3 = 7.3$ Hz, $J^2 = 10.5$ Hz, $\text{CHCH}^{\text{A}}\text{H}^{\text{B}}\text{O-C=O}$), 4.19 (2H, t, $J^3 = 7.3$ Hz, $\text{CHCH}_2\text{O-C=O}$), 3.72 (6H, s, OCH_3), 3.24 (2H, s, CH_2ODMTr), 3.02 (4H, m, $2 \times \text{CH}_2\text{-NHCO}$); IR ν 3500–3300 (O-H, N-H), 1704 ($\nu_{\text{C=O}}$, amid I), 1509 cm^{-1} (N-H, amid II); TLC R_f 0.8 (19/1 $\text{CH}_2\text{Cl}_2/\text{MeOH}$). Anal. ($\text{C}_{56}\text{H}_{52}\text{N}_2\text{O}_8$) C, H, N.

3-[(4,4'-Dimethoxytrityl)oxy]-2,2-bis[[N-(4-methoxytrityl)amino]methyl]propanol (14c). Trimethylsilyl chloride (0.5 mL, 4.2 mmol) was added to a solution of crude **13** (600 mg, 1.4 mmol) in dry pyridine (10 mL). After 2 h, MMTr-Cl (1.73 g, 5.6 mmol) in dry pyridine (10 mL) was added to the mixture, the mixture was stirred overnight at room temperature, and the reaction was quenched with MeOH (10 mL). The resulting solution was evaporated to an oil, and the residue was treated with 5% aqueous NaHCO_3 (50 mL) and extracted with CH_2Cl_2 (2×50 mL). The organic phase was washed in succession with 5% aqueous NaHCO_3 (50 mL) and saturated aqueous NaCl (2×50 mL), dried over Na_2SO_4 , and evaporated. The residue was separated on a silica gel column, eluting with a gradient from 1/1 hexane/ CH_2Cl_2 to 95/5 $\text{CH}_2\text{Cl}_2/\text{MeOH}$ to give **14c** (950 mg, 70%) as a yellow foam: ^1H NMR (CDCl_3) δ 7.35–7.10 (33H, m, aromatic + NH), 7.01 (3H), 6.83 (1H, m), 6.75 (3H, m), 6.66 (3H, m, aromatic), 3.77 (6H, s), 3.74 (6H, s, CH_3O), 3.68 (2H, s, $\text{CH}_2\text{-OH}$), 2.84 (2H, s, C-O- CH_2), 2.50 (2H, d, $J^2 = 15.9$ Hz, $2 \times \text{CH}^{\text{A}}\text{H}^{\text{B}}\text{NH}$), 2.41 (2H, d, $J^2 = 15.9$ Hz, $2 \times \text{CH}^{\text{A}}\text{H}^{\text{B}}\text{NH}$); TLC R_f 0.5 (99/1 $\text{CH}_2\text{Cl}_2/\text{MeOH}$), 0.1 (CH_2Cl_2). Anal. ($\text{C}_{66}\text{H}_{64}\text{N}_2\text{O}_6$) C, H, N.

3-[(4,4'-Dimethoxytrityl)oxy]-2,2-bis[[N-[6-(trifluoroacetamido)hexanoyl]amino]methyl]propanol (14d). Crude diamine **13** (1.6 g, 3.7 mmol) and *N*-oxysuccinimido 6-(trifluoroacetamido)hexanoate (**23**) (2.65 g, 8.2 mmol) in dry pyridine (50 mL) were stirred for 3 h at room temperature and treated with 5% aqueous NaHCO_3 (25 mL). The resulting mixture was concentrated to one-third of the initial volume, diluted with ethyl acetate (100 mL), washed with water (2×50 mL) and saturated aqueous NaCl (2×50 mL), and dried over Na_2SO_4 . The solvent was evaporated, and the product was isolated by silica gel column chromatography, eluting with a gradient of MeOH (0 to 6%) in $\text{CH}_2\text{Cl}_2/\text{pyridine}$ (99/1) to obtain **14d** (1.20 g, 38%) as a colorless oil: ^1H NMR (CDCl_3) δ 7.46–7.15 (9H, m, aromatic), 6.90–6.83 (4H, m, aromatic), 6.78 (4H, br m, NH), 5.95 (4H, br m, NH), 4.41 (1H, br, m, OH), 3.80 (6H, s, CH_3O), 3.42–3.30 (8H, m, $\text{CH}_2\text{-OH}$, $2 \times \text{CH}_2\text{NHCOCF}_3$, $2 \times \text{CH}^{\text{A}}\text{H}^{\text{B}}\text{-NHCO}$), 3.04 (2H, s, C-O- CH_2), 2.07 (4H, t, $J^3 = 6.8$ Hz, $2 \times \text{CH}_2\text{CONH}$), 2.50 (2H, dd, $J^2 = 14.2$ Hz, $J^3 = 7.3$ Hz,

$2 \times \text{CH}^{\text{A}}\text{H}^{\text{B}}\text{NH}$), 1.61–1.53 (8H, m), 1.39–1.28 (4H, m, $6 \times \text{C-CH}_2\text{-C}$); IR ν 3600–3300 (O-H, N-H), 1710, 1690 ($\nu_{\text{C=O}}$, amid I), 1550 (N-H, amid II), 1218 and 1170 cm^{-1} ($\nu_{\text{C-F}}$); TLC R_f 0.6 (9/1 $\text{CH}_2\text{Cl}_2/\text{MeOH}$). Anal. ($\text{C}_{42}\text{H}_{52}\text{F}_6\text{N}_4\text{O}_8$) C, H, N.

General Procedure for the Preparation of Phosphoramidites: 2-Cyanoethyl 3-[(4,4'-Dimethoxytrityl)oxy]-2,2-bis[[N-[(3-trifluoroacetamido)propyl]carboxamidopropyl] (*N,N*-Diisopropylamino)phosphoramidite (**1a**). Dry 1*H*-tetrazole (0.46 M in MeCN, 1.30 mmol, 2.9 mL) was added to a solution of **10a** (960 mg, 1.25 mmol) predried by coevaporation with MeCN (3×20 mL) and 2-cyanoethyl *N,N,N',N'*-tetraisopropylphosphorodiamidite (**24**) (490 mg, 1.6 mmol) in dry MeCN (5 mL). The reaction mixture was left at ambient temperature, and the reaction was found to be completed in 30 min (TLC, 19/1 $\text{CH}_2\text{Cl}_2/\text{MeOH}$). The reaction was stopped by addition of 5% aqueous NaHCO_3 (10 mL). The viscous product was extracted with CH_2Cl_2 (2×20 mL), washed with saturated aqueous NaCl, and dried over Na_2SO_4 . The solvent was removed under reduced pressure, and the residue was dried on an oil pump and dissolved in dry toluene (3 mL). The solution was added dropwise to well-stirred hexane (100 mL) at room temperature. The precipitate was collected on a sintered glass filter and washed with hexane (2×20 mL). Final drying *in vacuo* gave **1a** (1163 mg, 95%) as a white powder: ^{31}P NMR (CDCl_3) δ 149.2; TLC R_f 0.70 (19/1 $\text{CH}_2\text{Cl}_2/\text{MeOH}$), 0.45 (49/1 $\text{CH}_2\text{Cl}_2/\text{MeOH}$). Phosphoramidites **1b,c** and **2a–d** were prepared and isolated in 90–97% yield by a similar procedure.

Phosphoramidite 1b: ^{31}P NMR (CDCl_3) δ 147.2; TLC R_f 0.85 (19/1 $\text{CH}_2\text{Cl}_2/\text{MeOH}$), 0.55 (49/1 $\text{CH}_2\text{Cl}_2/\text{MeOH}$).

Phosphoramidite 1c: ^{31}P NMR (CDCl_3) δ 147.0; TLC R_f 0.9 (19/1 $\text{CH}_2\text{Cl}_2/\text{MeOH}$), 0.6 (49/1 $\text{CH}_2\text{Cl}_2/\text{MeOH}$).

Phosphoramidite 2a: ^{31}P NMR (CDCl_3) δ 149.5; TLC R_f 0.85 (19/1 $\text{CH}_2\text{Cl}_2/\text{MeOH}$), 0.5 (49/1 $\text{CH}_2\text{Cl}_2/\text{MeOH}$).

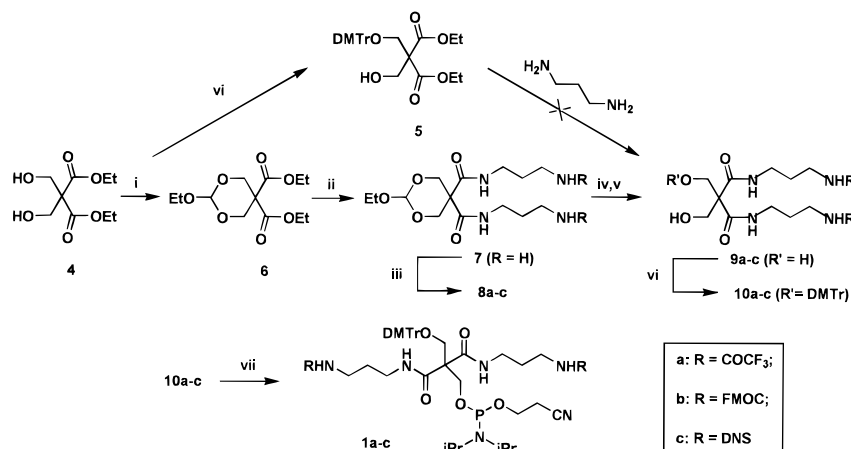
Phosphoramidite 2b: ^{31}P NMR (CDCl_3) δ 147.4; TLC R_f 0.9 (19/1 $\text{CH}_2\text{Cl}_2/\text{MeOH}$), 0.6 (49/1 $\text{CH}_2\text{Cl}_2/\text{MeOH}$).

Phosphoramidite 2c: ^{31}P NMR (CDCl_3) δ 146.9; TLC R_f 0.95 (19/1 $\text{CH}_2\text{Cl}_2/\text{MeOH}$), 0.65 (49/1 $\text{CH}_2\text{Cl}_2/\text{MeOH}$).

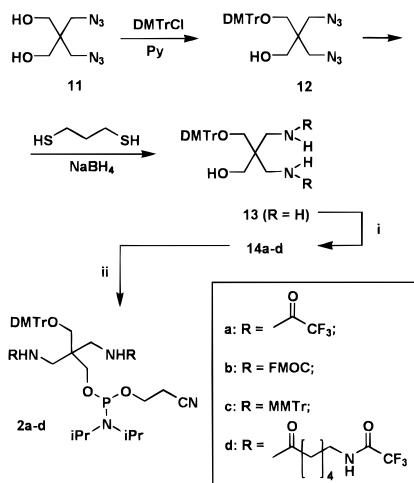
Phosphoramidite 2d: ^{31}P NMR (CDCl_3) δ 148.6; TLC R_f 0.8 (19/1 $\text{CH}_2\text{Cl}_2/\text{MeOH}$), 0.55 (49/1 $\text{CH}_2\text{Cl}_2/\text{MeOH}$).

RESULTS AND DISCUSSION

Synthesis of Phosphoramidites 1 and 2. Building blocks **1a–c** were synthesized from commercially available diethyl 2,2-bis(hydroxymethyl)malonate (**4**) (**21**), as depicted in Scheme 2. The synthetic use of **4** is hampered by its rapid degradation in basic media, in particular in the presence of primary amines. We have recently found that the mono-*O*-DMTr derivative of **4** (**5**) when treated with a catalytic amount of aqueous ammonia or butylamine is decomposed, and a stoichiometric amount of DMTr-OH is released (**25**). This precludes straightforward preparation of any aminoalkyl derivatives from **5**. To overcome this problem, **4** was converted to its ethoxymethylene derivative **6**, which gave the bis[*N*-(3-amino-propyl)amide] **7** upon treatment with 1,3-propanediamine in THF. The primary amino groups were subsequently acylated with methyl trifluoroacetate, Fmoc-Cl, or DNS-Cl to obtain **8a–c**, respectively, and the ethoxymethylene protection was removed by successive acid-catalyzed hydrolysis and triethylamine-catalyzed methanolysis. The use of a tertiary amine as a catalyst is crucial, since ammonia and primary and secondary amines degrade the deprotected products **9a–c**. Selective protection of one of the hydroxy functions of **9a–c** with DMTr-Cl gave **10a–c**. They were finally converted into corresponding phosphoramidites **1a–c** with 2-cyanoethyl *N,N,N',N'*-

Scheme 2^a

^a (i) (EtO)₃CH/H₂SO₄; (ii) 1,3-diaminopropane/THF; (iii) for **8a**, methyl trifluoroacetate/Py; for **8b**, Fmoc-Cl/NaHCO₃/aqueous dioxane; for **8c**, DNS-Cl/Py; (iv) 80% aqueous AcOH; (v) Et₃N/EtOH; (vi) DMTr-Cl/Py; (vii) 2-cyanoethyl *N,N,N',N'*-tetraisopropylphosphorodiamidite/1*H*-tetrazole. DNS, 5-(dimethylamino)-1-naphthalenesulfonyl.

Scheme 3^a

^a (i) for **14a**, methyl trifluoroacetate/Py; for **14b**, Fmoc-Cl/NaHCO₃/aqueous dioxane; for **14c**, MMTr-Cl/Py; for **14d**, *N*-oxysuccinimido 6-(trifluoroacetamido)hexanoate; (ii) 2-cyanoethyl *N,N,N',N'*-tetraisopropylphosphorodiamidite/1*H*-tetrazole. DMTr, 4,4'-dimethoxytrityl; MMTr, 4-monomethoxytrityl; Fmoc, 9-fluorenylmethoxycarbonyl.

tetraisopropylphosphorodiamidite (24) in the presence of 1*H*-tetrazole, followed by aqueous workup and precipitation from toluene solution to hexane.

Building blocks **2a–d** were prepared from easily available 2,2-bis(azidomethyl)-1,3-propanediol (**11**) (**22**) as outlined in Scheme 3. Treatment of **11** with a deficient amount of 4,4'-dimethoxytrityl chloride afforded **12** which was isolated by column chromatography in a moderate yield. The azido groups of **12** were subsequently reduced to amino functions with a mixture of NaBH₄ and 1,3-propanedithiol (**26**), yielding 85% of **13**. Reduction with H₂S (**27**) and 2-mercaptoethanol was unsuccessful, and reaction with triphenylphosphine followed by hydrolysis or ammonolysis gave **13** only in 30–40% yield.

Diamine **13** was *N*-acylated with either methyl trifluoroacetate or Fmoc-Cl to give **14a** and **14b** in a high yield, respectively. Compound **14c**, having an acid-labile 4-monomethoxytrityl (MMTr) protection at the amino functions instead of *N*-acyl groups, was obtained by treating **13** with MMTr-Cl. Compound **14d**, bearing *N*-[6-(trifluoroacetamido)hexanoyl] side arms, was prepared by *N*-acylation with *N*-oxysuccinimido 6-(trifluoroacetamido)hexanoate (**23**). Compounds **14a–d** were

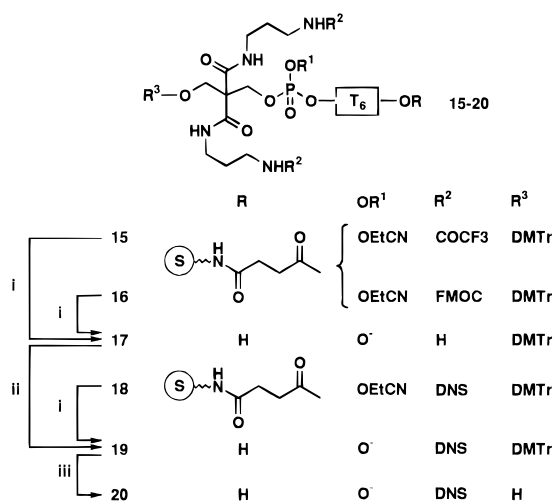
finally converted into corresponding phosphoramidites **2a–d** as described above.

Synthesis of Oligonucleotide Conjugates. The compatibility of phosphoramidites **1** and **2** with oligonucleotide synthesis by phosphoramidite strategy was first examined by assembling model oligonucleotides 5'-XT₆, where X stands for the non-nucleosidic unit. For reference purposes, a small part of the solid support bearing the T₆ sequence (20 nmol) was withdrawn from each synthesis column before the attachment of the non-nucleosidic unit. After conventional deprotection and cleavage from the solid support, the high yield of T₆ was verified by HPLC. All non-nucleosidic building blocks were attached at the final step of chain elongation by using 0.1 M solutions of **1** and **2** and unaltered coupling time (Applied Biosystems 392 DNA synthesizer, 0.2 μmol scale). The yield of the last coupling measured by DMTr assay was found to be 95–96% with **2a**, 97–98% with **1c**, and more than 98% with other phosphoramidites, indicating an acceptable efficiency.

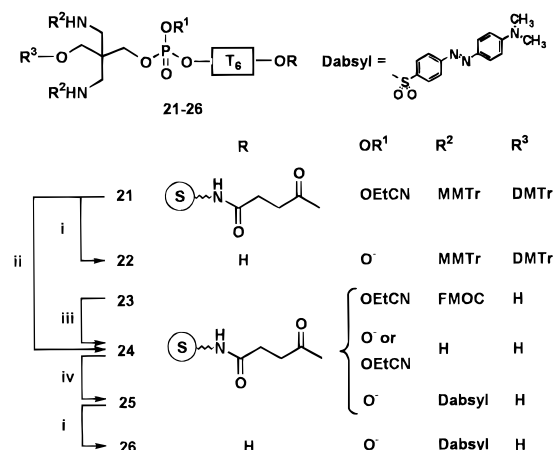
Phosphoramidites **1a–c** were shown to be compatible with the standard protocol of oligonucleotide synthesis and ammonolytic deprotection. No side reactions were detected when the oligonucleotide conjugates prepared from **1a** (**15**) or **1b** (**16**) were subjected to ammonia treatment (Scheme 4). Both conjugates (**15** and **16**) gave the same deprotected oligomer (**17**), as indicated by ion exchange and RP HPLC. Labeling **17** with DNS-Cl gave the fluorescent oligonucleotide, **19**. The identity of **19** was verified by insertion of a DNS-labeled building block (**1c**) into the 5'-terminus of a hexathymidylate on a solid support. The oligonucleotide conjugate obtained (**18**) gave **19** upon ammonolysis. Finally, **19** was detritylated in solution to **20** and isolated by RP HPLC.

It is essential to note that the 5'-DMTr group may be removed only after the deprotection and labeling of the amino functions. While the deprotected oligonucleotide conjugate **20** is stable for several weeks at pH <8.5, *i.e.*, a period long enough for most diagnostic purposes, it is at higher pH converted to hexathymidyl 5'-phosphate. We have shown previously that the unsubstituted hydroxymethyl group undergoes retrograde aldol reaction under alkaline conditions, and this leads to elimination of the non-nucleosidic structure (**25**). For example, in the presence of 0.01 M butylamine, the half-life of this reaction was observed to be less than 30 min with **20**.

The same problem concerns the 3'-labeled oligonucleotides, which may be obtained by conventional attachment

Scheme 4^a

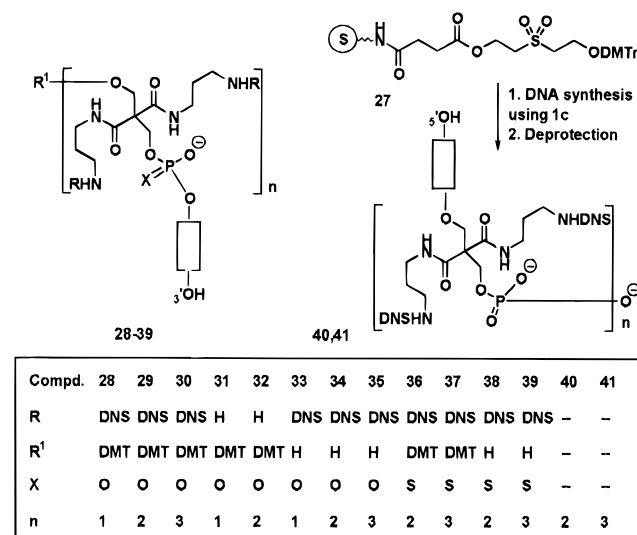
^a (i) concentrated aqueous NH₃; (ii) DNS-Cl; (iii) 80% aqueous AcOH.

Scheme 5^a

^a (i) concentrated aqueous NH₃; (ii) 3% Cl₂HCCO₂H/CH₂Cl₂; (iii) 10% piperidine/DMF; (iv) dabsyl chloride.

of compounds **14a–c** to aminoalkylated controlled pore glass via a succinyl linker and subsequent oligonucleotide synthesis. Cleavage from the solid support with aqueous ammonia creates a free hydroxymethyl function, and this leads to formation of oligonucleotide 3'-monophosphate instead of the desired 3'-labeled oligonucleotide conjugate. Therefore, the synthesis must be carried out on a modified support, from which the 3'-terminal hydroxy group is released in a protected form upon ammonolysis. For example, the hydroxy function of the non-nucleosidic moiety derived from **14a–c** may be kept protected with a phosphate group. This is readily achieved by carrying out the synthesis on the solid support **27** (Scheme 6) described previously (19).

Apparently, the phosphoramidites, **2a–d**, are also compatible with the conventional oligonucleotide synthesis. However, ammonolysis of the hexathymidylates derivatized with **2a**, **b**, or **d**, i.e., building blocks having base-labile amino protections, resulted in rather complicated reaction mixtures that contained a great number of truncated oligonucleotides, even when ammonolyzed in the DMTr-on mode. Only the conjugate of **2c**, having a base stable MMTr protection at the primary amino functions, gave a single product, **22**. Accordingly, oligonucleotide conjugates derived from non-nucleosidic building blocks that contain two unprotected primary amino functions appear to undergo an efficient self-cleavage

Scheme 6. Solid Support **27** and Structures of Poly-labeled Oligonucleotides **28–41**^a

^a The DNA fragments are marked as rectangles.

upon ammonolysis. This is somewhat unexpected, since the 5'-terminal tails consisting of several mono(aminoalkyl) units, such as 3-amino-1,2-propanediol, do not result in a similar instability (14).

For the reasons indicated above, building blocks **2a–d** may be used for multilabeling of oligonucleotides only if the amino protections are removed under mild conditions and the oligonucleotide conjugate is labeled when still attached to the solid support (Scheme 5). To demonstrate the feasibility of this approach, the oligonucleotide conjugate derived from **2c** (**21**) was converted to **24** by treatment with 3% dichloroacetic acid in acetonitrile, and the amino functions released were labeled with dabsyl chloride in organic medium to give **25**. Ammonolysis then led to a bis-labeled oligonucleotide **26** in a high yield. Similarly, the oligonucleotide conjugate of **2b** (**23**) was treated with 10% piperidine (20) to remove the FMOC protections. After labeling with dabsyl chloride and ammonolysis was complete, a bis-labeled product (**26**) was again obtained in a moderate yield, along with monolabeled oligonucleotides. The latter are probably formed by partial cleavage of the diamino oligonucleotide (**24**) during the removal of the FMOC groups and the subsequent labeling.

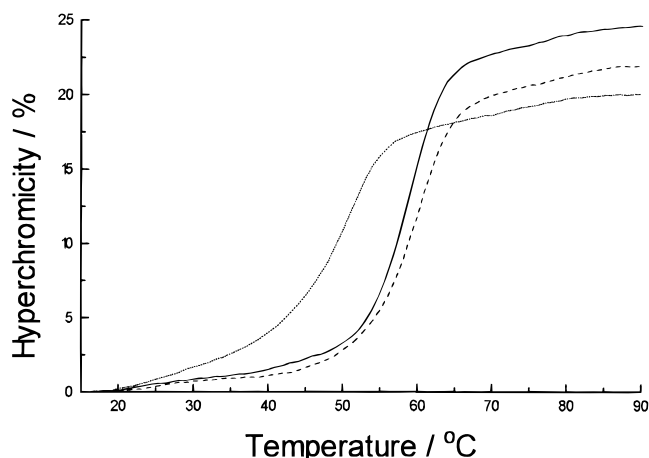
In summary, while **1a–c** may be conveniently used for multilabeling of oligonucleotides, **2b,c** suffer from serious limitations, and **2a,d** turned out to be useless for this purpose.

Synthesis of Polylabeled Oligonucleotide Conjugates by Building Blocks 1a–c. Since **1a–c** were observed to allow an efficient introduction of either amino or DNS groups, they were applied to preparation of both 3'- and 5'-multiderivatized oligonucleotides and their phosphorothioate analogues. The oligonucleotide part of these structures, 5'-GAA CAT CAT GGT CGT-3', is known to be an efficient antisense sequence against *c-fos* mRNA (28).

5'-Labeled oligonucleotides **28–32** were assembled as outlined in Scheme 6. The standard synthetic protocol was only slightly modified. A longer detritylation step (2 min, 3% dichloroacetic acid in dichloromethane) was used to detritylate the non-nucleosidic units. After DMTr-on ammonolysis, **28–32** were isolated in a yield typical for a routine synthesis (16–20 AU from 0.2 μmol column). The di- and tetraamino-derivatized oligomers obtained (**31** and **32**) were subsequently labeled with

Table 1. RP HPLC Retention Times (RT)^a of the Multilabeled Oligonucleotides and Melting Temperatures (T_m) of Their Complexes with the 27-Nucleotide Complement

oligonucleotide	28	29	30	31	32	33	34	35	38	39	40	41
RT, min	23.0	31.0	37.6	21.7	21.8	13.0	24.6	31.0	24.7	30.2	24.56	31.01
T _m , °C	—	—	—	—	—	58.0	57.5	59.5	—	50.3	—	55.1

^a For conditions, see Experimental Procedures.**Figure 1.** Melting curves for the complexes of unmodified (solid line) 5'-hexalabeled **35** (dashed line), and 5'-hexalabeled phosphorothioate **39** (dotted line) oligonucleotides with complementary oligonucleotide sequence. For structures, consult Scheme 6 and the text.

DNS-Cl to give **28** and **29** by an independent route. The synthesis was completed by conventional cleavage of the 5'-terminal DMTr group (80% aqueous AcOH, 20 min). This contrasts with the enhanced resistance of the DMTr group of non-nucleosidic units toward acidic treatment under nonaqueous conditions. The deprotected polylabeled oligonucleotides **33–35** were isolated by RP HPLC (Table 1).

When the iodine oxidation step within the DNA synthetic cycle was replaced by treatment with tetraethylthiuram disulfide (**29**), the same methodology allowed an efficient preparation of the 5'-polylabeled phosphorothioate oligonucleotide analogues, **36–39** (Table 1).

Oligonucleotides labeled at the 3'-terminus with either four or six DNS groups (**40** and **41**) were analogously synthesized on support **27** with the aid of **1c** (Table 1).

Hybridization Properties of the Polylabeled Oligonucleotides. In order to characterize the hybridization properties of the labeled oligonucleotides, melting experiments were carried out for the 5'-dansylated oligonucleotides, **33–35**, phosphorothioate analogue, **39**, and the 3'-labeled oligomer, **41**. A longer, 27-nucleotide, complementary synthetic oligonucleotide, 5'-CAG TCT ACG ACC ATG ATG TTC GTT CAG-3', was used as a hybridization target. The noncomplementary sites (six nucleotides at both termini) were included in its structure to find out whether the heavily labeled non-nucleosidic tails intercalate or, *vice versa*, destabilize the base pairing by creating steric hindrances. The melting points observed are listed in Table 1, and the thermal denaturation curves for the unmodified oligonucleotide and its hexalabeled analogues **35** and **39** are presented in Figure 1. As seen, the T_m values for the multilabeled oligonucleotides **33–35** remain practically unchanged compared to that of the underivatized oligonucleotide (T_m = 58.4 °C), whereas 3'-hexalabeled **41** exhibits a decrease of the melting point (Δ = −3.3 °C). For hexalabeled phosphorothioate analogue **39**, the melting point is lower (T_m = 50.3 °C). Expectedly, the low melting point results from

the modified phosphate backbone (**30**) rather than from the non-nucleosidic moiety.

CONCLUSIONS

Among the phosphoramidites **1** and **2** synthesized, the derivatives of *N,N'*-bis(3-aminopropyl)-2,2-bis(hydroxymethyl)malonodiamide **1a–c** allow the preparation of polyamino- and polydansyl-labeled oligonucleotides and their phosphorothioate analogues. The labeling may be accomplished via routine synthetic cycles at both the 5'- and 3'-termini of the oligonucleotide. In the 3'-labeling, a solid support that caps the 3'-terminal hydroxy group must, however, be used. The introduction of the reporter groups has no or only minor effect on the hybridization properties of the multilabeled oligonucleotides.

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LITERATURE CITED

- (1) For reviews, see: (a) Uhlman, E., and Peyman, A. (1990) Antisense oligonucleotides: A new therapeutic principle. *Chem. Rev.* 90, 543–584; (b) Goodchild, J. (1990) Conjugates of oligonucleotides and modified oligonucleotides: A review of their synthesis and properties. *Bioconjugate Chem.* 1, 165–187; (c) Beaucage, S. L., and Iyer, R. P. (1993) The functionalization of oligonucleotides via phosphoramidite derivatives. *Tetrahedron* 49, 1925–1963.
- (2) Bazin, H., Roget, A., and Teoule, R. (1991) Phosphoramidite reagents for the easy preparation of polylabeled oligonucleotide probes. *Nucleosides Nucleotides* 10, 363–366.
- (3) Teigelkamp, S., Ebel, S., Will, D. W., Brown, T., and Beggs, J. D. (1993) Branched polylabeled oligonucleotides: enhanced specificity of fork-shaped biotinylated oligoribonucleotides for antisense affinity selection. *Nucleic Acids Res.* 21, 4651–4652.
- (4) De Vos, M. J., Van Elsen, A., and Bollen, A. (1994) New non nucleosidic phosphoramidites for the solid phase multilabeling of oligonucleotides: comb- and multifork-like structures. *Nucleosides Nucleotides* 13, 2245–2265.
- (5) Nelson, P. S., Sherman-Gold, R., and Leon, R. (1989) A new and versatile reagent for incorporating multiple primary aliphatic amines into synthetic oligonucleotide. *Nucleic Acids Res.* 17, 7179–7186.
- (6) Korshun, V. A., Pestov, N. B., Birikh, K. R., and Berlin, Y. A. (1992) Reagent for introducing pyrene residues in oligonucleotides. *Bioconjugate Chem.* 3, 559–562.
- (7) Burmeister, J., Azzawi, A., and von Kiedrowski, G. (1995) Synthesis of novel phosphoramidite derivatives bearing pyrenyl and dansyl groups. *Tetrahedron Lett.* 36, 3667–3668.
- (8) Kane, R. R., Drechsel, K., and Hawthorne, M. F. (1993) Automated synthesis of carborane-derived homogeneous oligophosphates: reagents for use in the immunoprotein-mediated boron neutron capture therapy (BNCT) of cancer. *J. Am. Chem. Soc.* 115, 8853–8854; Drechsel, K., Lee, C. S., Leung, E. W., Kane, R. R., and Hawthorne, M. F. (1994) Synthesis of new building blocks for boron-rich oligomers in boron neutron capture therapy (BNCT). I. *Tetrahedron Lett.* 35, 6217–6220.
- (9) Grzybowski, J., Will, D. W., Randall, R. E., Smith, C. A., and Brown, T. (1993) Synthesis and antibody-mediated

- detection of oligonucleotides containing multiple 2,4-dinitrophenyl reporter groups. *Nucleic Acids Res.* 21, 1705–1712.
- (10) Misiura, K., Durrant, I., Evans, M. R., and Gait, M. J. (1990) Biotinyl and phosphotyrosinyl phosphoramidite derivatives useful in the incorporation of multiple reporter groups on synthetic oligonucleotides. *Nucleic Acids Res.* 18, 4345–4354.
- (11) Theisen, P., McCollum, C., Upadhy, K., Jacobson, K., Vu, H., and Andrus, A. (1992) Fluorescent dye phosphoramidite labeling of oligonucleotides. *Tetrahedron Lett.* 33, 5033–5036.
- (12) Thaden, J., and Miller, P. S. (1993) Automated synthesis of oligodeoxyribonucleoside methylphosphonates having [N-(3-aminoprop-1-yl)-N-(2-hydroxyethyl)-2-aminoethyl] phosphate or methylphosphonic acid at the 3' end using a modified controlled pore glass support. *Bioconjugate Chem.* 4, 395–401.
- (13) Reynolds, M. A., Beck, T. A., Hogrefe, R. I., McCaffrey, A., Arnold, L. J., Jr., and Vaghefi, M. M. (1992) A non-nucleotide-based linking method for the preparation of psoralen-derivatized methylphosphonate oligonucleotides. *Bioconjugate Chem.* 3, 366–374.
- (14) Nelson, P. S., Kent, M., and Muthini, S. (1992) Oligonucleotide labeling methods 3. Direct labeling of oligonucleotides employing a novel, non-nucleosidic, 2-aminobutyl-1,3-propanediol backbone. *Nucleic Acids Res.* 20, 6253–6259.
- (15) Endo, M., Saga, Y., and Komiyama, M. (1994) A novel phosphoramidite for the site-selective introduction of functional groups into oligonucleotides via versatile tethers. *Tetrahedron Lett.* 35, 5879–5882.
- (16) Hébert, N., Davis, P. W., DeBaets, E. L., and Acevedo, O. L. (1994) Synthesis of N-substituted hydroxyprolinol phosphoramidites for the preparation of combinatorial libraries. *Tetrahedron Lett.* 35, 9509–9512.
- (17) Behrens, C., Petersen, K. H., Egholm, M., Nielsen, J., Buchardt, O., and Dahl, O. (1995) A new achiral reagent for the incorporation of multiple amino groups into oligonucleotides. *Bioorg. Med. Chem. Lett.* 5, 1785–1790.
- (18) Will, D. W., Pritchard, C. E., and Brown, T. (1991) The synthesis of oligonucleotides that contain 2,4-dinitrophenyl reporter groups. *Carbohydr. Res.* 216, 315–322.
- (19) Kuusela, S., Azhayev, A., Guzaev, A., and Lönnberg, H. (1995) The effect of 3'-terminal monophosphate group on metal-ion-promoted hydrolysis of phosphodiester bonds of short oligonucleotides. *J. Chem. Soc., Perkin Trans. 2*, 1197–1202.
- (20) Tong, G., Lawlor, J. M., Tregear, G. W., and Haralambidis, J. (1993) The synthesis of oligonucleotide-polyamide conjugate molecules suitable as PCR primers. *J. Org. Chem.* 58, 2223–2231.
- (21) Block, P., Jr. (1960) Diethyl bis(hydroxymethyl)malonate. *Org. Synth.* 40, 27–28; *Coll. Vol. V*, pp 381–383.
- (22) Bitha, P., Carvajal, S. G., Citarella, R. V., Child, R. G., Delos Santos, E. F., Dunne, T. S., Durr, F. E., Hlavka, J. J., Lang, S. A., Lindsay, H. L., Morton, G. O., Thomas, J. P., Wallace, R. E., Lin, Y., Haltivanger, R. C., and Pierport, C. G. (1989) Water-soluble third generation antitumor platinum complexes, [2,2-bis(aminomethyl)-1,3-propanediol-N,N']-[1,1-cyclobutanedicarboxylato(2-)-O,O']platinum(II) and [1,1-cyclobutanedicarboxylato(2-)-O,O']tetrahydro-4H-pyran-4,4-dimethanamine-N,N']platinum(II). *J. Med. Chem.* 32, 2015–2020.
- (23) Urdea, M. S., Warner, B. D., Running, J. A., Stempien, M., Clyne, J., and Horn, T. (1988) A comparison of non-radioisotopic hybridization assay methods using fluorescent, chemiluminescent and enzyme labeled synthetic oligodeoxyribonucleotide probes. *Nucleic Acids Res.* 16, 4937–4956.
- (24) Nielsen, J., and Dahl, O. (1987) Improved synthesis of 2-cyanoethyl N,N,N',N'-tetraisopropylphosphorodiamidite (iPr₂N)₂POCH₂CH₂CN. *Nucleic Acids Res.* 15, 3626.
- (25) Guzaev, A., Salo, H., Azhayev, A., and Lönnberg, H. (1995) A new approach for chemical phosphorylation of oligonucleotides at the 5'-terminus. *Tetrahedron* 51, 9375–9384.
- (26) Pei, Y., and Wickman, B. O. S. (1993) Regioselective syntheses of 3-aminomethyl-5-substituted isoxazoles: a facile and chemoselective reduction of azide to amine by sodium borohydride using 1,3-propanedithiol as a catalyst. *Tetrahedron Lett.* 34, 7509–7512.
- (27) Adachi, T., Yamada, Y., Inoue, I., and Saneyoshi, M. (1977) An alternative method for the selective reduction of unsaturated nucleoside azides to the amines. *Synthesis*, 45–46.
- (28) Sommer, W., Bjelke, B., Ganten, D., and Fuxe, K. (1993) Antisense oligonucleotide to *c-fos* induces ipsilateral rotational behaviour to *d-amphetamine*. *NeuroReport* 5, 277–280.
- (29) Vu, H., and Hirschbein, B. L. (1991) Internucleotide phosphite sulfurization with tetraethylthiuram disulfide. Phosphorothioate oligonucleotide synthesis via phosphoramidite chemistry. *Tetrahedron Lett.* 32, 3005–3008.
- (30) Eckstein, F., and Jovin, T. M. (1983) Assignment of resonances in the phosphorus-31 nuclear magnetic resonance spectrum of poly[d(A-T)] from phosphorothioate substitution. *Biochemistry* 22, 4546–4550.

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Protein Patterning with a Photoactivatable Derivative of Biotin

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A method is described for the covalent immobilization of macromolecules at defined locations on polymer surfaces. A thin film of a photoactivatable analogue of biotin, *N*-(4-azido-2-nitrophenyl)-*N'*-(*N*-biotinyl-3-aminopropyl)-*N'*-methyl-1,3-propanediamine (photobiotin, in salt form) was dried onto polystyrene or nitrocellulose surfaces and then exposed to intense white light through a mask to yield patterns of biotin covalently bound to the polymers. The subsequent addition of avidin resulted in the formation of surfaces to which biotinylated molecules could then be bound through a biotin–avidin–biotin bridge. To develop the pattern of avidin on the surfaces, biotinylated enzymes (alkaline phosphatase and horseradish peroxidase) were specifically immobilized on the surface. These enzymes still retained their catalytic activities and were visualized through the formation of colored or fluorescent products. Various factors such as concentration, irradiation time, and light intensity were shown to determine the amount of active enzyme that could be bound and so by implication the degree of photobiotinylation that had occurred.

INTRODUCTION

The controlled immobilization of biomolecules on to both organic and inorganic surfaces has important implications in many areas of science and technology. In biosensors the patterning of different molecules in a spatially resolved manner allows for the construction of multianalyte sensors as well as “on sensor” calibration. Production of bioelectronic devices will undoubtedly demand much better control of the positioning and assembly of biomolecular materials. Even in conventional affinity assays the ability to covalently bind molecules to polymer surfaces may give better control of the performance of the assay. Protein patterning, the defined spatial localization of molecules on a surface, is a potentially powerful approach to generating molecular arrays for analytical or bioelectronic applications. Several different strategies for protein patterning have appeared in the literature during the past few years, including entrapment in electrogenerated polymers (Nakamoto *et al.*, 1988), ink-jet printing (Kimura *et al.*, 1988), and variations on photolithographic activation, deposition, or passivation of surfaces. It is this latter group of methods which appears to offer the greatest versatility and spatial resolution in patterning. Examples include photolithographic deposition of aminosilanes (Britland *et al.*, 1992), photooxidation of thiol groups on mercaptosilane-modified surfaces to render them unreactive (Bhatia *et al.*, 1993), photoactivation of surfaces modified with benzophenones (Rozsnyai *et al.*, 1992), nitroveratryloxy-carbonyl groups (Fodor *et al.*, 1991; Kiederowski *et al.*, 1991), or the photochemical uncaging of biotin (Sundberg *et al.*, 1992). Alternatively photodeposition methods using 4-substituted perfluorophenyl azides (Yan *et al.*, 1993) or proteins modified with (trifluoromethyl)aryl-diazirines (Sigrist *et al.*, 1992) have also been employed. Photobiotin was also employed in the patterning of proteins by Pritchard *et al.* (1995) who reported that avidin was first covalently immobilized on the surface through carbodiimide or silanization methods. Photobiotin was then added and incubated, and the surface

was exposed to light through a mask. The reactive nitrene generated by photolysis reacted with proteins present in solution and cross-linked them to the avidin.

Turning to the immobilization chemistry itself there are numerous reagents available for covalently cross-linking proteins to the modified surface (Sharma *et al.*, 1982); however, arguably the mildest and most generic approach is to use biotin–avidin complexes (Wilchek and Bayer, 1988). There are many advantages in using the avidin–biotin system for protein immobilization; for example, the complex that is formed has an extremely high affinity ($K_a = 10^{15} \text{ M}^{-1}$) (Green, 1975). Moreover the tetrameric structure of avidin means that it can simultaneously bind both to biotin groups on surfaces and to biotinylated molecules. Avidin adsorbed or covalently bound to solid phases has been used to immobilize biotinylated molecules, and a recent abstract describes the use of avidin–biotin technology for protein immobilization in conjunction with photolithography (Sundberg *et al.*, 1992) to obtain a patterned surface. The authors reported that a modified, non-avidin-binding derivative of biotin (a caged biotin) could be covalently bound to a solid surface and the avidin binding properties subsequently revealed photochemically by uncaging the biotin. Chemical biotinylation of many macromolecules has been extensively described in the literature, and in general it appears that biological activity and physical characteristics are often retained after this procedure is performed (Wilchek and Bayer, 1990).

Many biotinylation reagents have been described, and in the context of protein patterning, the derivative *N*-(4-azido-2-nitrophenyl)-*N'*-(*N*-biotinyl-3-aminopropyl)-*N'*-methyl-1,3-propanediamine (“photobiotin” in salt form, shown in Figure 1), is particularly suitable as this compound, upon irradiation, generates a nitrene which can insert readily into not only C–H bonds but also other chemical groups (OH, NH, NO₂, etc.). This reagent has been widely employed for the nonselective labeling of proteins and nucleic acids and would be expected to also react with organic surfaces. In the present work immobilization of enzymes at defined locations on polymer surfaces has been demonstrated. Scheme 1 demonstrates the general patterning procedure which involves the irradiation of a dried film of photobiotin with intense visible light (350–370 nm) on the polymer surface. This

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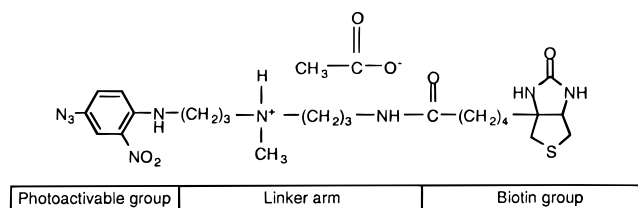
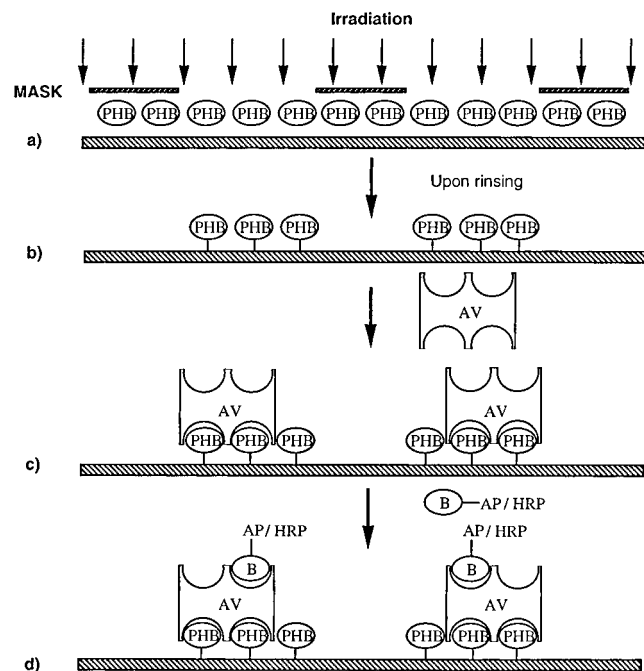


Figure 1. The structure of photobiotin (acetate salt), showing the different parts of the molecule involved in binding to avidin, the photoreactivity, and a linker joining these two.

Scheme 1. Strategy of Protein Patterning Using the Avidin and Biotin System^a



^a (a) A dried photobiotin (PHB) film on the surface is exposed to light in selected areas through a mask. (b) Unreacted photobiotin is removed by washing. (c) Avidin (AV) is then specifically bound to the photobiotin surface. (d) A biotinylated macromolecule is then immobilized on the patterned surface through avidin and biotin.

leads to the conversion of the aryl azide to a reactive aryl nitrene (Forster *et al.*, 1985), which then inserts into the bonds of the polymer (Smith, 1984) resulting in the formation of a covalent linkage between the two. Avidin is then bound specifically to the biotinylated surface. Finally the avidin captures biotinylated macromolecules. This method is distinct from that of Sundberg *et al.* (1992), who covalently bound a caged biotin to a silane-modified surface and then exposed the avidin binding function photolithographically, and from that of Pritchard *et al.* (1995), who chemically coupled avidin to the surface and used photobiotin as a cross-linking agent to a second protein.

EXPERIMENTAL PROCEDURE

Materials. Polystyrene microtitre plates (Linbro) for absorption measurements were purchased from ICN-Flow, and for fluorescence measurements (MicroFluor) from Dynatech. Nitrocellulose membranes (Hybond-C) were from Amersham International, and Photoprobe biotin (acetate salt) and Vector Red were purchased from Vector Laboratories. Avidin was a gift from STC Laboratories (Winnipeg, Canada); it had a reported binding capacity of 14.0 BBU (biotin binding unit) per milligram of protein and was used without further purification.

Biotinylated alkaline phosphatase (BAP),¹ biotinyl *N*-hydroxysuccinimide ester (BNHS), *p*-nitrophenyl phosphate (pNPP), 4-methylumbelliferyl phosphate (4MUP), diaminobenzidine (DAB), *o*-phenylenediamine (OPD), bovine serum albumin (BSA), phosphate-buffered saline (PBS) tablets, and Tween 20 were from Sigma. Horseradish peroxidase (HRP) was obtained from Biozyme. Copper grids (50 mesh) were purchased from Agar Scientific Ltd. A 500-W lamp (code number G 174) from Phillips was used as a light source for photobiotinylation of microtitre plates. For patterning at a microscopic level, the mercury lamp and optics of a Zeiss Axioplan microscope were used. Infrared spectra were obtained on a Matson Research Series FTIR. A microplate reader from TechGen International Ltd. was used to measure the activity of the immobilized enzymes with chromogenic substrates, and fluorescence measurements were performed using a Perkin-Elmer luminescence spectrometer (LS50) connected to an Epson PC AX2 computer. A Zeiss Axioplan microscope operated in epifluorescence mode was used to visualize the patterns of either fluorescein-labeled avidin (450–490 nm bandpass excitation filter, 510 nm dichroic mirror, and 515–565 nm bandpass emission filter) or AP activity (540–550 nm bandpass excitation filter, 580 nm dichroic mirror, and 590 nm longpass emission filter). Images were captured by a LabStar CCD camera and analyzed on a Macintosh IIfx computer using the public domain NIH Image program (written by Wayne Rasband at the U.S. National Institutes of Health and available from the Internet by anonymous ftp from zippy.nimh.nih.gov or on floppy disk from NTIS, 5285 Port Royal Rd., Springfield, VA 22161, part number PB93-504868).

Labeling Avidin with Fluorescein Isothiocyanate (FITC). The method used was that of Wilchek and Bayer (1990) except that a Sephadex G-25 column was used instead of Sephadex G-50 and a Productiv DE column obtained from bps Ltd was used instead of DEAE-cellulose column. The labeling ratio of fluorescein to avidin tetramer (F/A) was calculated from the following equation (Wilchek and Bayer, 1990):

$$F/A = \frac{0.3(A_{495})}{A_{280} - 0.35(A_{495})}$$

The labeling ratios obtained were typically 1.1.

Biotinylation of Horseradish Peroxidase. A solution of biotinyl *N*-hydroxysuccinimide ester (BNHS) in dimethylformamide (55 μ L, 0.1 M) was added to HRP (11 mg) dissolved in sodium bicarbonate solution (1.1 mL, 0.1 M). The mixture was incubated at room temperature for 3 h. The solution was then applied to a G-25 column, pre-equilibrated with PBS, to separate the excess BNHS and biotinylated protein.

Photoimmobilization to Polystyrene. The general procedure for protein immobilization is described for AP, although the same procedure was also used for HRP. A solution of photobiotin in purified water (50 μ L, 1 μ g) was added to the wells of a microtitre plate, and the photobiotin solution was dried under vacuum. The plate was then irradiated for different periods of time with unfiltered light from the 500-W Phillips bulb. The distance

¹ Abbreviations: BAP, biotinylated alkaline phosphatase; BNHS, biotinyl *N*-hydroxysuccinimide ester; pNPP, *p*-nitrophenyl phosphate; 4MUP, 4-methylumbelliferyl phosphate; DAB, diaminobenzidine; OPD, *o*-phenylenediamine; BSA, bovine serum albumin; PBS, phosphate-buffered saline; HRP, horseradish peroxidase; FITC, fluorescein isothiocyanate; PHB, photobiotin; AV, avidin; AP, alkaline phosphatase.

from the bulb to the microtitre plate was approximately 7 cm. The wells were then washed three times with purified water followed by incubation with 3% (w/v) BSA in PBS for 1 h to block any nonspecific binding. A solution of avidin in PBS (50 μ L, 1 mg mL⁻¹) was then incubated in each well for 30 min followed by thorough washing with PBS. A solution of BAP in 3% BSA/PBS solution (50 μ L, 5 units mL⁻¹) was then added to the well, and the mixture was incubated for 30 min. After the plate was washed with PBS three times, the activity of AP immobilized on the surface was determined spectrophotometrically at 405 nm with pNPP in 1 M diethanolamine, 0.5 mM MgCl₂, pH 9.8 (50 μ L, 1 mg mL⁻¹). The reaction mixture was incubated for 10 min, then 50 μ L of a stopping solution (0.1 M EDTA, pH 9.5) was added to each well, and the absorbance in each well of the plate was then read. Controls to check for nonspecific binding were performed by irradiating plates in the absence of photobiotin and by adding photobiotin to plates kept in the dark. In the case of immobilizing biotinylated HRP (B-HRP), a solution of unmodified (used as a control) and modified HRP, diluted in PBS containing 0.5 M NaCl (50 μ L, 2 μ g), was used. The enzyme activity was then determined spectrophotometrically at 450 nm using OPD (at a concentration of 2 mg/mL) and 0.003% (v/v) H₂O₂ dissolved in sodium citrate buffer (100 mM, pH 5).

In some experiments MicroFluor plates were used instead of Linbro with a fixed irradiation time of 20 min. In these experiments control wells either contained photobiotin and were covered with black tape to prevent irradiation or did not contain photobiotin. The activity was developed with 4MUP as the substrate (0.1 M 4MUP in 10 mM DEA, 0.5 mM MgCl₂, pH 9.5), and the fluorescent product was measured using the plate reader attachment of a Perkin-Elmer LS50 spectrofluorimeter (λ_{ex} = 360 nm and λ_{em} = 450 nm). When HRP was immobilized, a solution of nonmodified and modified HRP, diluted in 0.5 M NaCl in PBS (50 μ L, 4 μ g), was added, and enzyme activity was visualized using DAB and H₂O₂ tablets dissolved in deionized water. In this case positive results appear as brown precipitates.

Light-Directed Spatial Photobiotinylation. A solution of photobiotin in purified water (20 μ L, 10 μ g) was applied to a nitrocellulose membrane and incubated for 5 min. The membrane was then dried in the dark in a vacuum oven. After drying, the photobiotin-treated membranes were irradiated, using the mercury lamp and optics of a fluorescence microscope, through a patterned mask (a 50 mesh copper grid) for 10 min. The unreacted photobiotin was removed by thoroughly rinsing the membrane with water. Then the membrane was incubated in 0.3% Tween 20 in PBS for 1 h to block nonspecific binding. Fluorescein-labeled avidin in PBS (1 mL, 1 mg mL⁻¹) was then added to the membrane, and the membrane was incubated for 30 min. The pattern of fluorescent avidin was then observed by examining the membrane under a fluorescence microscope.

Spatially-Selective Immobilization of AP and HRP. The photobiotinylation of nitrocellulose membranes was carried out exactly as described above. After the membrane had been photobiotinylated it was washed and blocked as described above, and avidin solution in PBS (1 mL, 1 mg mL⁻¹) was added and incubated with the membrane for 30 min. The membrane was then washed with 0.05% Tween 20/PBS, and a solution of BAP in 0.05% Tween 20 in PBS (1 mL, 0.5 unit mL⁻¹) was added and incubated for a further 30 min. Finally the membranes were washed thoroughly with 0.05% Tween 20 in PBS followed by PBS prior to exposing the mem-

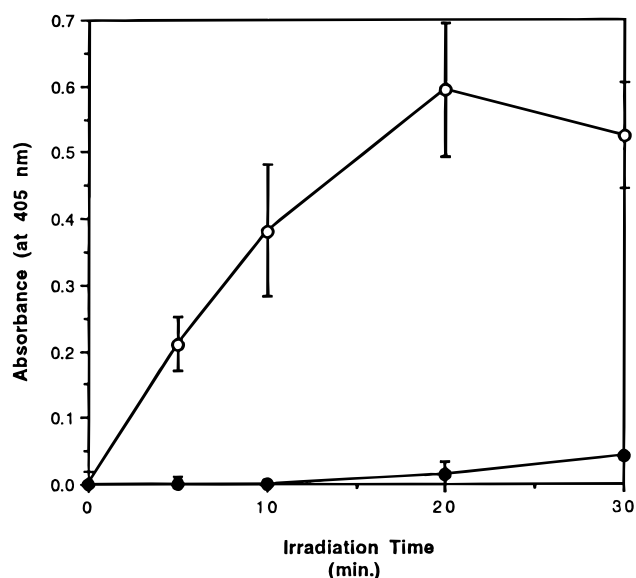


Figure 2. Effect of different irradiation times on the degree of photobiotinylation and hence the amount of active enzyme (AP) immobilized on the polystyrene surface: (○) irradiated, (●) unirradiated. Points: means of three assays. The error bars represent the standard deviation of three experiments.

branes to 5 mL of its substrate (Vector Red). Subsequently the membranes were examined under the fluorescence microscope. Immobilization of HRP followed the same procedure, but biotinylated HRP diluted in PBS containing 0.5 M NaCl (1 mL, 0.04 mg mL⁻¹) was added. DAB and H₂O₂ tablet were then used to visualize the HRP activity.

RESULTS AND DISCUSSION

Photobiotinylation of Polystyrene. In a recent publication (Elsner and Mouritsen, 1994) it was reported that photobiotin was not bound to solid phases when irradiated in solution, and the authors proposed that this may have been due to the nitrene rapidly reacting with water. In preliminary experiments we also observed that irradiation of photobiotin solutions in either aqueous or organic solvents gave low degrees of attachment to the solid phase. However when the irradiation was performed with dried films of photobiotin, considerably greater amounts were bound.

Initial experiments investigated the optimum conditions for photobiotinylation of polystyrene wells in a microtitre plate. After irradiation of the photobiotin films, avidin and BAP were then added followed by the substrate pNPP and subsequent measurement of the absorbance at 405 nm. Both the irradiation time and the amount of photobiotin in the wells were systematically varied, and the results are collected in Figure 2. Control experiments were performed to assess the levels of nonspecific adsorption.

The levels of AP activity immobilized in the wells reached a limiting value with both photobiotin loading and irradiation time, an observation consistent with essentially complete coverage of the bottom surface of the wells by the captured avidin as shown in Figure 3. It was observed that the amounts of AP activity bound after photobiotinylation and addition of avidin were comparable to those found when avidin was simply adsorbed in the wells. The tetrameric structure of avidin is particularly favorable for the capture of biotinylated molecules at a solid-liquid interface as the consequences of differences in orientation of the avidin at the surface are likely to be less pronounced than those in molecules

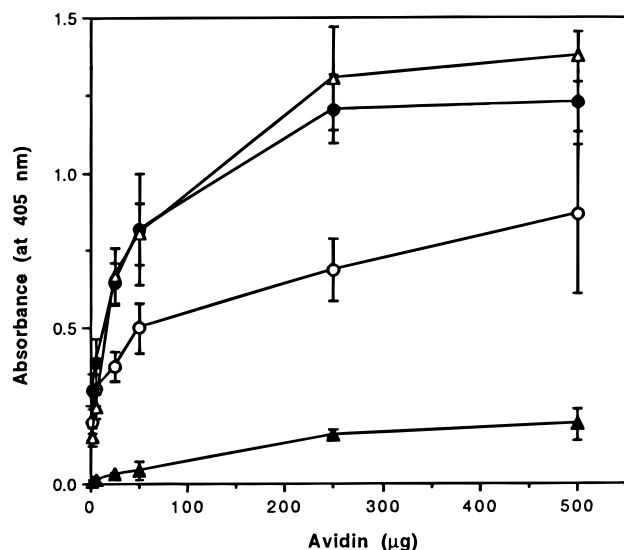


Figure 3. Amount of AP activity specifically bound onto avidin modified surfaces with and without photobiotin and with different amounts of photobiotin present: (○) 4 $\mu\text{g/mL}$, (●) 20 $\mu\text{g/mL}$ of photobiotin, (Δ) avidin adsorbed in the absence of photobiotin, and (▲) Avidin adsorbed in the presence of a blocking agent. Points: means of three assays. The error bars represent the standard deviation of three experiments.

where there is only a single ligand binding site; therefore, it is not surprising that both adsorbed and specifically bound avidin preparations have comparable biotin binding capacity. Although one could imagine using other methods such as ink-jet or screen printing or micro-pipetting to achieve patterning of passive adsorbed avidin, it should be noted that the surfaces employed in the work described in this paper have been specifically prepared to ensure high protein adsorption. In contrast, photobiotin should be more generally applicable for protein patterning on any kind of organic surface.

In both control experiments the nonspecific adsorption of avidin to suitably "blocked" surfaces was found to be only about 8% of the specifically bound value and was quite independent of either irradiation time (no photobiotin) or photobiotin level (no irradiation). No evidence was found for nonspecific adsorption of BAP to the plates irradiated in the absence of photobiotin. Biotinylated HRP was also immobilized on the polystyrene surface in the same way as AP except that biotinylated HRP was diluted in phosphate buffer containing 0.5 M NaCl. The higher ionic strength was necessary to disrupt the ionic interactions of HRP with immobilized avidin. The amount of active HRP immobilized on the photobiotin-avidin surface was determined as a function of PHB, and the results are shown in Figure 4.

The experiments described above were performed in separate plates; however, immobilization in specific wells on a single plate was also demonstrated using opaque plates (Micro Fluor). Black tape was used to mask some of the wells during irradiation, and the AP activity was subsequently developed with the fluorogenic substrate 4MUP as shown in Figure 5A. In the unmasked wells the mean fluorescence intensity was 24.5 ± 1.6 , and in the masked wells it was 3.8 ± 0.3 . Control experiments with no photobiotin again showed that minimal nonspecific binding was occurring (intensity, 3.4 ± 0.3). The fluorescent intensities in Figure 5A have been corrected for nonspecific adsorption of avidin. The optimum time for irradiation again found to be 20 min. We attempted to immobilize B-HRP in specific ways as described for AP. The HRP activity was developed, which appeared

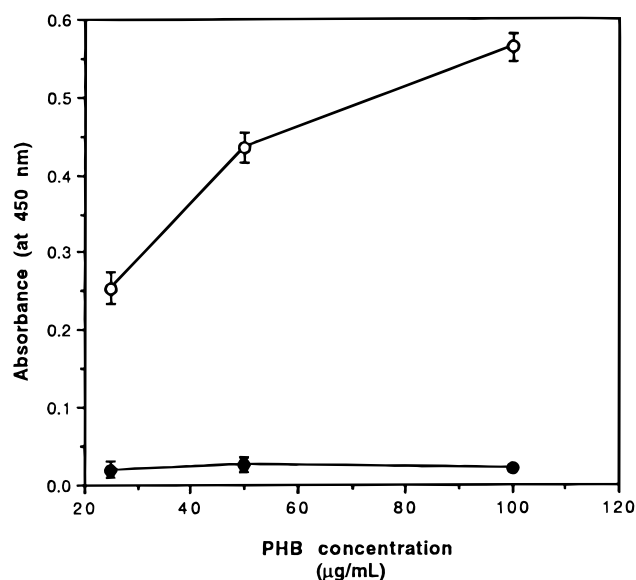


Figure 4. HRP activity immobilized on a photobiotin-avidin surface as a function of the amount of photobiotin present: (○) Photobiotin with irradiation and (●) photobiotin without irradiation. Points: means of three assays. The error bars represent the standard deviation of three experiments.

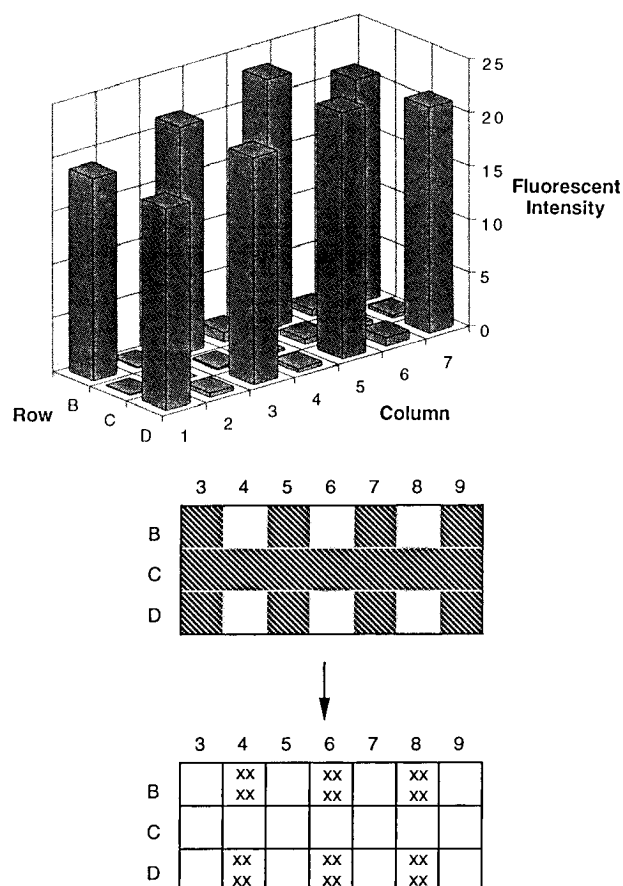


Figure 5. (A, Top) Amount of AP immobilized in different wells of the same microtitre plate depending upon whether they were exposed or masked. (B, Bottom) Pattern of HRP activity immobilized in different wells of a microtitre plate. Alternate rows and columns of the microtitre plate had wells covered by black tape. X represents the brown precipitates occurring upon enzyme catalysis.

as brown precipitates, in the exposed wells, and the pattern was obtained as shown in Figure 5B.

Protein Patterning on Nitrocellulose Membranes. Microscopic patterning was first attempted on polysty-

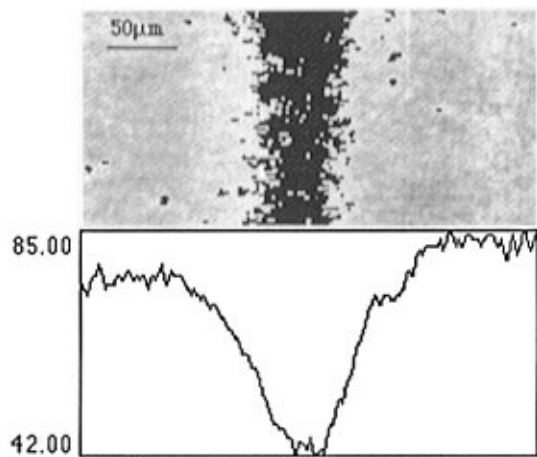


Figure 6. Fluorescence micrograph and plot profile of fluorescein-labeled avidin immobilized on a patterned nitrocellulose membrane through photobiotin. The dark line corresponds to one of the grid lines of the mask.

rene surfaces, but it was found that the pattern that resulted was not clearly and evenly observed. The reason for this could be the surface tension of the photobiotin solution in the well when dried could have resulted in the uneven deposition of a photobiotin film on the surface. This may be overcome by employing a spin-coating method.

As an alternative nitrocellulose membrane was chosen as a surface for photobiotinylation as we reasoned that the photobiotin solution would adsorb into its pores, giving a more even coverage. The photobiotinylation conditions, blocking agents and concentrations, and incubation times with AV and BAP were re-examined with this new surface. The optimum irradiation time was found to be 10 min. The light source was changed from the 500-W tungsten light source to a 100-W mercury source from a fluorescence microscope. It produces a focused light beam, thereby giving more control over exactly where the light irradiates the membrane. This was found to reduce the scattering of light into the masked areas.

In the next step, light-directed spatially controlled photobiotin patterning was developed on nitrocellulose membranes. The dried photobiotin film on the membrane was irradiated using a 50 mesh copper electron microscope grid as a mask, and after adding fluorescein-labeled avidin, it was thoroughly washed and visualized using an epifluorescence microscope. Regions which had been exposed were expected to bind the fluorescein-labeled avidin, and as shown in Figure 6, a pattern resulted in which quantitative determination of the intensity in exposed and unexposed regions yielded values of 89.5 ± 2.2 and 42.5 ± 3 , respectively, giving a contrast factor of 2.

Two control experiments were carried out; in the first, a photobiotin-treated membrane was not exposed to light. The membrane was then washed exhaustively, blocked, and incubated with fluorescein-labeled avidin solution in the same way as for the experimental samples. No pattern was detected. However, the intensity measured on the membrane was 19.4 ± 2.8 units, suggesting that nonspecific binding to the nitrocellulose was occurring. The intensity in this case compares to a value of 42.5 ± 3 for regions of the irradiation sample which were masked as described above.

A second control with no photobiotin present during the irradiation was similarly treated with fluorescein-labeled avidin and examined under the microscope; again

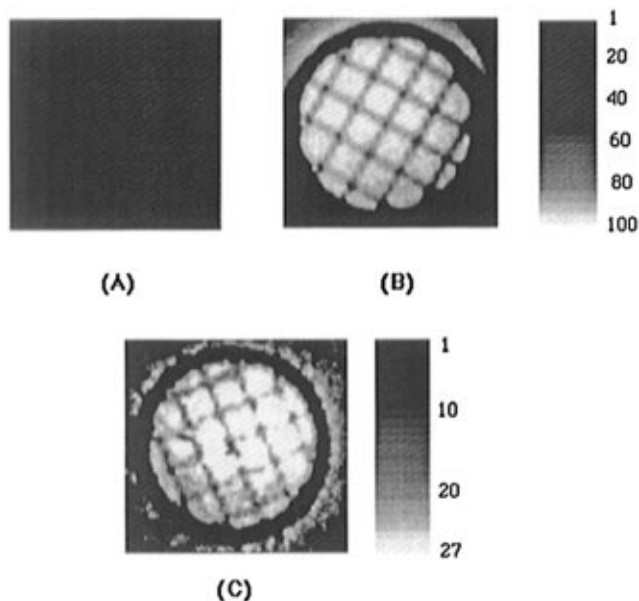


Figure 7. Vector Red image of AP activity immobilized on a patterned nitrocellulose membrane using 50 mesh grid as a mask (grid bar width, $50 \mu\text{m}$): (A) control where photobiotin was not irradiated, (B) irradiated photobiotin, and (C) DAB image of HRP activity immobilized on a patterned nitrocellulose membrane.

no pattern could be detected. The intensity measured was 15.2 ± 1.6 , again consistent with some nonspecific adsorption of fluorescein-labeled avidin to the polymer surface. Measurement of the untreated membranes in the absence of fluorescein-labeled avidin yield intensities of 1–2, implying that the background from light reflected or scattered from the membrane surface contributed relatively little to the overall signal.

Despite the masked areas being shielded from the light, their intensities were significantly higher than the control experiments, implying that photobiotin was still being activated. This could be due to the scattering of light at the grid line areas to partially expose the masked area and hence activate the photobiotin. However, the difference in fluorescence intensities between the grid lines and the holes was still sufficient to be able to distinguish between them as shown in Figure 6.

The pattern of photobiotinylation could also be developed by adding BAP to avidin bound to the irradiated surface. After incubation, the membrane was then thoroughly washed and incubated with the AP substrate (Vector Red). This reaction is known to result in an insoluble red deposit of the product (this product is also highly fluorescent and can be visualized using a rhodamine filter), which reveals the location of the enzyme. Figure 7B shows an image of a membrane containing patterned AP after a 4 min incubation with Vector Red. This is also consistent with AP being immobilized through the biotin–avidin–biotin bridge wherever photobiotin was patterned onto the membrane through the mask. Here again no pattern was observed in the absence of irradiation (Figure 7A).

Similar results were obtained in the case of HRP immobilization. The HRP activity was observed in the exposed area, and no pattern was detected in the absence of irradiation. The image of the membrane containing patterned HRP after a 10 min incubation with DAB and H_2O_2 is shown in Figure 7C.

Fourier transform infrared spectra were obtained from the membrane to confirm that the azide had been converted to a reactive nitrene and inserted into organic

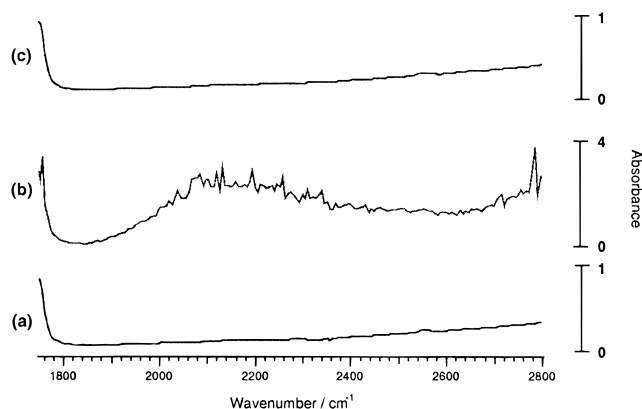


Figure 8. FTIR spectra of (a) a nitrocellulose membrane, (b) a nitrocellulose membrane with photobiotin added, and (c) the photolyzed membrane after a 10 min irradiation.

surfaces. An IR spectra showed the characteristic azide adsorption at 2138 cm^{-1} after photobiotin was dried on the membrane. After the photobiotin-coated membrane was exposed to intense white light for 10 min, complete decomposition of the azide group occurred as indicated by the disappearance of the azide adsorption peak (Figure 8).

CONCLUSIONS

Photobiotin, a well-established reagent for biotinylating macromolecules in solution, has also been shown to be capable of reacting with polymer surfaces during irradiation. The resulting materials can bind avidin, and by employing a mask to screen regions of the surface from photoactivation, the avidin can be patterned either macroscopically or microscopically. Although the long-chain version of photobiotin with a spacer arm between the photoactive group and biotin group has not been investigated, this compound may result in higher levels of discrimination between specific and nonspecific avidin binding on surface can be achieved. (We are grateful to one of the referees for this suggestion.)

Other bifunctional aryl azides could be employed as cross-linking agents between avidin and the surface instead of photobiotin. However, these would react nonselectively with the avidin and may result in either steric effects or inactivation of the binding capacity of the protein. The use of the specific interaction with biotin is simultaneously strong and occurs under mild conditions.

The strength of the avidin biotin interaction as well as the generic nature of biotinylation of proteins, DNA, or polysaccharides suggests that this approach could find wide applicability in patterning biological macromolecules on a variety of surfaces.

LITERATURE CITED

- Bhatia, S. K., Teixeira, J. L., Anderson, M., Shriver-Lake, L. C., Calvert, J. M., Georger, J. H., Hickman, J. J., Dulcey, C. S., Schoen, P. E., and Ligler, F. S. (1993) Fabrication of Surfaces Resistant to Protein Adsorption and Application to Two-Dimensional Protein Patterning. *Anal. Biochem.* 208, 197–205.
- Britland, S., Arnaud, E. P., Clarke, P., McGinn, B., Connolly, P., and Moores, G. (1992) Micropatterning Proteins and Synthetic Peptides on Solid Supports: A Novel Application for Microelectronics Fabrication Technology. *Biotechnol. Prog.* 8, 155–160.
- Elsner, H. I., and Mouritsen, S. (1994) Use of Psoralens for Covalent Immobilisation of Biomolecules in Solid Phase Assays. *Bioconjugate Chem.* 5, 463–467.
- Fodor, S. P. A., Reid, J. L., Pirrung, M. L., Stryer, L., Lu, A. T., and Olas, D. (1991) Light-Directed, Spatially Addressable Parallel Chemical Synthesis. *Science* 251, 767–773.
- Forster, A. C., McInns, J. L., Skingle, D. C., and Symons, R. (1985) Non-Radioactive Hybridisation Probes Prepared by the Chemical Labelling of DNA and RNA with a Novel Reagent Photobiotin. *Nucleic Acid Res.* 13, 745–761.
- Green, N. M. (1975) Avidin. *Advances in Protein Chemistry* 29 (M. L. Anson and J. T. Edsell, Eds.), pp 85–133, Academic Press, New York.
- Kiederowski, G. (1991) Light-Directed Parallel Synthesis of Up to 250,000 Different Oligopeptides and Oligonucleotides. *Angew. Chem., Int. Ed. Engl.* 30, 822–823.
- Kimura, J., Kawana, Y., and Kuriyama, T. (1988) An Immobilised Enzyme Membrane Fabrication Method Using an Ink-Jet Nozzle. *Biosensors* 4, 41–52.
- Nakamoto, S., Ito, N., Kuriyama, T., and Kimura, J. (1988) A Lift-Off Method for Patterning Enzyme-Immobilised Membranes in Multibiosensors. *Sens. Actuators* 13, 165–172.
- Pritchard, D. J., Morgan, H., and Cooper, J. M. (1995) Micron-Scale Patterning of Biological Molecules. *Angew. Chem., Int. Ed. Engl.* 34, 91–93.
- Rozsnyai, L. F., Benson, D. R., Fodor, S. P. A., and Schultz, P. G. (1992) Photolithographic Immobilisation of Biopolymers on Solid Supports. *Angew. Chem., Int. Ed. Engl.* 31, 759–761.
- Sharma, B. P., Bailey, L. F., and Messing, R. A. (1982) Immobilised Biomaterials—Techniques and Applications. *Angew. Chem., Int. Ed. Engl.* 21, 837–854.
- Sigrist, H., Gao, H., and Wegmüller, B. (1992) Light-Dependent, Covalent Immobilisation of Biomolecules on Inert Surfaces. *Bio/technology* 10, 1026–1028.
- Smith, P. A. S. (1984) In Azides and Nitrenes. *Reactivity and Utility* (E. F. V. Scriven, Ed.) p 95, Academic Press, London.
- Sundberg, S., Barrett, R., and Holmes, C. (1992) Spatially-Addressable Immobilisation of Macromolecules on Solid Substrates. *Biophys. J.* 61, A171.
- Wilchek, M., and Bayer, E. A. (1988) The Avidin-Biotin Complex in Bioanalytical Applications. *Anal. Biochem.* 171, 1–32.
- Wilchek, M., and Bayer, E. A. (1990) Avidin- and Streptavidin-Containing Probes. *Methods in Enzymology* 184 (M. Wilchek and E. A. Bayer, Eds.) pp 174–176, Academic Press Inc., New York.
- Wilchek, M., and Bayer, E. A. (1990) Introduction to Avidin-Biotin Technology. *Methods in Enzymology* 184 (M. Wilchek and E. A. Bayer, Eds.) pp 5–13, Academic Press Inc., New York.
- Yan, M., Cai, S. X., Wybourne, M. N., and Keana, J. F. W. (1993) Photochemical Functionalisation of Polymer Surfaces and the Production of Biomolecule-Carrying Micrometre-Scale Structures by Deep-UV Lithography Using 4-Substituted Perfluorophenyl Azides. *J. Am. Chem. Soc.* 115, 814–816.

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A Comparison of Cleavable and Noncleavable Hydrazinopyridine Linkers for the ^{99m}Tc Labeling of Fab' Monoclonal Antibody Fragments

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The design and synthesis of hydrazinopyridine bifunctional chelating agents (BCA's) featuring amide, ester, and disulfide groups are described. The BCA's site-specifically react with the free thiol groups of the tumor-specific monoclonal antibody fragment C46.3 using a one-pot *in situ* reduction and conjugation procedure from the F(ab')_2 to give Fab'–linker conjugates. Molar substitution ratios (MSR's) of the hydrazinopyridine conjugates were comparable to the theoretical (maximum) number of thiols per fragment determined by free hydrazine and residual thiol assays. The series of C46.3 Fab'–linker conjugates were ^{99m}Tc -labeled in greater than 95% radiochemical purity by incubation with ^{99m}Tc –tricine for 1 h at room temperature. In order to evaluate the conjugates for radiopharmaceutical applications, the tumor localization and biodistribution properties of the radiolabeled Fab'–linker conjugates, compared to the direct labeled fragment, were tested in nude mice bearing LS174T xenografts. Depending upon the structure of the linker connecting the radiolabeled hydrazinopyridine group to the antibody fragment, we observed a variation in kidney uptake and whole-body clearance. Diester- and monoester-linked conjugates exhibited lower kidney uptake and faster whole-body clearance than the corresponding linker containing amide groups. This result may be interpreted as evidence for rapid metabolism of ester compared to amide groups in the kidney following uptake. At 24-h postinjection, the monoester-linked conjugate ^{99m}Tc –C46.3 Fab'–BA displayed the highest tumor:blood ratio (16.2) compared to the directly labeled conjugate (6.6) and is therefore a potential clinical candidate for imaging breast and ovarian cancer.

INTRODUCTION

Monoclonal antibodies labeled with radionuclides have received considerable recent attention for the diagnosis and treatment of cancer. In this regard, several groups have developed "bifunctional chelating agents" (BCA's) which feature a protein reactive group that is capable of covalently attaching the chelating agent to the protein while maintaining the ligands necessary for strong radiometal complexation. Since the radiolabeling step is performed via a chelating agent linked to the antibody, one can consider this approach to be an "indirect" method. Alternatively, the antibody can be labeled by "direct" methods which involve the reduction of the available disulfide groups within the protein and subsequent reaction of the liberated free thiols with radiometals such as ^{99m}Tc . While direct labeling methods are straightforward, they suffer from the disadvantages of nonspecific and hence uncharacterizable ^{99m}Tc labeling and, in some cases, the sulfhydryl groups cannot be directly labeled without a significant loss in the biological specificity of the protein required for targeting *in vivo* (1–3).

The use of a hydrazinonicotinate ester BCA for modification of primary amine residues on a protein and subsequent labeling of the derived hydrazinonicotinamide conjugates with ^{99m}Tc –glucoheptanoate has been de-

scribed (4, 5). This indirect approach has been used to label polyclonal human IgG (6, 7), monoclonal IgG's (2), fragment E1 (8), and chemotactic peptides (9). More recently, we reported the preparation of ^{99m}Tc –tricine, a precursor complex that provides more efficient labeling of hydrazinonicotinamide–protein conjugates with higher specific activity than that of ^{99m}Tc –glucoheptanoate (10, 11). The advantages of these combined methods have been recently exemplified for the ^{99m}Tc labeling of a platelet GP IIb/IIIa receptor antagonist, intended as a potential thrombus imaging agent (12). As part of our ongoing efforts to develop radiopharmaceuticals that utilize the hydrazinonicotinamide labeling methodology, we have prepared a series of conjugates of the murine monoclonal antibody C46.3 (IgG2a) in which the hydrazinopyridine moiety is covalently appended to the sulfhydryl groups of the protein via a thioether or disulfide group containing combinations of ester and/or amide linkers. Our reasoning for the introduction of a variety of chemical linkages was based on the reports of Paik (13–15), Meares (16), Weber (17), and others (18, 19), who have previously shown that the linker functional groups have significant effects upon the biodistribution of ^{111}In - or ^{99m}Tc -labeled antibodies. Depending upon the radionuclide and chelating agent used in these studies, linker groups capable of rapid metabolism such as esters or disulfides can increase the clearance of labeled antibody from (a) the blood and/or (b) the kidneys, which are the main nontarget organs for uptake of radiolabeled antibody fragments. In either event, these observations provides us with an approach to optimize the overall target:background ratios for ^{99m}Tc -labeled hydrazinopyridine conjugates and ultimately give tumor images of

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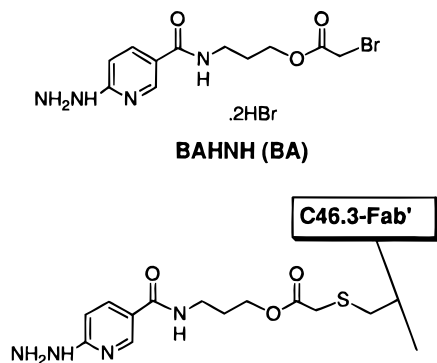


Figure 1. Structures of BAHNH and C46.3 Fab'-BAHNH conjugate.

superior quality. In the present study, we report the design and syntheses of a series of hydrazinopyridine containing BCA's, and their C46.3 Fab' antibody conjugates, in which the functional groups connecting the hydrazinopyridine moiety to the antibody were systematically varied. The biodistributions of both the hydrazinopyridine conjugates labeled with ^{99m}Tc -tricine and the directly labeled C46.3 Fab' were then compared in LS174T tumor-bearing mice. These experiments led to the identification of the ^{99m}Tc -labeled C46.3 Fab'-BAHNH conjugate (Figure 1) as a potential clinical candidate for imaging breast and ovarian cancer (20).

EXPERIMENTAL SECTION

General. ^1H NMR spectra were recorded on a Bruker AC-300 spectrometer operating at 300 MHz. Unless otherwise indicated, ^1H NMR spectra were recorded in $\text{DMSO}-d_6$. Chemical shifts are expressed as δ units downfield from TMS (in CDCl_3 or $\text{DMSO}-d_6$) or TSP [3-(trimethylsilyl)propionic acid- d_4 sodium salt in D_2O]. Fast atom bombardment (FAB) mass spectral analysis was carried out by M-Scan (West Chester, PA) on a VG Analytical ZAB 2-SE high-field spectrometer operating at $V_{\text{acc}} = 8$ kV using a *m*-nitrobenzyl alcohol (MNBA) or glycerol/thioglycerol (1:1) matrix. Mass calibration was performed using cesium iodide. Microanalyses for C, H, N, S, and halogen were carried out by Atlantic Microlabs (Norcross, GA) and were within $\pm 0.4\%$ of theoretical values. The presence and approximate stoichiometry of residual solvent in certain samples was confirmed by ^1H NMR. Merck silica gel 60 was used for all chromatographic purifications.

Analytical HPLC to determine compound purity was carried out on a Waters 600E instrument using the following conditions: 4.6×250 mm Selectosil C-18 column ($5 \mu\text{m}$, 300 Å, silica); mobile phases, A = 0.1% TFA in H_2O , B = 0.1% TFA in CH_3CN ; gradient 10–70% B over 30 min; flow rate, 1 mL/min; UV detection at 254 nm.

Chemistry. The hydrazinopyridine intermediates **3** and **5** and the linker BAHNH were prepared as previously described (6, 20).

General Procedure A: Coupling of Amino Acids with Boc-SHNH. *N*-[(6-[2-[(1,1-Dimethylethoxy)carbonyl]hydrazino]-3-pyridinyl)carbonyl]-3-(2-pyridinyldithio)-L-alanine (**4**). To a solution of *S*-(2-thiopyridyl)-L-cysteine hydrochloride (**2**) (369 mg, 1.37 mmol) and saturated aqueous NaHCO_3 solution (5 mL) and H_2O (3 mL) was added a solution of succinimidyl 2-(Boc-hydrazino)pyridine-5-carboxylate (**3**) (500 mg, 1.42 mmol) in dioxane (5 mL), and the reaction mixture was stirred at room temperature for 2.5 h. Water (25 mL) was added to the reaction mixture, and the aqueous

solution was extracted with EtOAc to remove unreacted ester. The aqueous phase was acidified to pH 3.7 with concentrated aqueous HCl, saturated with NaCl, and then extracted with EtOAc (2×25 mL). The combined organic extracts were dried (MgSO_4), filtered, and evaporated, and the residue was triturated with ether to give a white precipitate. The precipitate was collected by filtration to give **4** (550 mg, 82%) as a white solid: ^1H NMR δ 1.41 (s, 9H), 3.30 (m, 2H), 4.62 (m, 1H), 6.53 (d, 1H, $J = 8.6$ Hz), 7.20 (m, 1H), 7.73 (m, 2H), 7.95 (m, 1H), 8.43 (m, 1H), 8.56 (s, 1H), 8.72 (m, 2H), 8.95 (br s, 1H); FAB MS m/z 488 ($M + \text{Na}$, 17), 466 ($M + \text{H}$, 100), 223 (22), 180 (32), 136 (91). Anal. Calcd for $\text{C}_{19}\text{H}_{23}\text{N}_5\text{O}_5\text{S}_2 \cdot 0.5\text{H}_2\text{O}$: C, 48.10; H, 5.06; N, 14.77. Found: C, 47.83; H, 5.28; N, 14.50.

General Procedure B: HCl/Dioxane Deprotection. *N*-[(6-Hydrazino-3-pyridinyl)carbonyl]-3-(2-pyridinyldithio)-L-alanine Dihydrochloride (**PC**). A solution of hydrogen chloride(g) in dry dioxane was prepared by passing hydrogen chloride(g) through dry dioxane at a moderate rate for 5 min. To a solution of **4** (50 mg) in dioxane (2 mL) was added a solution of HCl/dioxane solution (5 mL), and the reaction mixture was stirred at room temperature for 2 h during which time a white precipitate formed. The solvent was evaporated under reduced pressure, and the residue was washed with ether by decantation followed by drying *in vacuo* overnight to give **PC** (35 mg, 70%) as a white amorphous solid: ^1H NMR δ 3.30 (m, 2H), 4.65 (m, 1H), 6.93 (d, 1H, $J = 8.6$ Hz), 7.25 (m, 1H), 7.75 (m, 1H), 8.15 (m, 1H, $J = 8.6$ Hz), 8.45 (d, 1H), 8.70 (s, 1H), 9.05 (m, 1H); FAB MS m/z 366 ($M + \text{H}$, 100). Anal. Calcd for $\text{C}_{14}\text{H}_{15}\text{N}_5\text{S}_2\text{O}_3 \cdot 2\text{HCl} \cdot 0.2\text{Et}_2\text{O}$: C, 39.22; H, 4.22; N, 15.45. Found: C, 39.61; H, 3.97; N, 15.54.

General Procedure C: Esterification with 3-Bromo-1-propanol. 6-[2-[(1,1-Dimethylethoxy)carbonyl]hydrazino]-3-pyridinecarboxylic Acid 3-Hydroxypropyl Ester (**6**). To a solution of 6-[2-[(1,1-dimethylethoxy)carbonyl]hydrazino]-3-pyridinecarboxylic acid (**5**) (3.0 g, 11.86 mmol) and K_2CO_3 (2.2 g, 15.9 mmol) in DMF (20 mL) was added 3-bromo-1-propanol (2.0 g, 14.39 mmol, 1.2 equiv), and the reaction mixture was stirred under argon at 70 °C for 16 h. The reaction mixture was concentrated to dryness under reduced pressure, and the brown residue was dissolved in EtOAc (100 mL) and washed with H_2O (2×50 mL). The organic phase was separated, dried (Na_2SO_4), and concentrated to give a pale yellow oil. Purification by column chromatography on silica gel (EtOAc/hexane, 3:1) gave **6** (2.28 g, 60%) as a white solid: ^1H NMR (CDCl_3) δ 1.46 (s, 9H), 1.98 (quint, 2H, $J = 6.1$ Hz), 3.75 (t, 2H, $J = 6.1$ Hz), 4.45 (t, 2H, $J = 6.1$ Hz), 6.68 (d, 1H, $J = 8.8$ Hz), 8.11 (dd, 1H, $J = 8.8$, 2.1 Hz), 8.76 (d, 1H, $J = 2.2$ Hz); FAB MS m/z 312 ($M + \text{H}$, 40), 256 (100). Anal. Calcd for $\text{C}_{14}\text{H}_{21}\text{N}_3\text{O}_5$: C, 54.01; H, 6.80; N, 13.50. Found: C, 53.92; H, 6.86; N, 13.17.

General Procedure D: Bromoacetylation 6-[2-[(1,1-Dimethylethoxy)carbonyl]hydrazino]-3-pyridinecarboxylic Acid 3-[(Bromoacetyl)oxy]propyl Ester (**7**). To a stirred solution of **6** (935 mg, 3.0 mmol) and anhydrous Na_2CO_3 (381 mg, 1.2 equiv) in anhydrous THF (30 mL) cooled to -30 to -40 °C under argon was added dropwise bromoacetyl bromide (290 μL , 3.6 mmol, 1.2 equiv), and the reaction mixture was allowed to stir at -30 to -40 °C for a further 4 h and then evaporated to dryness. The residue was partitioned between EtOAc and H_2O , and the organic layer was then separated, dried (MgSO_4), and evaporated to give a colorless oil. Purification of the crude product by column chromatography on silica gel (EtOAc/hexane, 2:1) gave **7** (360 mg, 28%) as a white solid: ^1H NMR (CDCl_3) δ 1.47 (s, 9H), 2.14 (quint,

2H, $J = 6.3$ Hz), 3.84 (s, 2H), 4.34 (t, 2H, $J = 6.3$ Hz), 4.40 (t, 2H, $J = 6.2$ Hz), 6.72 (d, 1H, $J = 8.6$ Hz), 7.05 (br m, 2H), 8.12 (dd, 1H, $J = 8.6, 2.2$ Hz), 8.79 (d, 1H, $J = 2.2$ Hz); FAB MS m/z 434 ($M^{81}\text{Br} + \text{H}$, 25), 432 ($M^{79}\text{Br} + \text{H}$, 25), 376 (17), 374 (17), 201 (17), 185 (100). Anal. Calcd for $\text{C}_{16}\text{H}_{22}\text{N}_3\text{BrO}_6 \cdot 0.15\text{CH}_2\text{Cl}_2$: C, 43.59; H, 5.05; N, 9.44. Found: C, 43.83; H, 5.29; N, 9.09.

General Procedure E: HBr/Acetic Acid Deprotection. 6-Hydrazino-3-pyridinecarboxylic Acid 3-[(Bromoacetyl)oxy]propyl Ester Dihydrobromide (BP). A solution of hydrogen bromide in acetic acid was prepared by passing hydrogen bromide(g) through glacial acetic acid at a moderate rate for 5 min. *Without stirring*, an aliquot of the HBr/acetic acid solution (1 mL) was added to a cooled ($5-10^\circ\text{C}$) solution of **7** (75 mg) in acetic acid (2 mL), and the reaction mixture was allowed to stand at room temperature for 3 min, during which time a white precipitate formed. A large volume of ether was added (50 mL), the solid was allowed to settle to the bottom of the flask, and the supernatant solution was decanted off. The solid was then washed by decantation with ether 5–10 times, and the remaining volatiles were removed by evaporation followed by drying (and storage) *in vacuo* at room temperature to give BP (40 mg, 56%) as a hygroscopic white solid: ^1H NMR (D_2O) δ 2.18 (quint, 2H, $J = 6.0$ Hz), 4.01 (s, 2H), 4.39 (t, 2H, $J = 6.0$ Hz), 4.44 (t, 2H, $J = 6.0$ Hz), 6.89 (d, 1H, $J = 9.0$ Hz), 8.13 (dd, 1H, $J = 9.2, 2.0$ Hz), 8.62 (d, 1H, $J = 2.0$ Hz); HPLC $t_R = 17.3$ min, 95.7%; FAB MS m/z 334 ($M^{81}\text{Br} + \text{H}$, 100), 332 ($M^{79}\text{Br} + \text{H}$, 100), 212 (76), 194 (38), 154 (48), 136 (45); FAB HRMS calcd for $\text{C}_{11}\text{H}_{15}\text{N}_3^{79}\text{BrO}_4$ 332.0246, found 332.0230.

N-[[6-[2-[(1,1-Dimethylethoxy)carbonyl]hydrazino]-3-pyridinyl]carbonyl]-L-glutamic Acid 5-(1,1-Dimethylethyl) Ester (9). Using general procedure A, γ -tert-butyl-L-glutamic acid (**8**) (173 mg) and **3** (300 mg, 1.0 equiv) gave **9** (360 mg, 96%) as a white powder: ^1H NMR δ 1.37 (s, 9H), 1.41 (s, 9H), 1.80–2.14 (m, 2H), 2.31 (t, 2H, $J = 7.5$ Hz), 4.36 (m, 1H), 6.51 (d, 1H, $J = 8.7$ Hz), 7.95 (d, 1H, $J = 8.7$ Hz), 8.40 (br d, 1H, $J = 7.6$ Hz), 8.56 (s, 1H), 8.71 (br s, 1H), 8.92 (br s, 1H). Anal. Calcd for $\text{C}_{20}\text{H}_{30}\text{N}_4\text{O}_7$: C, 54.78; H, 6.89; N, 12.77. Found: C, 54.57; H, 6.79; N, 12.72.

N-[[6-[2-[(1,1-Dimethylethoxy)carbonyl]hydrazino]-3-pyridinyl]carbonyl]-L-glutamic Acid 5-(1,1-Dimethylethyl) 1-(3-Hydroxypropyl) Ester (10). Using general procedure C, reaction of **9** (1.0 g, 2.29 mmol) and 3-bromo-1-propanol (227 μL , 1.1 equiv) followed by purification of the crude product by column chromatography on silica gel (EtOAc/hexane, 9:1) gave **10** (900 mg, 78%) as a white solid: ^1H NMR δ 1.37 (s, 9H), 1.41 (s, 9H), 1.69 (quint, 2H, $J = 6.3$ Hz), 1.80–2.20 (m, 2H), 2.33 (t, 2H, $J = 7.3$ Hz), 3.42 (q, 2H, $J = 6.2$ Hz), 4.10 (m, 2H), 4.39 (m, 1H), 4.51 (t, 1H, $J = 5.0$ Hz), 6.51 (d, 1H, $J = 8.6$ Hz), 7.96 (d, 1H, $J = 8.6$ Hz), 8.50 (d, 1H, $J = 7.2$ Hz), 8.56 (s, 1H). Anal. Calcd for $\text{C}_{23}\text{H}_{36}\text{N}_4\text{O}_8$: C, 55.63; H, 7.30; N, 11.28. Found: C, 55.41; H, 7.32; N, 11.28.

N-[[6-[2-[(1,1-Dimethylethoxy)carbonyl]hydrazino]-3-pyridinyl]carbonyl]-L-glutamic Acid 1-3-[(Bromoacetyl)oxy]propyl 5-(1,1-Dimethylethyl) Ester (11). Using general procedure D, reaction of **10** (200 mg, 0.40 mmol) with bromoacetyl bromide (40 μL , 1.2 equiv) followed by purification of the crude product by column chromatography on silica gel (EtOAc/hexane, 1:1) gave **11** (40 mg, 16%) as a colorless oil: ^1H NMR (CDCl_3) δ 1.42 (s, 9H), 1.47 (br s, 9H), 2.08–2.40 (quint overlapping multiplet, 6H, $J = 6.3$ Hz), 2.41 (t, 2H, $J = 7.0$ Hz), 3.85 (s, 2H), 4.28 (m, 4H), 4.71 (m, 1H), 6.50–6.64 (m, 2H), 6.92 (br s, 1H), 7.43 (br m, 1H), 7.95 (br d, 1H), 8.60 (s, 1H). Anal. Calcd for $\text{C}_{25}\text{H}_{37}\text{N}_4\text{BrO}_9 \cdot 0.25\text{EtOAc}$: C,

48.83; H, 6.14; N, 8.76; Br, 12.49. Found: C, 49.22; H, 6.15; N, 8.74; Br, 12.19.

N-[[6-(Hydrazino-3-pyridinyl)carbonyl]-L-glutamic Acid 1-3-[(Bromoacetyl)oxy]propyl Ester Dihydrobromide (BG). Using general procedure E, deprotection of **11** (40 mg, 0.06 mmol) gave BG (25 mg, 77%) as a white solid: ^1H NMR (D_2O) δ 2.07 (quint, 2H, $J = 6.2$ Hz), 2.09–2.31 (m, 2H), 2.38 (t, 2H, $J = 6.9$ Hz), 4.02 (s, 2H), 4.25 (t, 2H, $J = 6.1$ Hz), 4.31 (t, 2H, $J = 5.9$ Hz), 4.53 (m, 1H), 6.96 (d, 1H, $J = 9.3$ Hz), 8.07 (dd, 1H, $J = 9.3, 2.1$ Hz), 8.41 (d, 1H, $J = 2.0$ Hz); HPLC $t_R = 9.24$ min, 91.6%; FAB MS m/z 463 ($M^{81}\text{Br} + \text{H}$, 100), 461 ($M^{79}\text{Br} + \text{H}$, 100), 383 (22), 339 (10), 237 (10); FAB HRMS calcd for $\text{C}_{16}\text{H}_{22}\text{N}_4^{79}\text{BrO}_7$ 461.0672, found 461.0668.

N-[(Benzyloxy)acetyl]propanolamine (12). To a stirred solution of propanolamine (5.17 g, 0.069 mol) and NaHCO_3 (14.47 g, 0.17 mol) in a mixture of dioxane (50 mL) and H_2O (100 mL) cooled to 0°C was added dropwise a solution of (benzyloxy)acetyl chloride (20.6 g, 0.11 mol, 1.5 equiv) in dioxane (36 mL), and the mixture was allowed to stir for 3 h. The mixture was evaporated to dryness and the residue dissolved in CH_2Cl_2 . The solution was washed with saturated aqueous NaHCO_3 and brine, then dried (MgSO_4), and evaporated to give **12** (11.15 g, 73%) as a colorless oil: ^1H NMR (CDCl_3) δ 1.69 (m, 2H), 3.45 (m, 2H), 3.61 (m, 2H), 4.01 (s, 2H), 4.57 (s, 2H), 6.90 (br s, 1H), 7.26–7.37 (m, 5H). Anal. Calcd for $\text{C}_{12}\text{H}_{17}\text{NO}_3$: C, 64.55; H, 7.67; N, 6.27. Found: C, 63.90; H, 7.53; N, 5.95. This was used without further purification.

6-[2-[(1,1-Dimethylethoxy)carbonyl]hydrazino]-3-pyridinecarboxylic Acid 3-[(Benzyloxy)acetyl]aminopropyl Ester (13). To a stirred solution of **5** (5.0 g, 19.7 mmol), **12** (4.4 g, 19.7 mmol), and DMAP (2.48 g, 19.7 mmol) in DMF (25 mL) was added a solution of DCC (4.48 g, 21.7 mmol) in DMF (10 mL) dropwise, and the mixture was allowed to stir overnight at room temperature during which time a copious white precipitate of dicyclohexylurea formed. The urea was removed by filtration, and the filtrate was evaporated to dryness. The residue was redissolved in EtOAc, and the solution was washed with dilute HCl, saturated aqueous NaHCO_3 , brine, and H_2O , then dried (MgSO_4), and evaporated. The crude product was purified by column chromatography on silica gel (EtOAc) to give **13** (5.1 g, 56%) as a white foam: ^1H NMR (CDCl_3) δ 1.45 (s, 9H), 1.97 (m, 2H), 3.42 (m, 2H), 3.97 (s, 2H), 4.35 (t, 2H, $J = 6.1$ Hz), 4.57 (s, 2H), 6.69 (d, 1H, $J = 8.8$ Hz), 6.71–7.10 (br m, 3H), 7.26–7.40 (m, 5H), 8.11 (dd, 1H, $J = 8.8, 2.1$ Hz), 8.80 (d, 1H, $J = 2.1$ Hz); FAB MS m/z 459 ($M + \text{H}$, 100), 403 (75). Anal. Calcd for $\text{C}_{23}\text{H}_{30}\text{N}_4\text{O}_6 \cdot 0.7\text{H}_2\text{O}$: C, 58.64; H, 6.72; N, 11.89. Found: C, 58.99; H, 6.48; N, 11.51.

6-[2-[(1,1-Dimethylethoxy)carbonyl]hydrazino]-3-pyridinecarboxylic Acid 3-[(Hydroxyacetyl)amino]propyl Ester (14). A solution of **13** (1.6 g, 3.48 mmol), ammonium formate (1.1 g, 17.4 mmol, 5.0 equiv), and Pd/C (Aldrich, 10%, 1.0 g) in MeOH (25 mL) was stirred overnight at room temperature. The mixture was filtered through Celite to remove the Pd/C, and the filtrate was evaporated to dryness. The residue was dissolved in EtOAc, and the solution was washed with H_2O , then dried (MgSO_4), and evaporated to give **14** (1.0 g, 78%) as a white foam: ^1H NMR (CDCl_3) δ 1.46 (s, 9H), 2.00 (m, 2H), 3.45 (m, 2H), 4.09 (s, 2H), 4.39 (t, 2H, $J = 6.0$ Hz), 6.65 (br m, 1H), 6.72 (d, 1H, $J = 8.8$ Hz), 6.80–7.00 (br m, 2H), 8.12 (dd, 1H, $J = 8.8, 2.2$ Hz), 8.79 (d, 1H, $J = 2.2$ Hz). This was used without further purification.

6-[2-[(1,1-Dimethylethoxy)carbonyl]hydrazino]-3-pyridinecarboxylic Acid 3-[(methylsulfonyl)acetyl]aminopropyl Ester (15). To a stirred solution of **14**

(1.0 g, 2.71 mmol) and Et₃N (415 μ L, 1.1 equiv) in CH₂-Cl₂ (20 mL) cooled to 0 °C under argon was added methanesulfonyl chloride (231 μ L, 1.1 equiv), and the mixture was allowed to stir at 0 °C for a further 1.5 h. The solution was washed with H₂O, dried (MgSO₄), and evaporated to give **15** (1.2 g, 98%) as a white solid: ¹H NMR (CDCl₃) δ 1.47 (s, 9H), 1.99 (m, 2H), 3.15 (s, 3H), 3.44 (m, 2H), 4.40 (t, 2H, *J* = 6.0 Hz), 4.65 (s, 2H), 6.50 (br m, 1H), 6.72 (d, 1H, *J* = 9.2 Hz), 6.78 (br m, 1H), 8.12 (dd, 1H, *J* = 9.2, 2.2 Hz), 8.80 (d, 1H, *J* = 2.2 Hz). Anal. Calcd for C₁₇H₂₆N₄O₈·0.2EtOAc: C, 46.04; H, 6.00; N, 12.06; S, 6.90. Found: C, 46.40; H, 6.04; N, 12.36; S, 6.86.

6-[2-[(1,1-Dimethylethoxy)carbonyl]hydrazino]-3-pyridinecarboxylic Acid 3-[(Bromoacetyl)amino]propyl Ester (16). A solution of **15** (1.2 g, 2.68 mmol) and LiBr (1.7 g, 10.0 equiv) in acetone (30 mL) was heated to reflux with stirring for 1.5 h. Upon cooling, the solvent was evaporated and the residue was partitioned between EtOAc and H₂O. The organic layer was separated, washed with H₂O, dried (MgSO₄), and evaporated. The crude product was purified by column chromatography on silica gel (EtOAc) to give **16** (0.8 g, 69%) as a colorless oil: ¹H NMR δ 1.41 (s, 9H), 1.82 (m, 2H), 3.22 (m, 2H), 3.82 (s, 2H), 4.20 (t, 2H, *J* = 6.3 Hz), 6.53 (d, 1H, *J* = 8.8 Hz), 7.98 (dd, 1H, *J* = 8.8, 2.1 Hz), 8.36 (br m, 1H), 8.61 (d, 1H, *J* = 2.1 Hz), 9.00 (br m, 2H); FAB MS *m/z* 433 (M⁸¹Br, 100), 431 (M⁷⁹Br, 431).

6-Hydrazino-3-pyridinecarboxylic Acid 3-[(Bromoacetyl)amino]propyl Ester Dihydrobromide (BH). Using general procedure E, deprotection of **16** (50 mg) gave **BH** (35 mg, 74%) as a white solid: ¹H NMR (D₂O) δ 1.95 (quint, 2H, *J* = 6.5 Hz), 3.38 (t, 2H, *J* = 6.6 Hz), 3.82 (s, 2H), 4.36 (t, 2H, *J* = 6.3 Hz), 6.95 (d, 1H, *J* = 8.8 Hz), 8.25 (dd, 1H, *J* = 8.8, 2.1 Hz), 8.59 (d, 1H, *J* = 2.1 Hz); FAB MS *m/z* 373 (M⁸¹Br + K, 75), 371 (M⁷⁹Br + K, 75), 333 (M⁸¹Br + H, 100), 331 (M⁷⁹Br + H, 100). Anal. Calcd for C₁₁H₁₅N₄O₃Br·2HBr·0.4HOAc: C, 27.39; H, 3.62; N, 10.83. Found: C, 27.00; H, 3.69; N, 10.72.

6-[2-[(1,1-Dimethylethoxy)carbonyl]hydrazino]-3-(3-aminopropyl)pyridinecarboxamide (17). To a solution of 1,3-propanediamine (7.41 g, 0.1 mol) in DMF (20 mL) was added **3** (3.5 g, 10 mmol, 0.1 equiv), and the mixture was allowed to stir at room temperature overnight during which time a solid precipitated. The solvent was evaporated under reduced pressure, and the residue was partitioned between H₂O and EtOAc. The aqueous layer was separated, saturated with Na₂CO₃ until the pH ~11.5, and then extracted twice with EtOAc. The combined organic extracts were dried (MgSO₄) and evaporated to give **17** (1.1 g, 36%) as a white solid: ¹H NMR δ 1.42 (s, 9H), 1.55 (m, 2H), 2.56 (t, 2H, *J* = 6.6 Hz), 3.24 (m, 2H), 6.51 (d, 1H, *J* = 8.7 Hz), 7.93 (dd, 1H, *J* = 8.9, 1.8 Hz), 8.33 (br t, 1H, *J* = 5.1 Hz), 8.52 (d, 1H, *J* = 2.0 Hz). Anal. Calcd for C₁₄H₂₃N₅O₃·0.25H₂O: C, 53.57; H, 7.55; N, 22.31. Found: C, 53.53; H, 7.33; N, 22.33.

6-[2-[(1,1-Dimethylethoxy)carbonyl]hydrazino]-3-[3-[(bromoacetyl)amino]propyl]pyridinecarboxamide (18). Using general procedure D, reaction of the amine **17** (510 mg, 1.62 mmol) and bromoacetyl bromide (170 μ L, 1.95 mmol, 1.2 equiv) in DMF (due to the solubility of **17**, 10 mL) followed by purification of the crude product by column chromatography on silica gel (CH₂Cl₂/MeOH, 95:5) gave **18** as a colorless oil (110 mg, 15%): ¹H NMR (CDCl₃) δ 1.46 (s, 9H), 1.74 (m, 2H), 3.30 (m, 2H), 3.40 (m, 2H), 3.88 (s, 2H), 6.65 (d, 1H, *J* = 8.7 Hz), 7.07 (br s, 1H), 7.16 (br s, 1H), 7.36 (m, 2H), 7.96 (d, 1H, *J* = 8.8 Hz), 8.58 (s, 1H); FAB MS *m/z* 432 (M⁸¹Br + H, 100), 430 (M⁷⁹Br + H, 100), 376 (60), 374 (60).

6-Hydrazino-3-[3-[(bromoacetyl)amino]propyl]pyridinecarboxamide Dihydrobromide (BD). Using general procedure E, deprotection of **18** (110 mg, 0.26 mmol) gave **BD** (90 mg, 86%) as a white solid: ¹H NMR (D₂O) δ 1.64 (quint, 2H, *J* = 6.6 Hz), 3.10 (t, 2H, *J* = 6.6 Hz), 3.21 (t, 2H, *J* = 6.6 Hz), 3.68 (s, 2H), 6.85 (d, 1H, *J* = 9.5 Hz), 7.89 (d, 1H, *J* = 9.6 Hz), 8.14 (s, 1H); FAB MS *m/z* 372 (M⁸¹BrH + K, 40), 370 (M⁷⁹BrH + K, 40), 332 (M⁸¹Br + H, 100), 330 (M⁷⁹Br + H, 100). Anal. Calcd for C₁₁H₁₆N₅BrO₂·2HBr·0.6HOAc: C, 27.75; H, 3.89; N, 13.26. Found: C, 28.02; H, 4.25; N, 13.20.

Monoclonal Antibody C46.3. The murine monoclonal antibody C46.3 (IgG2a) is a product of Cytogen. C46.3 was developed by Amersham International (Amersham, U.K.) (22). F(ab')₂ fragments of C46.3 were generated by pepsin digestion as previously described (23).

Conjugation of Fab' Fragments. For conjugation with the linkers, a solution of C46.3 F(ab')₂ fragment in PBS-EDTA (10 mM phosphate, 150 mM NaCl, 1 mM EDTA, pH 7) was first concentrated to 5–20 mg/mL using either a Centricon-10 concentrator (Amicon, Beverly, MA) or an Amicon stirred ultrafiltration cell with a YM10 membrane. The F(ab')₂ was reduced with an 8 molar excess of dithiothreitol (DTT, Calbiochem, La Jolla, CA) for 14–18 h at room temperature in the dark. The reduction was monitored by isocratic size exclusion HPLC using either a TSK-3000SW_{XL} column (TOSOHASS, Philadelphia, PA) or a Biosep S3000 column (Phenomenex, Torrance, CA) equilibrated with PBS and 1 mM EDTA, pH 6. Without prior purification to remove the excess DTT, the reduced F(ab')₂-DTT mixtures were reacted with a 2–4-fold molar excess of linker to the DTT thiol content. Linker was added as a 30–100 mM solution in water and the pH adjusted to pH 6.0 with 1 N NaOH. The F(ab')₂-DTT-linker mixtures were allowed to react for 16–23 h at 15–30 °C after the reaction vessel was purged with nitrogen or argon to prevent oxidation.

The C46.3 Fab'-linker conjugates were purified from their respective reaction mixtures by size exclusion chromatography on a column of either Zorbax SE250 (DuPont, Wilmington, DE) or Superose-12 (Pharmacia, Piscataway, NJ). The maximal reaction mixture load volumes were 3% of total bed volume. The column was eluted at a linear flow rate of 0.5 cm/min with 10–20 mM sodium citrate pH 5.0 containing 1–2 mM EDTA. The Fab'-linker conjugates were concentrated to 2–5 mg/mL and stored at 4 °C.

Protein Concentration Determination. The protein concentration of the antibody samples were determined by calculation from the UV absorbance using extinction coefficients of *E*₂₈₀ (mg/mL) of 1.51 for C46.3 Fab' (22). For the conjugates, protein concentration was determined by the Bio-Rad Protein Determination Assay (Bio-Rad, Richmond, CA) as described by the manufacture using the C46.3 Fab' protein reference standards.

Residual Thiol Assay. Residual thiols remaining after reaction were determined by reaction with 4,4'-dithiodipyridine (24) [DTDP (Aldrich, Milwaukee, WI)]. Samples to be analyzed were diluted to a total volume of 0.9 mL in buffer (100 mM phosphate, 1 mM EDTA, pH7), and the UV absorbances at 280 nm and 324 nm were determined. One hundred microliters (100 μ L) of 2 mM 4,4'-dithiodipyridine in H₂O was added to the diluted sample, and the absorbance at 324 nm was again determined. The thiol concentration was calculated using the published extinction coefficient of 4-pyridinethiol (*E*_{324,M} = 23 000) after subtraction of the pre-

DTDP OD₃₂₄ absorbance from the post-DTDP OD₃₂₄ absorbance, correcting for dilution.

The number of thiols per antibody fragment is expressed as the ratio of the thiol concentration (μM) to the protein concentration (μM).

o-Sulfonic Benzaldehyde Hydrazine Assay. Hydrazine content of the linker modified Fab' was determined by reaction with *o*-sulfonic acid benzaldehyde. Samples to be analyzed were diluted into 1 mL of *o*-sulfonic benzaldehyde (Eastman Kodak, Rochester, NY; 10.4 mg/100 mL, 0.1 M sodium acetate, pH 4.7). The reaction was incubated at room temperature in the dark overnight. The absorption of the hydrazone adduct was read at 343 nm. The hydrazine concentration was calculated using an extinction coefficient of $E_{343,\text{M}} = 26\,500$ for the adduct.

The molar substitution ratio (MSR) of hydrazine conjugated to antibody was defined as the ratio of the hydrazine concentration (μM) to the protein concentration (μM) as determined by a Bio-Rad protein determination assay.

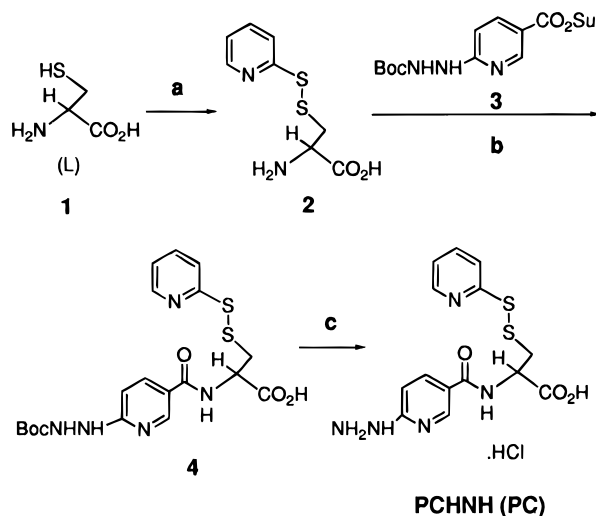
Radiolabeling. Direct radiolabeling of the C46.3 Fab' fragment was carried out at 50 mCi/mg using $^{99\text{m}}\text{Tc}$ –glucoheptonate (Glucoscan Kit, Dupont, North Billerica, MA). Briefly, a solution of 50 mCi of $^{99\text{m}}\text{TcO}_4$ in saline (~ 1.25 mL) was added to the Glucoscan vial and allowed to stand at room temperature for 15 min. The radiopurity of the $^{99\text{m}}\text{Tc}$ –glucoheptonate solution was determined by TLC (ITLC-SG, 1×8 cm, Gelman, Ann Arbor, MI) using saline as the mobile phase to determine colloid, and acetone as the mobile phase to determine free pertechnetate. The $^{99\text{m}}\text{Tc}$ solution was transferred by syringe into a solution of the C46.3 Fab' fragment, and the mixture was incubated for 1 h at room temperature.

Solutions of C46.3 Fab'–linker conjugates were radiolabeled at 20–50 mCi/mg by mixing with a $^{99\text{m}}\text{Tc}$ –tricine solution (36 mg/mL tricine, 50 $\mu\text{g/mL}$ $\text{SnCl}_2 \cdot 2\text{H}_2\text{O}$, 50 mCi/mL $^{99\text{m}}\text{TcO}_4$, pH7, preincubated for 15 min at room temperature). The conjugate solutions were incubated for 1 h at room temperature. Radiopurity of the $^{99\text{m}}\text{Tc}$ –tricine solution was determined by TLC using saline as the mobile phase to determine colloid, and methyl ethyl ketone as the mobile phase to determine free pertechnetate.

The radiopurity of the radiolabeled C46.3 Fab'–linker fragments was determined by ITLC using saline as the mobile phase (ITLC strips were cut at $R_f = 0.5$). Greater than 95% $^{99\text{m}}\text{Tc}$ incorporation was achieved for all conjugates used in this study.

Immunoreactivity Analysis. The immunoreactivity of C46.3 Fab'–linker conjugates was determined by the RhoChek (Rhomed, Albuquerque, NM) solid phase binding assay as described by the manufacturer (25). Briefly, a suspension of the beads coated with tumor extract, or control beads, were incubated with the radiolabeled conjugate for 1 h in duplicate sealed tubes with end-over-end mixing. The tubes were then counted in a γ counter, their beads were washed twice with saline, and the tubes were then recounted in the γ -counter. The immunoreactivity was calculated from the bound and the total radioactivity using the control beads to correct for nonspecific binding. To ensure the condition of antigen excess needed for a valid assay, the procedure is performed at two concentrations of radiolabeled conjugate (at a 2-fold concentration range), and the results must be within 5% for a valid assay. Immunoreactivities of other antibodies measured by this assay have ranged from 35 to 65% (25).

Scheme 1^a

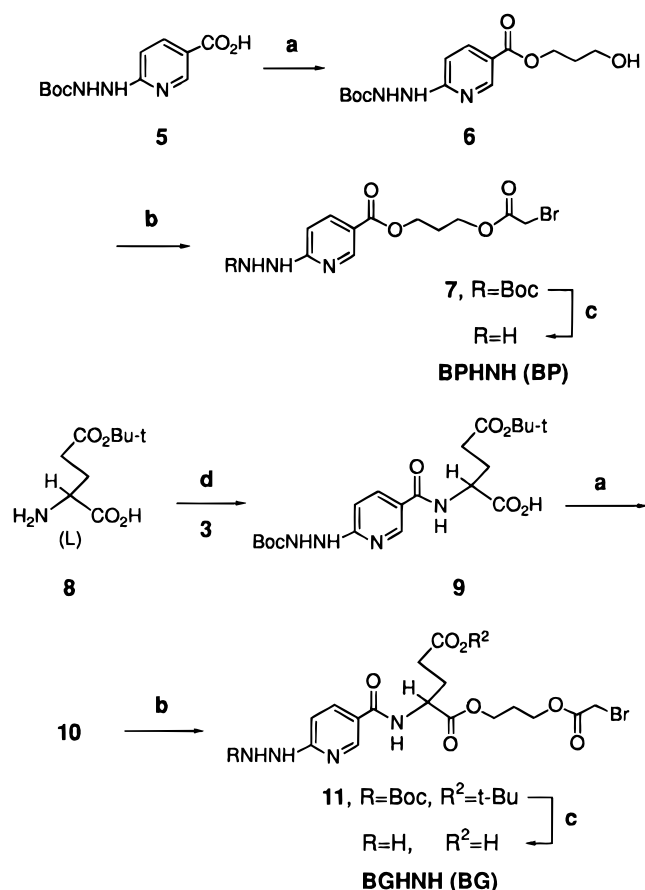


^a Reagents: (a) Aldrithiol-2, HOAc, MeOH; (b) saturated NaHCO_3 , dioxane, room temperature; (c) HCl, dioxane.

Biodistribution Determination. Nude mice bearing LS174T xenografts were used to determine the biodistribution, pharmacokinetics, and tumor imaging of $^{99\text{m}}\text{Tc}$ -labeled C46.3 Fab'–linker fragments (26). Tumor sizes varied (as is typical for the LS174T xenograft) and were as follows, in mm^2 of surface area (mean $\pm$ standard deviation) for the 4 h and 24 h group of mice, respectively: direct = 53 ± 26 , 65 ± 21 ; PC = 111 ± 17 , 97 ± 3 ; BG = 160 ± 144 , 77 ± 56 ; BA = 62 ± 39 , 142 ± 35 ; BP = 64 ± 17 , 117 ± 45 ; BH = 61 ± 14 , 180 ± 126 ; BD = 74 ± 18 , 146 ± 58 . Mice were injected via the retro-orbital sinus with 5–50 μg of Fab' fragments containing 150–800 μCi of $^{99\text{m}}\text{Tc}$ in a volume of 300 μL of phosphate-buffered saline. The amount injected per mouse was quantified by both the loss of weight and radioactivity in each syringe and by the radioactivity taken up by the mouse. Mice were dose calibrated and bled for initial blood and whole-body pharmacokinetics immediately after injection of the $^{99\text{m}}\text{Tc}$ -labeled Fab' fragments. Blood pharmacokinetics and whole-body clearances were determined by bleeding or dose calibrating the mice at 0.5, 2, 4, and 23 h postinjection. Tissue biodistribution was determined at 4 and 24 h postinjection. Dissected tissues were weighed, and their radioactivity was determined in a γ counter. All γ counter values were corrected for radioactive decay by counting retained aliquots of the injected dose at the same time as the organs. Final data are expressed as the mean of three to five mice per group $\pm$ standard deviation.

RESULTS AND DISCUSSION

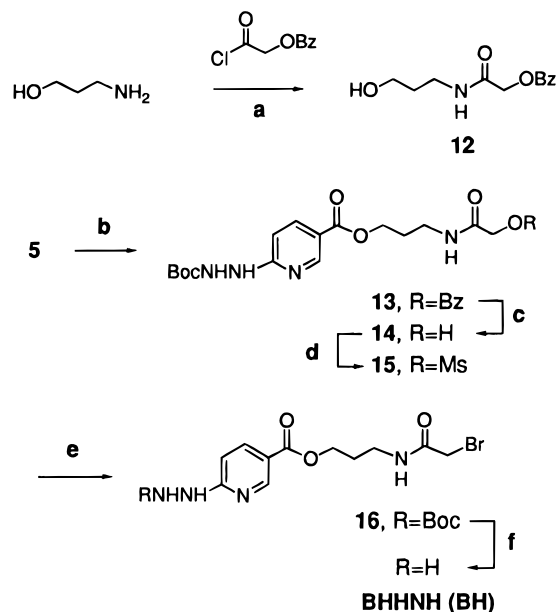
A series of hydrazinopyridine compounds containing cleavable/metabolizable linkers were designed and prepared. Initially, the disulfide–linker PC was synthesized which, upon reaction with the C46.3 Fab' fragment, would form a disulfide bridge connecting the hydrazinopyridine moiety to the monoclonal antibody. The synthesis of PC was accomplished in a straightforward manner as shown in Scheme 1. Reaction of L-cysteine (1) with Aldrithiol-2 (Aldrich) according to a previously published procedure gave the pyridyl disulfide intermediate 2. The hydrazinopyridine group was introduced by the reaction of 2 with Boc-SHNH (3) in a mixture of saturated aqueous sodium bicarbonate and dioxane to give the Boc-protected disulfide 4 in 82% yield. Boc deprotection of 4 with HCl(g)/dioxane over 2 h proved

Scheme 2^a

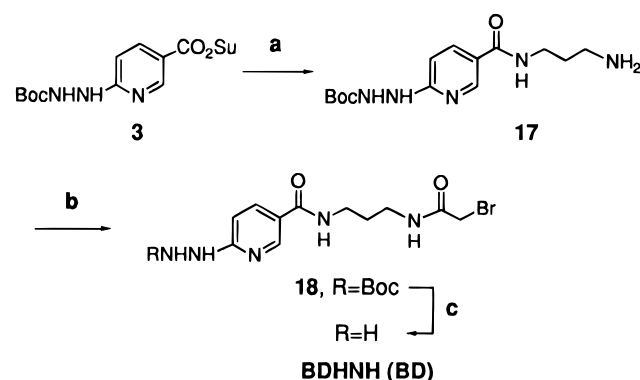
^a Reagents: (a) 3-bromopropanol, K₂CO₃, DMF, 70 °C; (b) 1.5 equiv of BrCH₂COBr, Na₂CO₃, THF, -35 °C, 4 h; (c) HBr/HOAc; (d) saturated NaHCO₃, dioxane, room temperature.

uncomplicated by the presence of the linking disulfide group, giving PC in a 70% yield.

A variety of ester functionalities were also introduced into the linker connecting the hydrazinopyridine ligand to the protein (thiol) reactive moiety. From a design perspective, the bromoacetate group in BP and BG (and BA, Figure 1) is a particularly attractive linking group since it combines the thiol reactive portion for conjugation with the C46.3 Fab' fragment (to form a thioether), and a cleavable (ester) group, into a single, low molecular weight entity. To this end, the linker BA incorporates a single ester group, BP a diester functionality, and BG a diester functionality in which the ⁹⁹Tc-labeled hydrazinopyridine moiety formed upon cleavage of either ester group generates a hydrophilic, carboxylate-containing fragment intended for rapid clearance (in a similar manner to PC). BA was synthesized as previously described (20), and the syntheses of BP and BG are illustrated in Scheme 2. Esterification of the Boc-hydrazino carboxylate intermediate (**5**) (**6**) with 3-bromopropanol in DMF containing Na₂CO₃ gave the ester **6** in a 60% yield following purification by column chromatography on silica gel. Low-temperature bromoacetylation of **6** with bromoacetyl bromide in THF gave the diester **7** which was subsequently deprotected with HBr(g)/acetic acid for 3 min to give BP as a hygroscopic white solid. The corresponding glutamic acid containing linker, BG was prepared in a similar manner to BP following isolation of the *tert*-butyl ester intermediate **9** (Scheme 2). In the final deprotection step of the bromoacetate **11** with HBr/acetic acid, a 3 min reaction time was sufficient to simultaneously deprotect the Boc and *tert*-butyl ester groups to give the desired linker BG.

Scheme 3^a

^a Reagents: (a) Et₃N, CH₂Cl₂; (b) DCC, DMAP, DMF; (c) Pd/C, NH₄·CO₂H, MeOH; (d) MeSO₂Cl, Et₃N, CH₂Cl₂; (e) LiBr, acetone; (f) HBr/HOAc.

Scheme 4^a

^a Reagents: (a) 10.0 equiv of propane-1,3-diamine, DMF; (b) BrCH₂CO₂Br, Na₂CO₃, THF, -40 °C; (c) HBr/HOAc.

In order to evaluate the regiospecificity requirements of the ester groups in BA and BP on the biodistribution and tumor uptake of the ⁹⁹Tc-labeled C46.3 Fab' conjugates, we synthesized two additional linkers. Firstly, BH which features a hydrazinonicotinate ester connected to a bromoacetamide moiety (or the "reversed" ester of BA; compare Figure 1 to BH, Scheme 3) and secondly, BD in which both functional groups of the linker are amides. In this manner, significant differences in the biodistribution properties of the Fab'-linker conjugates (BA, BH, BP, and BD) would provide information on the optimum position of the ester group for enhanced tumor: background ratios and rapid blood clearance. The syntheses of BH and BD are illustrated in Schemes 3 and 4. In order to assemble the core linker structure of BH, the acid **5** was esterified with the hydroxy intermediate **12** (prepared from propanolamine and benzyloxyacetyl chloride) using DCC/DMAP to give the ester **13** in a 56% overall yield following purification by column chromatography on silica gel. Transfer hydrogenolysis of the benzyl group with Pd/C in the presence of ammonium formate gave the hydroxy amide **14** which was subsequently reacted with methanesulfonyl chloride (to give the mesylate **15**) followed by LiBr in refluxing acetone to provide the desired bromoacetamide **16**. The alterna-

Table 1. Physical Properties of C46.3 Fab'-Linker Conjugates

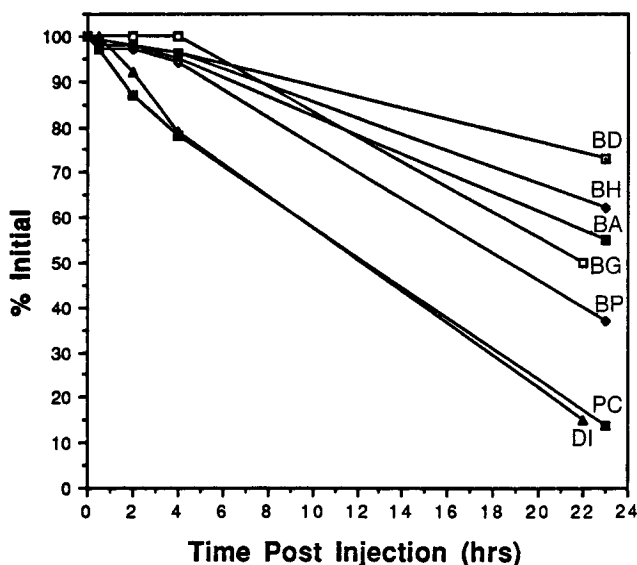
linker	C46.3 Fab'-linker		^{99m} Tc–tricine labeled C46.3 Fab'-linker	
	MSR ^a	res thiol ^b	% radiopurity ^c	preinjection % immunoreactivity ^d
PC	3.1	0.01	98	53
BG	4.5	0.16	98	36
BA	3.5	0.20	95	59
BP	3.6	0.05	95	67
BH	3.0	0.66	96	62
BD	2.4	0.43	95	68
direct	NA	(4.1) ^e	99 ^f	57

^a MSR = molar substitution ratio, the number of hydrazines incorporated per Fab' fragment. ^b Determined postconjugation with the linker. ^c Preinjection; measured by ITLC following (in some cases) purification of the ^{99m}Tc–tricine-labeled Fab' conjugate by gel filtration chromatography (G25 Sephadex column). ^d Determined by the RhoChek solid phase binding assay. ^e Number of free thiols in the starting C46.3 Fab' fragment. ^f Labeled with ^{99m}Tc–glucoheptonate.

tive "shorter" route to prepare the bromoacetamide of BH, namely, esterification of the acid **5** with an amino-protected alcohol, deprotection, and bromoacetylation of the intermediate amino ester, suffered from the potential pitfall of an intramolecular rearrangement of the amino ester to give the amido alcohol of BA and was thus avoided. The bromoacetamide **16** was deprotected with HBr/acetic acid to give the corresponding linker BH. Finally, we synthesized the diamide linker BD starting with Boc-SHNH (**3**). Reaction of **3** with a 10-fold excess of 1,3-propanediamine avoided complete formation of the symmetrical Boc-SHNH dimer to give a modest (36%) yield of the corresponding amine (**17**). Due to the insolubility of the amine in THF or CH₂Cl₂, **17** was bromoacetylated in DMF at low temperature to give **18** in a 15% overall yield. However, we obtained sufficient material for the deprotection step with HBr/acetic acid, which gave BD.

The series of thiol-reactive linkers were conjugated to the C46.3 Fab' fragment by reduction of the C46.3 F(ab')₂ fragment with DTT followed by reaction with the appropriate linker. Initially, the Fab' fragment generated from the reduction step was purified by gel filtration chromatography (G25 Sephadex Column) in order to remove the excess DTT, thus eliminating the possibility of cross-reaction with the linker. However, it was subsequently discovered that this purification step can be avoided by a one-step *in situ* reduction and conjugation procedure in which the linker is added in a 3 molar excess to the DTT thiol content used for the reduction. Under these conditions, conjugation of the linker to the free thiols of the C46.3 Fab' fragment was highly efficient as shown in Table 1. Using the *o*-sulfonic acid benzaldehyde hydrazine assay on the purified C46.3 Fab'–linker conjugates, the ratio of the free hydrazine concentration to the protein concentration (the molar substitution ratio, MSR) was found to be in the range 2.4–4.5 hydrazines per fragment, and furthermore, by addition of the measured residual thiol content to the MSR for each conjugate, we found a reasonable agreement with the number of available thiols in the starting C46.3 Fab' (determined experimentally to be 4.1 thiols per fragment).

The C46.3 Fab'–linker conjugates were radiolabeled by incubation with ^{99m}Tc–tricine at 20–50 mCi/mg for 1 h at room temperature. Using our standard formulation for the preparation of ^{99m}Tc–tricine (36 mg/mL tricine, 50 μg/mL SnCl₂·2H₂O, 20–50 mCi ^{99m}TcO₄ in saline), the radiopurity of the ^{99m}Tc–tricine complex prior to labeling of the conjugates was consistently greater than 90%.

**Figure 2.** Whole-body clearance of ^{99m}Tc-labeled C46.3 Fab' conjugates.

However, at the highest specific activity, ^{99m}Tc incorporation was dependent upon the concentration of the Fab'–linker conjugate. For example, at a final protein concentration of 0.41–0.43 mg/mL, the ^{99m}Tc incorporation of BA, BP, BD, and BH derived conjugates labeled at 50 mCi/mg with ^{99m}Tc–tricine was 88–91%. The series of labeled conjugates were therefore purified by gel filtration chromatography (G25 Sephadex column) which increased the radiopurity to >98%, and were used for the 24 h group of mice. In contrast, concentration of the protein solutions to 0.72–0.73 mg/mL, followed by radiolabeling, improved the initial radiopurities to 95–96%, and the resulting labeled conjugates were sufficiently pure to be used for the 4 h group of mice without further purification. The labeled conjugates were analyzed by size exclusion HPLC and fractions collected for γ counter detection. All conjugates exhibited a single peak of radioactivity with less than 3% aggregate.

For direct radiolabeling of the C46.3 Fab' fragment, we initially attempted to label with ^{99m}Tc–tricine under a variety of previously established conditions. To our surprise, tricine proved to be a poor precursor ligand for the radiolabeling of free sulfhydryl groups with ^{99m}Tc (unpublished data). While this result precluded the use of ^{99m}Tc–tricine for the directly labeled biodistribution experiment, it confirmed the labeling specificity of ^{99m}Tc–tricine for the hydrazinopyridine groups of the C46.3 Fab'–linker conjugates, which would be particularly important for those conjugates with a residual thiol content (see BH and BD, Table 1). Thus, direct labeling was accomplished with ^{99m}Tc–glucoheptonate at a specific activity of 50 mCi/mg.

Finally, the ^{99m}Tc-labeled C46.3 Fab'–linker conjugates were tested for their preinjection immunoreactivity by the RhoChek solid phase binding assay (Table 1). Apart from the somewhat lower immunoreactivity of the radiolabeled BG conjugate, the conjugation of radiolabeled hydrazinopyridine groups in general appears to maintain the percent immunoreactivity of the antibody fragment comparable to direct labeling.

Biodistributions and Pharmacokinetics. The ^{99m}Tc-labeled C46.3 Fab'–linker conjugates and the direct ^{99m}Tc-labeled C46.3 Fab' fragment were injected via the retroorbital sinus into nude mice bearing LS174T xenografts. Blood pharmacokinetics and whole body clearances were determined immediately after injection of the

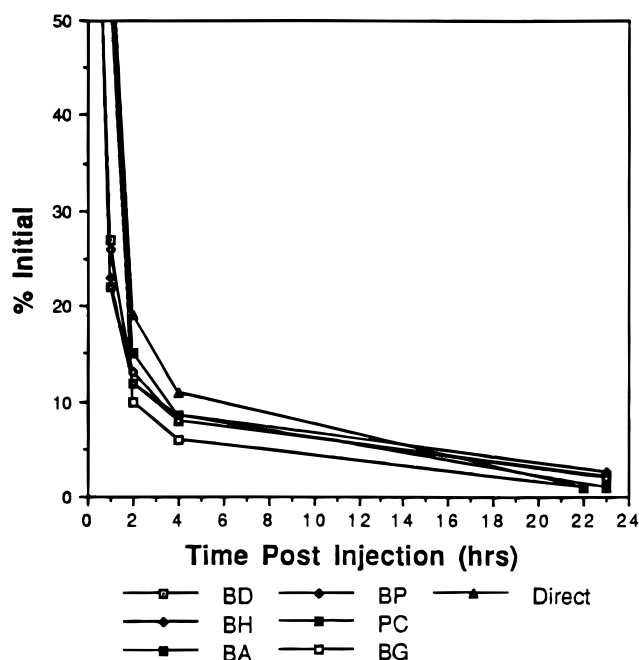


Figure 3. Blood clearance of ^{99m}Tc -labeled C46.3 Fab' conjugates.

conjugates (0.5–1.0 h) by bleeding and dose-calibrating, at two of three selected time points (1, 2, or 4 h) and finally at 22–23 h postinjection (prior to sacrifice). The results are shown in Figures 2 and 3. The whole-body radioactivity clearance of the conjugates was found to be dependent upon the composition and position of the functional groups connecting the ^{99m}Tc -labeled hydrazinopyridine moiety to the antibody fragment. The disulfide-linked conjugate, PC, exhibited similar whole-body clearance to the directly labeled fragment. These two conjugates by far cleared most rapidly from the body. In order of decreasing clearance, PC was followed by the diester-linked conjugates BP and BG, the monoesters BA and BH, and the diamide conjugate BD. Interestingly, the order of clearance was consistent with a specificity for the position of the ester group, since BA was cleared more rapidly than the corresponding reversed ester BH. The significantly faster clearance of the disulfide-linked conjugate (PC) compared to that of the ester-linked conjugates can be explained by the greater reactivity of disulfides to other thiol nucleophiles *in vivo*. That the whole-body clearance profile of the linker conjugates was not simply due to varying rates of disulfide cleavage/ester hydrolysis in serum was confirmed by studying blood clearance (Figure 3). All conjugates, including direct (DI), showed similar blood clearances at 4 h (6–12% of the injected counts remained in circulation) and at 22–23 h (2–5%). These observations are consistent with the findings of Weber (17) for ^{99m}Tc -labeled N_3S (mercaptoacetyltriglycine) BCA's linked to the antimyosin F(ab') via ester or amide groups.

Tissue biodistributions were determined at 4 and 24 h postinjection. The results are shown in Table 2, expressed as percent injected dose per gram of tissue (%ID/g) at dissection time $\pm$ the standard deviation. Independent of conjugate or time point, the kidney exhibited the highest uptake (%ID/g) of all the organs studied. At 4 h postinjection, the diamide-linked BD conjugate had significantly higher kidney uptake (246%) compared with the monoester-linked conjugates BA and BH (167–181%) and the diester-linked conjugates BP and BG (146–154%). However, the kidney uptake of the disulfide-linked conjugate, PC, and the DI fragment were

Table 2. Percent Injected Dose per Gram of Tissue at Dissection Time for Tumor-Bearing Mice Given C46.3 Fab' ^{99m}Tc -Labeled via Linkers with ^{99m}Tc -Tricine versus Direct Labeled with ^{99m}Tc -Glucosephosphate

organ	direct Fab'		PC-Fab'		BG-Fab'		BA-Fab'		BP-Fab'		BH-Fab'		BD-Fab'	
	4 h	24 h	4 h	24 h	4 h	24 h	4 h	24 h	4 h	24 h	4 h	24 h	4 h	24 h
blood	1.54 $\pm$ 0.28	0.30 $\pm$ 0.07	1.29 $\pm$ 0.13	0.23 $\pm$ 0.09	2.63 $\pm$ 0.09	0.35 $\pm$ 0.06	1.51 $\pm$ 0.22	0.17 $\pm$ 0.02	1.67 $\pm$ 0.32	0.20 $\pm$ 0.04	1.55 $\pm$ 0.15	0.24 $\pm$ 0.09	2.31 $\pm$ 0.44	0.30 $\pm$ 0.04
lung	1.31 $\pm$ 0.28	0.29 $\pm$ 0.07	0.76 $\pm$ 0.11	0.30 $\pm$ 0.06	2.10 $\pm$ 0.06	0.51 $\pm$ 0.12	1.47 $\pm$ 0.42	0.33 $\pm$ 0.07	1.70 $\pm$ 0.48	0.31 $\pm$ 0.08	1.18 $\pm$ 0.19	0.40 $\pm$ 0.03	1.94 $\pm$ 0.43	0.48 $\pm$ 0.12
spleen	0.73 $\pm$ 0.17	0.18 $\pm$ 0.10	0.40 $\pm$ 0.02	0.28 $\pm$ 0.09	1.03 $\pm$ 0.09	0.64 $\pm$ 0.12	1.10 $\pm$ 0.28	0.44 $\pm$ 0.10	1.31 $\pm$ 0.15	0.35 $\pm$ 0.12	0.86 $\pm$ 0.14	0.69 $\pm$ 0.10	1.23 $\pm$ 0.26	0.81 $\pm$ 0.25
liver	0.91 $\pm$ 0.10	0.39 $\pm$ 0.20	0.81 $\pm$ 0.10	0.54 $\pm$ 0.07	2.14 $\pm$ 0.46	1.43 $\pm$ 0.20	1.17 $\pm$ 0.28	0.54 $\pm$ 0.10	1.53 $\pm$ 0.12	0.58 $\pm$ 0.14	1.18 $\pm$ 0.17	1.05 $\pm$ 0.36	1.99 $\pm$ 0.38	1.18 $\pm$ 0.21
kidney	34.22 $\pm$ 2.76	20.08 $\pm$ 2.79	70.1 $\pm$ 6.6	62.3 $\pm$ 11.3	154 $\pm$ 15	87.6 $\pm$ 11.5	181 $\pm$ 41	100 $\pm$ 21	146 $\pm$ 18	48 $\pm$ 13	167 $\pm$ 23	127 $\pm$ 14	246 $\pm$ 42	141 $\pm$ 25
tumor	5.01 $\pm$ 0.64	1.99 $\pm$ 0.26	1.99 $\pm$ 0.71	0.96 $\pm$ 0.16	5.38 $\pm$ 0.83	5.18 $\pm$ 1.60	4.98 $\pm$ 2.98	2.76 $\pm$ 1.41	4.66 $\pm$ 1.19	1.63 $\pm$ 0.53	3.29 $\pm$ 1.23	2.88 $\pm$ 0.57	4.12 $\pm$ 1.18	3.36 $\pm$ 0.70
muscle	0.48 $\pm$ 0.13	0.10 $\pm$ 0.04	0.44 $\pm$ 0.29	0.18 $\pm$ 0.13	0.44 $\pm$ 0.08	0.12 $\pm$ 0.01	0.77 $\pm$ 0.32	0.42 $\pm$ 0.25	0.87 $\pm$ 0.22	0.2 $\pm$ 0.12	1.17 $\pm$ 0.69	0.56 $\pm$ 0.27	0.97 $\pm$ 0.55	0.45 $\pm$ 0.19

Table 3. Selected Organ:Blood Ratios at Dissection Time for Tumor-Bearing Mice Given C46.3 Fab' ^{99m}Tc Labeled via Linkers with ^{99m}Tc –Tricine versus Direct Labeled with ^{99m}Tc –Glucoheptonate

organ	DI		PC		BG		BA		BP		BH		BD	
	4 h	24 h	4 h	24 h	4 h	24 h	4 h	24 h	4 h	24 h	4 h	24 h	4 h	24 h
kidney	22.2	66.9	54.3	271	58.6	250	120	588	87.4	240	108	529	106	470
tumor	3.3	6.6	1.5	4.2	2.0	14.8	3.3	16.2	2.8	8.2	2.1	12	1.8	11.2

approximately 2-fold and 4-fold lower, respectively, than either BP or BG.

At 24 h postinjection, all conjugates showed reduced kidney uptake, but the comparative pattern of conjugate uptake in the kidney was similar to that of the 4 h time point: BD > BH > BA > BG > BP, and furthermore, this pattern directly correlated with the whole-body clearance of the respective conjugates (Figure 2). On the basis of the assumption that the stability of the ^{99m}Tc –hydrazinopyridine complex is identical for all ^{99m}Tc –labeled C46.3 Fab'–linker conjugates, the linker-structure/kidney-uptake relationship, corroborated by the whole-body clearance data, strongly suggests that ester linkers are more easily metabolized in the kidney than the corresponding amide linkers to give a ^{99m}Tc –labeled metabolite that is rapidly cleared from the body.

While the disulfide-linked conjugate PC exhibited the lowest %ID/g of the ^{99m}Tc –labeled hydrazinopyridine conjugates in the kidney at 4 h, the kidney uptake of PC was similar to that of BP at 24 h. In order to explain this anomalous result, we compared the radioactivity retained in the kidney at 24 h expressed as a percentage of the 4 h uptake. In striking contrast, the kidney retention of PC was significantly higher than BP: for PC, 89% of the radioactivity in the kidneys at 4 h remained after 24 h, compared to 33% for BP. These observations suggest that while the disulfide linker in PC is also rapidly metabolized following uptake in the kidney, a significant portion of the ^{99m}Tc –labeled metabolite remains covalently cross-linked to the tissue, possibly via disulfide bonds. Finally, the lower *in vivo* stability of the ^{99m}Tc –thiol radiometal complex in the direct labeled conjugate compared to the ^{99m}Tc –hydrazinopyridine complexes most likely explains the significantly lower kidney uptake exhibited by direct at both time points, consistent with the findings of Hnatowich (2).

The remainder of the nontarget organs, blood, lung, spleen, liver, and muscle, were also compared for uptake of radioactivity at two time points. In general, relatively small differences between the respective conjugates were observed, with the following exceptions. At 4 h postinjection, BD and BG exhibited significantly higher blood, lung, and liver uptake (1.9–2.6%ID/g) compared to all other conjugates (0.8–1.7%). Uptake of BD and BG in these organs remained high at 24 h. The radioactivity in muscle was approximately 2-fold lower for BG, PC, and DI than for the other conjugates at both time points.

Tumor uptake of the conjugates at 4 and 24 h was the highest non-kidney tissue uptake, accounting for 2.0–5.4%ID/g at 4 h and 1.0–5.2% at 24 h. At 4 h postinjection, there were no significant differences between the conjugates in terms of tumor:blood ratios as shown in Table 3. Kidney:blood ratios were higher for BD, BH, and BA (106–120) than for the diester- and disulfide-linked conjugates BG, BP, and PC (54–87). The direct labeled conjugate showed the lowest kidney:blood ratio (22) at 4 h. By 24 h postinjection, tumor:blood ratios for hydrazinopyridine-linked conjugates had improved significantly over the direct labeled conjugate: BA had the highest tumor:blood ratio (16.2) followed by BG (14.8), and both were greater than 2-fold higher than the tumor: blood ratio exhibited by the direct labeled conjugate (6.6).

Although BA had a 2-fold higher kidney: blood ratio than BG at 24 h, the lower uptake of BA in other nontarget organs combined with the highest tumor: blood ratio make ^{99m}Tc –labeled C46.3 Fab'–BA a potential candidate for further clinical development.

In conclusion, a series of hydrazinopyridine BCA's containing ester, amide, and disulfide groups have been prepared and conjugated to the tumor-specific monoclonal antibody C46.3 Fab' via the free sulfhydryl groups. Using ^{99m}Tc –tricine as a precursor complex, the hydrazinopyridine groups of the conjugates can be labeled in a short incubation time (1 h) in high radiochemical purity, thereby avoiding a postlabeling purification step. The biodistribution and pharmacokinetic properties of the ^{99m}Tc –labeled C46.3 Fab'–linker conjugates versus the C46.3 Fab' fragment direct labeled with ^{99m}Tc –glucoheptonate were compared in nude mice bearing LS174T xenografts. Although higher kidney uptake was observed for the linker conjugates compared to direct, the tumor: blood ratio for ^{99m}Tc C46.3 Fab'–BA at 24 h postinjection was approximately 2.5-fold higher than direct. Tumor images of mice given BA or direct are reported elsewhere (20).

ACKNOWLEDGMENT

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LITERATURE CITED

- Reilly, R. M. (1993) Immunoscintigraphy of Tumors Using ^{99m}Tc –Labelled Monoclonal Antibodies: A Review. *Nucl. Med. Commun.* 14, 347–359.
- Hnatowich, D. J., Mardirossian, G., Rusckowski, M., Forgarasi, M., Virzi, F., and Winnard, P. (1993) Directly and Indirectly Technetium-99m Labelled Antibodies. A Comparison of *in vitro* and *in vivo* Properties. *J. Nucl. Med.* 34, 109–119.
- Parker, D., and Jankowski, K. J. (1993) Diagnosis and Therapy with Antibody Conjugates of Metal Radioisotopes. In *Advances in Metals in Medicine* (M. J. Abrams and B. A. Murrer, Eds.) pp 29–75, JAI Press Inc.
- Schwartz, D. A., Abrams, M. J., Hauser, M. M., Gaul, F. E., Larsen, S. K., Rauh, D., and Zubieta, J. A. (1991) Preparation of Hydrazino-Modified Proteins and Their Use for the Synthesis of ^{99m}Tc –Protein Conjugates. *Bioconjugate Chem.* 2, 333–336.
- Schwartz, D. A., Abrams, M. J., Giandomenico, C. M., and Zubieta, J. A. (1993) Preparation of Succinimido Hydrazinoarylcarboxylates and Analogues as Conjugating Agents for Biological Macromolecules. U.S. Patent 5,206,370.
- Abrams, M. J., Juweid, M., Tenkate, C. I., Schwartz, D. A., Hauser, M. M., Gaul, F. E., Fuccello, A. J., Rubin, R. H., Strauss, H. W., and Fischman, A. J. (1990) Technetium-99m Human Polyclonal IgG Radiolabelled via the Hydrazino Nicotinamide Derivative for Imaging Focal Sites of Infection in Rats. *J. Nucl. Med.* 31, 2022–2028.
- Fischman, A. J., Solomon, H. F., Babich, J. W., Abrams, M. J., Callahan, R. J., Strauss, H. W., and Rubin, R. H. (1994) Imaging of Focal Sites of Inflammation in Rhesus Monkeys with ^{99m}Tc –Labelled Human Polyclonal IgG. *Nucl. Med. Biol.* 21, 111–116.
- Knight, L. C., Abrams, M. J., Schwartz, D. A., Hauser, M. M., Kollman, M., Gaul, F. E., Rauh, D. A., and Maurer, A. H. (1992) Preparation and Preliminary Evaluation of Technetium-99m

- tium-99m-Labelled Fragment E₁ for Thrombus Imaging. *J. Nucl. Med.* 33, 710–715.
- (9) Babich, J. W., Solomon, H., Pike, M. C., Kroon, D., Graham, W., Abrams, M. J., Tompkins, R. G., Rubin, R. H., and Fischman, A. J. (1993) Technetium-99m Labelled Hydrazino Nicotinamide Derivatized Chemotactic Peptide Analogs for Imaging Focal Sites of Bacterial Infection. *J. Nucl. Med.* 34, 1964–1974.
- (10) Larsen, S. K., Solomon, H. F., Caldwell, G., and Abrams, M. J. (1995) ^{99m}Tc-Tricine: A Useful Precursor Complex for the Radiolabeling of Hydrazinonicotinate Protein Conjugates. *Bioconjugate Chem.* 6, 635–638.
- (11) Bridger, G. J., Hernandez, P. E., Higgins, J. D., and Larsen, S. K. (1994) Complexes with ^{99m}Tc Suitable for Radiolabelling Monoclonal Antibodies or Other Macromolecules. U.S. Patent 5,350,837.
- (12) Liu, S., Edwards, D. S., Looby, R. J., Harris, A. R., Poirer, M. J., Barrett, J. A., Heminway, S. J., and Carroll, T. R. (1996) Labelling a Hydrazino Nicotinamide-Modified Cyclic II_b/III_a Receptor Antagonist with ^{99m}Tc Using Aminocarboxylates as Co-Ligands. *Bioconjugate Chem.* 7, 63–71.
- (13) Paik, C. H., Quadri, S. M., and Reba, R. C. (1989) Interposition of Different Chemical Linkages Between Antibody and In-111 DTPA to Accelerate Clearance from Non-target Organs and Blood. *Nucl. Med. Biol.* 16, 475–481.
- (14) Paik, C. H., Yokoyama, K., Reynolds, J. C., Quadri, S. M., Min, C. Y., Shin, S. Y., Maloney, P. J., Larsen, S. M., and Reba, R. C. (1989) Reduction of Background Activities by Introduction of a Diester Linkage Between Antibody and a Chelate in Radioimmunoassay of Tumor. *J. Nucl. Med.* 30, 1693–1701.
- (15) Subramanian, R., Paik, C. H., Quadri, S. M., Kim, H. J., Pomato, N., Haspel, M. V., Reba, R. C., Hanna, M. G., and McCabe, R. P. (1990) Tissue Distribution of In-111 Labeled Human Monoclonal Antibody (16.88) in Nude Mice Bearing Tumor Xenografts: Effect of Diester Linkage. *Antibody, Immunoconjugates, Radiopharm.* 3, 127–136.
- (16) Deshapande, S. V., DeNardo, S. J., Meares, C. F., McCall, M. J., Adams, G. P., and DeNardo, G. L. (1989) Effect of Different Linkages Between Chelates and Monoclonal Antibodies on Levels of Radioactivity in the Liver. *Nucl. Med. Biol.* 16, 587–597.
- (17) Weber, R. W., Boutin, R. H., Nedelman, M. A., Lister-Jones, J., and Dean, R. T. (1990) Enhanced Kidney Clearance with an Ester-Linked ^{99m}Tc-Radiolabeled Antibody Fab'-Chelator Conjugate. *Bioconjugate Chem.* 1, 431–437.
- (18) Gustavson, L. M., Srinivasan, A., Kasina, S., Reno, J. M., Fitzner, J. N., and Jones, D. S. (1992) Radiolabeled Proteins for Diagnostic or Therapeutic Use. U.S. Patent 5,112,953.
- (19) Brixner, D. I. (1992) Cleavable Diphosphate and Amidated Diphosphate Linkers. U.S. Patent 5,094,848.
- (20) Ultee, M. E., Abrams, M. J., Longley, C. B., Burton, C. A., Bridger, G. J., Larsen, S. K., Henson, G. W., Padmanabhan, S., Gaul, F. E., and Schwartz, D. A. (1996) Tumor Targeting and Imaging by Two ^{99m}Tc-Labelled Fab' Fragments: A Comparison of a Hydrazinonicotinamide Analog and Direct Labelling. *J. Nucl. Med.*, submitted.
- (21) Chong, P. C. S., and Hodges, R. S. (1981) A New Hetero-bifunctional Cross-linking Reagent for the Study of Biological Interactions Between Proteins. *J. Biol. Chem.* 256, 5064–5070.
- (22) Armitage, N. C., Perkins, A. C., Durran, L. G., Ellis, I. O., Ballantyne, K. C., Harrison, R. C., Riley, A. M. L., Robins, R. A., Wastie, M. L., and Hardcastle, J. D. (1986) *In vitro* binding and *in vivo* localization in colorectal cancer of a high affinity monoclonal antibody to carcinoembryonic antigen. *Br. J. Surg.* 73, 965–969.
- (23) Rea, R. W., and Ultee, M. E. (1993) A novel method for controlling the pepsin digestion of antibodies. *J. Immunol. Methods* 157, 165–173.
- (24) Grassetti, D. R., and Murray, J. F. (1967) Determination of Sulfhydryl Groups with 2,2'- or 4,4'-Dithiodipyridine. *Arch. Biochem. Biophys.* 119, 41–49.
- (25) Rhodes, B. A., Buckelew, J. M., Pant, K. D., and Hinkle, G. H. (1990) Quality control test for immunoreactivity of radiolabelled antibody. *Biotechniques* 8, 70–73.
- (26) Alvarez, V. L., Lopes, A. D., Lee, C., Rodwell, J. D., and McKearn, T. J. (1988) Site-Specific Modification of 111-Indium Labeled Monoclonal Antibodies using Nude Mouse Xenograft Systems. In *Covalent Modification of Monoclonal Antibodies* (J. D. Rodwell, Ed.) p 283, Marcel Dekker, New York.

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Benzoylecgonine Hydrazides: Synthesis, Coupling to Horseradish Peroxidase, and Characterization of the Conjugates

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Benzoylecgonine–horseradish peroxidase conjugate (BE–HRP) can be used as a diagnostic reagent for the detection of cocaine in illicit drug samples and in biological fluids. This paper describes the preparation and characterization of BE–HRP. Two hydrazide derivatives of benzoylecgonine, *N*-2-(*tert*-butyloxycarbonyl)benzoylecgonine hydrazide and mono(*N*-2'-benzoylecgoninoyl)adipic dihydrazide, were synthesized by carbodiimide-activated coupling of benzoylecgonine to *N*-2-(*tert*-butyloxycarbonyl)hydrazide and adipic dihydrazide, respectively. Removal of the *tert*-butyloxycarbonyl protecting group in *N*-2-(*tert*-butyloxycarbonyl)benzoylecgonine hydrazide with anhydrous HCl yielded benzoylecgonine hydrazide hydrochloride. NMR and high-resolution mass spectral analyses demonstrated that the benzoyl group of benzoylecgonine remained intact under the conditions of both carbodiimide coupling and anhydrous HCl treatment. By aldehyde–hydrazide condensation, the hydrazides were covalently conjugated to the carbohydrate residues of horseradish peroxidase (HRP). Dot blot analysis of the conjugates employing antibodies specific to benzoylecgonine demonstrated the presence of bound benzoylecgonine in HRP. The stoichiometry of benzoylecgonine residues to HRP was determined by matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS). Mono(*N*-2'-benzoylecgoninoyl)adipic dihydrazide gave a 2.5–3-fold higher coupling compared with benzoylecgonine hydrazide. Conjugates were also prepared by the coupling of the carbodiimide-activated benzoylecgonine to HRP that was derivatized with adipic dihydrazide.

INTRODUCTION

Benzoylecgonine is a key metabolite of cocaine, and structurally, they are closely related (1). Monoclonal and polyclonal antibodies raised against benzoylecgonine cross-react with cocaine. Benzoylecgonine has a free carboxylic group which can be used as a site for conjugation to various proteins for immunological characterization of cocaine. Benzoylecgonine–horseradish peroxidase conjugate (BE–HRP)¹ can be used as a diagnostic reagent for detection of cocaine employing standard immunological assay procedures. Currently, BE–HRP is prepared by biochemical companies using proprietary procedures; thus, published methods are not available.

Generally, primary amine or carboxylic group-containing haptens are conjugated to antibodies and enzymes by using water-soluble carbodiimides (2, 3). The reactions are invariably random and may lead to drastic reductions in antibody and enzyme activity. An alterna-

tive and a better approach would be aldehyde–amine condensation utilizing amino groups of haptens and aldehyde groups generated on the carbohydrate moieties of glycoproteins (see reviews in refs 4 and 5). Since the carbohydrate moieties of antibodies and enzymes are generally not required for their activities, conjugation of haptens through the carbohydrate moieties should not affect their activities (4–6). However, condensation of a primary amine to an aldehyde group yields an unstable Schiff base which has to be reduced using sodium cyanoborohydride to obtain stable conjugates (5). The yields of conjugates are often low because Schiff bases are unstable at the pH employed for the cyanoborohydride reduction (5). Moreover, the use of cyanoborohydride may lead to a decrease in the biological activity (7). These disadvantages can be eliminated by introduction of a hydrazide functional group into the hapten. Due to the lower pK of the amino group of hydrazides compared with those of primary amines (5, 8), they readily react with aldehydes to form stable hydrazone bonds and give conjugates in high yields. In the present study, we used this approach for the preparation of BE–HRP.

In this study, we synthesized two hydrazide derivatives of benzoylecgonine. One has no spacer arm, and the other has an adipic acid hydrazide spacer arm. Under the conditions employed for the preparation of these hydrazides, the alkali-labile benzoyl group remained intact. Conjugation of these hydrazides to horseradish peroxidase (HRP) was accomplished using aldehyde–amine condensation after generation of aldehyde groups on the carbohydrate moieties of HRP by periodate oxidation. Conjugation was also achieved by direct coupling of benzoylecgonine to adipic dihydrazide-derivatized HRP employing a water-soluble carbodiimide. The conjugates were characterized by Western blotting and by matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS).

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¹ Abbreviations: BE–HRP, benzoylecgonine–horseradish peroxidase conjugate; HRP, horseradish peroxidase; MALDI-MS, matrix-assisted laser desorption/ionization mass spectrometry; HBT, 1-hydroxybenzotriazole; EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride; NHS, *N*-hydroxysuccinimide; DCC, dicyclohexylcarbodiimide; biotin-LC hydrazide, 6-biotinamido hexanoic hydrazide; BSA, bovine serum albumin; PVDF, poly(vinylidene difluoride); FAB-MS, fast atom bombardment mass spectrometry; mAb, monoclonal antibody; MES, 4-morpholineethanesulfonic acid.

EXPERIMENTAL PROCEDURES

Materials. Cocaine hydrochloride was provided by Dr. Richard A. Gillis, Department of Pharmacology, Georgetown University Medical Center. 1-Hydroxybenzotriazole (HBT), 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride (EDC), adipic dihydrazide, *tert*-butyl carbazate (*N*-2-*tert*-butyloxycarbonyl hydrazide), dimethylacetamide, dimethyl sulfoxide, *N*-hydroxysuccinimide (NHS), dicyclohexylcarbodiimide (DCC), sinapinic acid, and silica gel thin-layer plates were purchased from Aldrich Chemical Co. (Milwaukee, WI). Biotin hydrazide and 6-biotinamido hexanoic hydrazide (biotin-LC hydrazide) were from Pierce Chemical Co. (Rockford, IL). HRP (type VI), 4-chloro-1-naphthol, 4-morpholineethanesulfonic acid (MES), and bovine serum albumin (BSA) were obtained from Sigma Chemical Co. (St. Louis, MO). Protein A-purified murine monoclonal antibody (PO1-99-11M-P, 3.39 mg/mL) and a rabbit polyclonal antibody (PO1-99-13R-1F, 3.61 mg/mL) that were raised against benzoylecgonine were purchased from Biotride Inc. (Palo Alto, CA). Poly(vinylidene difluoride) (PVDF) membranes were from Millipore (Bedford, MA).

General Methods. Unless otherwise mentioned, drying and concentration of solutions was performed by rotary vacuum evaporation at 30–40 °C. Compounds on thin-layer chromatograms were visualized by a hand-held UV lamp and/or by exposing to iodine vapor.

IR spectra of compounds in KBr pellets were recorded with a Nicolet 170SX FT-IR spectrometer (Nicolet Instrument Corp., Madison, WI) with an on-line microcomputer data station. ¹H-NMR studies were performed with a 270 MHz Nicolet model NT270 spectrometer (Nicolet Instrument Corp.) coupled to a Tecmeg data system. The spectra were recorded at 24 °C using tetramethylsilane as the internal standard.

Positive ion mode fast atom bombardment mass spectrometry (FAB-MS) analysis was performed with a JEOL SX102 mass spectrometer (JEOL U.S.A., Inc., Peabody, MA) using 6 keV xenon atoms as the ionization source. Samples (1 μ L of 1 mg/mL solutions in methanol or water) were mixed with 1 μ L of Magic Bullet matrix (dithiothreitol/dithioerythritol, 5:1) on the probe tip. Spectra were acquired at a resolution of 3000 and calibrated against an external standard.

High-resolution FAB-MS analysis was performed with a JEOL SX102 mass spectrometer as described above at a resolution of 10 000.

For carbohydrate analysis, untreated HRP (10 μ g) and periodate-oxidized HRP (10 μ g) were hydrolyzed with 400 μ L of 2.5 M trifluoroacetic acid at 100 °C for 5 h. The hydrolysates were evaporated in a Speed-Vac, and the residues were dissolved in water and analyzed for neutral sugars and hexosamines using a Dionex BioLC system with amperometric detection (Dionex Corp., Sunnyvale, CA) (9). Analysis was performed on a CarboPac PA1 high-pH anion exchange column (4 $\times$ 250 mm) (Dionex) at a flow rate of 0.8 mL/min using 20 mM sodium hydroxide (9).

Preparation of Benzoylecgonine. To a solution of cocaine hydrochloride (400 mg in 5 mL of ice-cold distilled water) was added 2 M sodium hydroxide until the solution was slightly alkaline (pH $\sim$ 8.0). The cocaine base that precipitated as a white solid mass was recovered by centrifugation at 5000g and washed two times with 3 mL of ice-cold water. The pellet either was used directly for the preparation of benzoylecgonine or was lyophilized and stored in a desiccator over solid sodium hydroxide.

The cocaine base (300 mg) was suspended in 10 mL of distilled water and heated under reflux in an oil bath at 90 °C for 18 h. The solution was then concentrated under

reduced pressure to 1.5 mL and was then lyophilized. The product was crystallized from hot water and its melting point determined. Benzoylecgonine was further characterized by IR, ¹H-NMR, and high-resolution FAB-MS analyses.

Preparation of Benzoylecgonine Hydrazide Hydrochloride. Benzoylecgonine (149 mg, 0.05 mmol), HBT (68 mg, 0.5 mmol), and *tert*-butyl carbazate (66 mg, 0.5 mmol) were dissolved in dimethylacetamide (1.5 mL), and the mixture was stirred to obtain a clear solution. EDC (97 mg, 0.5 mmol) was added. After the mixture was stirred overnight at room temperature, water (1.5 mL) was added and the solution was extracted with ethyl acetate (5 $\times$ 2.5 mL). The combined extracts were washed successively with water (8 mL) and saturated saline (8 mL) and then dried over anhydrous magnesium sulfate. The solution was applied to a silica gel (60–200 mesh) column (0.7 $\times$ 15 cm) equilibrated with ethyl acetate/*n*-hexane (1:1, v/v) and washed with the same solvent (150 mL). The bound *N*-2-(*tert*-butyloxycarbonyl)-benzoylecgonine hydrazide (identified by mass spectral analysis) was eluted with chloroform/methanol (9:1, v/v), and the elution was monitored by TLC using the same solvent. Fractions containing *N*-2-(*tert*-butyloxycarbonyl)-benzoylecgonine hydrazide were combined and evaporated (yield, 90 mg). The compound was characterized by ¹H-NMR (in CDCl₃/CD₃OD, 9:1, v/v) and high-resolution FAB-MS analyses.

The *N*-2-(*tert*-butyloxycarbonyl)-benzoylecgonine hydrazide (90 mg) was dissolved in dry ethyl acetate (0.5 mL) and cooled in an ice bath. To the solution was added cold ethyl acetate (2 mL) saturated with anhydrous HCl. After 30 min at room temperature, excess HCl was removed by passing a jet of nitrogen gas through the solution. The resulting benzoylecgonine hydrazide hydrochloride was precipitated with anhydrous ethyl ether, collected by centrifugation, dried in a vacuum desiccator, and then characterized by high-resolution FAB-MS.

Preparation of Mono(*N*-2'-benzoylecgoninoyl)-adipic Dihydrazide. Benzoylecgonine (100 mg, 0.34 mmol), HBT (45 mg, 0.34 mmol), and adipic dihydrazide (232 mg, 1.32 mmol) were suspended in a mixture of dimethylacetamide (5 mL) and dimethyl sulfoxide (5 mL). The mixture was stirred for 30 min at room temperature. To the cloudy solution was added EDC (97 mg, 0.5 mmol), and the mixture was stirred overnight at room temperature. Water (20 mL) was added, and the solution was extracted with ethyl acetate (5 $\times$ 20 mL). The aqueous phase was then deionized with AG 4 $\times$ 4 (free base) ion exchange resin and lyophilized. The solid was dispersed in ethanol and stirred for 2 h to obtain a fine suspension and centrifuged. The precipitate contained unreacted adipic dihydrazide and was discarded. The ethanol solution was chromatographed on a silica gel column (1 $\times$ 15 cm) using ethanol/water (85:15, v/v). Fractions (2 mL) were collected and analyzed by TLC using the above solvent. Mass spectral analysis demonstrated that the desired compound, mono(*N*-2'-benzoylecgoninoyl)-adipic dihydrazide, was eluted in fractions 10 through 25 along with significant amounts of other compounds. The product, mono(*N*-2'-benzoylecgoninoyl)-adipic dihydrazide (*R*_f value relative to adipic dihydrazide = 0.27), was purified (yield, 49 mg) by preparative TLC using ethanol/water (85:15, v/v) and characterized by high-resolution FAB-MS.

Conjugation of Benzoylecgonine Hydrazides to HRP. To a solution of HRP (2.4 mg) in 50 mM sodium acetate (2.16 mL), pH 5.0 at 4 °C, was added 100 mM sodium metaperiodate in 50 mM sodium acetate (0.24 mL), pH 5.0. The solution was allowed to stand at 4 °C for 30 min in the dark, dialyzed against 50 mM sodium

acetate, pH 5.0 (24 h, several changes), and then concentrated to 0.6 mL using a Centricon-10 centrifugal microconcentrator (Amicon, Danvers, MA). Benzoylecgonine hydrazide hydrochloride (3.8 mg in 100 μ L of water, 200-fold molar excess) was added and allowed to react at room temperature for 4 h. The solution was then dialyzed against 50 mM sodium phosphate, pH 7.2. Mono(*N*-2'-benzoylecgoninoyl)adipic dihydrazide, biotin hydrazide, and biotin-LC hydrazide were conjugated, as above, to the carbohydrate residues of HRP.

Direct Conjugation of Benzoylecgonine to Adipic Dihydrazide-Derivatized HRP. HRP (2 mg) was oxidized with periodate as described above and dialyzed against 50 mM sodium acetate, pH 5.0. The solution was concentrated (final volume, 1 mL) using a Centricon-10 microconcentrator, treated with adipic dihydrazide (1.8 mg in 100 μ L of 50 mM sodium acetate, pH 5.0), and allowed to react at room temperature for 4 h. The solution was dialyzed against 100 mM MES, pH 4.7, and then concentrated to 400 μ L using a Centricon-10 microconcentrator. In a separate test tube, EDC (5.0 mg in 100 μ L of 100 mM MES, pH 4.7) was added to a solution of benzoylecgonine (2.5 mg in 100 μ L of 100 mM MES, pH 4.7) and allowed to react at room temperature. After 10–15 min, adipic dihydrazide-modified HRP was added to EDC-activated benzoylecgonine and allowed to react at room temperature. Aliquots (200 μ L) of this solution drawn at 30 min, 1 h, and 2 h were dialyzed extensively against 20 mM sodium phosphate, pH 7.4, and analyzed by MALDI-MS.

Dot Blot Analysis of Benzoylecgonine–HRP Conjugates. The benzoylecgonine residues covalently bound to HRP were identified by dot blot analysis using mAb and a polyclonal antibody that are specific to benzoylecgonine. The benzoylecgonine antibodies (amounts ranging from 4 to 0.03 μ g per spot) were spotted on PVDF membranes using a dot blot apparatus. The membranes were either stained with Amido black to visualize the amounts of applied antibody (data not shown) or blocked with a 1% solution of BSA in 10 mM Tris-HCl and 150 mM NaCl, pH 8.0, at room temperature for 2 h. The blocked membranes were treated with BE–HRP (4 μ g/mL) in 50 mM Tris-HCl and 150 mM NaCl, pH 8.0, containing 0.05% Tween 20 at room temperature for 1 h. The membranes were washed three to four times with 50 mM Tris-HCl and 150 mM NaCl, pH 8.0, containing 0.05% Tween 20 to remove unbound BE–HRP and then treated with 4-chloro-1-naphthol reagent (10). After 5–10 min of color development, the membranes were washed with excess distilled water.

Mass Spectrometry of Benzoylecgonine–HRP and Biotin–HRP Conjugates. MALDI-MS (11) of the conjugates was performed using a Kratos MALDI III mass spectrometer (Shimadzu Scientific Instruments, Columbia, MD) operated in the linear mode at an accelerating voltage of 22 keV with detection of positive ions. Conjugates (5–10 μ M in 0.5 μ L of water) were placed on the sample slide, and sinapinic acid (0.8 μ L of a 10 mg/mL solution in 50% aqueous acetonitrile and 0.05% trifluoroacetic acid) was added. The slide was then dried in a vacuum desiccator for 10 min. Desorption/ionization was accomplished by a nitrogen UV laser (337 nm). Spectra (100) were accumulated over a 2 mm stripe across the sample spot. The instrument was calibrated against BSA as an external standard [(M + H)⁺ = 66 431 and (M + 2H)²⁺ = 33 216] (12); the error in mass measurements was about 0.1%. Unless otherwise stated, the reported *m/z* values of HRP and BE–HRP correspond to the centers of the whole peak.

RESULTS AND DISCUSSION

Preparation of Benzoylecgonine. Benzoylecgonine was prepared by heating an aqueous solution of cocaine base under optimal reaction conditions to obtain quantitative conversion without any degradation. FAB-MS analysis showed that the (M + H)⁺ ion of cocaine (304 amu) completely disappeared while a new (M + H)⁺ ion of benzoylecgonine (290 amu) appeared. The quantitative conversion of cocaine to benzoylecgonine was further demonstrated by IR and NMR analyses. Benzoylecgonine showed IR absorption peaks at 1631 cm⁻¹ (carboxylic group), 1724 cm⁻¹ (aryl ester carbonyl), and 3450 cm⁻¹ (broad, carboxylic OH), whereas cocaine gave absorption peaks at 1737 cm⁻¹ (methyl ester carbonyl) and 1710 cm⁻¹ (aryl ester carbonyl). The ¹H-NMR (in CDCl₃) of benzoylecgonine contained resonance signals at δ 8.20–7.31 (5H, m, C₆H₅) and 2.58–2.44 (3H, s, NCH₃); the methyl ester resonance at δ 3.85–3.66 (3H, s, COOCH₃) that was observed for cocaine was absent (13). Upon recrystallization from hot water, benzoylecgonine was obtained as white needles: mp 88–90 °C (lit. 86–92 °C) (14). High-resolution mass spectral analysis of the purified product gave the (M + H)⁺ ion at 290.1388 (calcd 290.1392, C₁₆H₂₀NO₄).

Synthesis of Benzoylecgonine Hydrazide. To accomplish a facile conjugation of benzoylecgonine to HRP by aldehyde–hydrazide condensation reaction, we synthesized and characterized two hydrazide derivatives of benzoylecgonine. The carbodiimide-activated benzoylecgonine was reacted with *tert*-butyloxycarbonyl hydrazide using HBT as a catalyst to obtain *N*-2-(*tert*-butyloxycarbonyl)benzoylecgonine hydrazide (Figure 1) in 58% yield. The product was purified on a silica gel column and characterized by NMR and FAB-MS. ¹H-NMR analysis showed resonance signals at δ 9.32–8.27 (2H, b, CONHNHCOO-*t*-Bu), 1.76–1.06 (9H, s, OC(CH₃)₃), and 8.24–7.06 (5H, m, COC₆H₅). These results demonstrated that the benzoyl moiety remained intact under the reaction conditions employed for hydrazide formation. High-resolution FAB-MS analysis gave the (M + H)⁺ ion at 404.2169 (calcd, 404.2185, C₂₁H₃₀N₃O₅).

The protective *tert*-butoxy group in *N*-2-(*tert*-butoxycarbonyl)benzoylecgonine hydrazide was removed with anhydrous HCl, and the product, benzoylecgonine hydrazide hydrochloride, was obtained in almost quantitative yield. High-resolution FAB-MS analysis gave (M + H)⁺ at 304.1662 (calcd, 304.1661, C₁₆H₂₂N₃O₃).

Synthesis of Mono(*N*-2'-benzoylecgoninoyl)adipic Dihydrazide. The observed coupling efficiency of benzoylecgonine to periodate-oxidized HRP was much lower than that expected, considering the number of available aldehyde groups (see below) and the ease of aldehyde–hydrazide condensation. This is presumably due to steric hindrance from the bulky benzoyl group on the adjacent carbon atom. To overcome this disadvantage, an adipic acid spacer arm-containing hydrazide was synthesized. Carbodiimide-activated benzoylecgonine was condensed with adipic dihydrazide using HBT as a catalyst. The product, mono(*N*-2'-benzoylecgoninoyl)adipic dihydrazide, was obtained in about 32% yield after purification by ethanol precipitation to remove unreacted adipic dihydrazide followed by silica gel chromatography and preparative TLC. FAB-MS showed (M + H)⁺ and (M + Na)⁺ ions at 446 and 468 amu, respectively. High-resolution mass spectral analysis gave (M + H)⁺ at 446.2381 (calcd, 446.2403, C₂₂H₃₂N₅O₅).

Conjugation of Benzoylecgonine Hydrazides to the Carbohydrate Moieties of HRP. HRP contains eight N-linked oligosaccharides with Xyl(Man)₂Man(Fuc)-

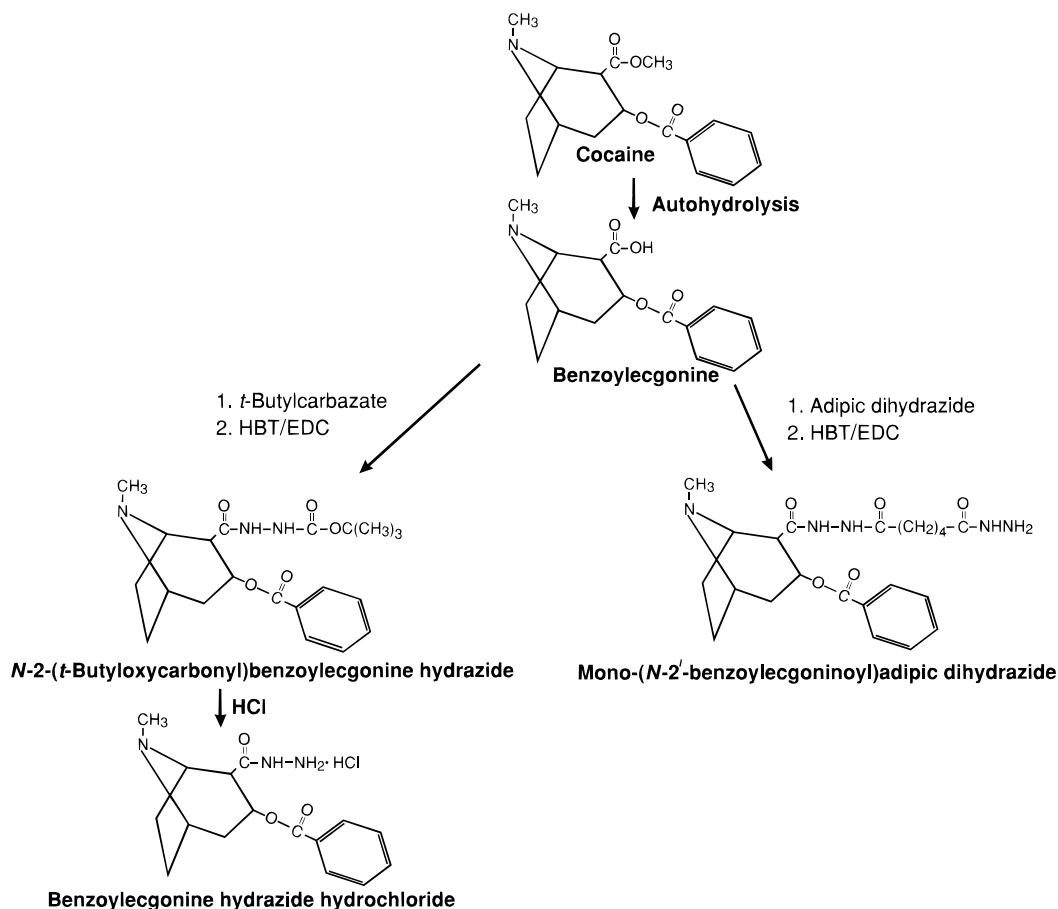


Figure 1. Scheme for the synthesis of benzoylecgonine hydrazide and mono(*N*-2'-benzoylecgoninoyl)adipic dihydrazide.

GlcNAc₂ structures (15, 16). The fucose, xylose, and two out of three mannose residues of each HRP oligosaccharide structure are amenable to periodate oxidation. In all, there are approximately 32 unsubstituted terminal sugar residues that can potentially be oxidized by periodate. Generally, periodate oxidation of glycoproteins to generate aldehyde groups on terminal hexoses is performed using 10–20 mM periodate for 30–40 min in the dark (4, 5, 17–20). In the present study, HRP was oxidized with periodate under relatively mild conditions to generate aldehyde groups on the terminal sugar residues. The carbohydrate compositional analysis of the periodate-oxidized HRP demonstrated that approximately 80% of the fucose and 50% of the combined mannose and xylose (Figure 2) were oxidized. From this result, we estimate that 18 sugar residues of HRP were oxidized.

Approximately 4.5 mol of benzoylecgonine hydrazide was coupled to 1 mol of HRP even though a 200-fold molar excess of the hydrazide was used. Both untreated HRP and BE–HRP showed clusters of heterogeneous peaks by MALDI-MS analysis (Figure 3). At least three major HRP species were present in the HRP preparation used in this study (see Figure 3A). In several BE–HRP conjugates that we prepared, stoichiometries of benzoylecgonine coupling to the major HRP species ranged from 4.2 to 4.9 per mole, based on the observed molecular weight increase of about 1200 to 1400, respectively (Figure 2B). Within any one preparation, the majority of BE–HRP contained about 4.5 mol of benzoylecgonine per mole of HRP. Considering that as many as 36 aldehyde groups were generated on periodate oxidation of HRP (see above) and that the hydrazide–aldehyde additions are facile, the low efficiency of conjugation of benzoylecgonine hydrazide to HRP is likely due to the

steric hindrance caused by the neighboring bulky benzoyl moiety. In support of this conclusion, the DCC-activated benzoylecgonine failed to form a succinimide ester with NHS even after a prolonged reaction. However, FAB-MS of the reaction mixture indicated that a stable benzoylecgonine–DCC adduct was formed [$(M + H)^+ = 496$].

The amount of mono(*N*-2'-benzoylecgoninoyl)adipic dihydrazide that coupled to periodate-oxidized HRP (about 11.0 residues per mole of HRP) was about 2.5-fold higher compared with the amount of benzoylecgonine hydrazide. MALDI-MS analysis of the conjugates (Figure 3C) demonstrated a coupling stoichiometry of approximately 10.1 to 11.8 mol of benzoylecgonine residues per mole of the major HRP species, based on the observed molecular weight increase of about 4300 to 5050, respectively. This result taken together with those described above suggests that the steric hindrance caused by the benzoyl group can be considerably minimized by introduction of a spacer arm, and thus, more benzoylecgonine residues could be coupled to HRP.

The coupling of two commercially available hydrazides, biotin hydrazide and biotin-LC hydrazide, to periodate-oxidized HRP was studied to determine whether the length of the spacer arm is critical for efficient conjugation. We reasoned that any difference in coupling efficiency between these two compounds must be related to the steric effect exerted by the bulky fused ring system. The molecular weight of HRP was increased by approximately 1560 and 2890 after conjugation with biotin hydrazide and biotin-LC hydrazide, respectively, as demonstrated by MALDI-MS analysis (data not shown). These molecular weight shifts correspond to coupling of about 6.5 mol of biotin hydrazide and 8.2 mol of biotin-LC hydrazide to HRP. The data suggest that the

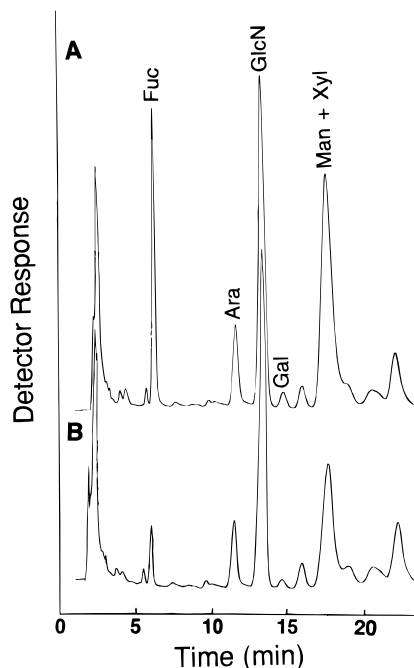


Figure 2. Carbohydrate compositional analysis of HRP (A) and periodate-oxidized HRP (B). HRP (10 μ g) and periodate-oxidized HRP (10 μ g) were hydrolyzed as described in Experimental Procedures. The hydrolysates corresponding to the 2 μ g protein were chromatographed on a CarboPac PA1 high-pH anion exchange column (4 $\times$ 250 mm). The elution was with 20 mM sodium hydroxide at a flow rate of 0.8 mL/min. Sugars were identified by comparison of their retention times with those of standards: Fuc, fucose; Ara, arabinose (could be from a polysaccharide contaminant in the HRP preparation); GlcN, glucosamine (derived from *N*-acetylglucosamine); Gal, galactose; Man, mannose; Xyl, xylose.

additional spacer arm in biotin-LC hydrazide has a significant contribution toward the coupling efficiency. From these data, we infer that a longer spacer arm, similar to the one used in this study, is necessary to achieve optimal coupling of benzoylecgonine to periodate-oxidized HRP.

Conjugation of Benzoylecgonine to Adipic Dihydrazide-Derivatized HRP. An alternative method involving in situ conjugation of EDC-activated benzoylecgonine to adipic dihydrazide-derivatized HRP was also studied. In this approach, periodate-oxidized HRP was first reacted with adipic dihydrazide. MALDI-MS analysis of the product revealed that as many as 15–17 adipic dihydrazide molecules (approximately two residues per oligosaccharide chain of HRP) were coupled to 1 mol of HRP (data not shown). The coupling did not increase with a further increase in reaction time, suggesting that each oligosaccharide chain of HRP can accommodate no more than two adipic dihydrazide residues even though considerably more aldehyde groups are available (see above). The resultant adipic dihydrazide-derivatized HRP was then allowed to react with the carbodiimide-activated benzoylecgonine. MALDI-MS analysis of the reaction mixture at different time intervals indicated that the coupling of benzoylecgonine residues to HRP was essentially complete within 30 min. Approximately 2.5–3 mol of benzoylecgonine was coupled to 1 mol of HRP (data not shown). The primary amine groups of the lysine side chain and amino terminal primary amino groups of HRP are highly protonated at pH 4.7, whereas those of the adipic dihydrazide moiety (pK_a of a hydrazide is ~ 2.6) (8) are not. Therefore, the coupling of the carbodiimide-activated benzoylecgonine to adipic dihydrazide-derivatized HRP is likely through the carbohydrate-linked adipic hydrazide groups, rather than the

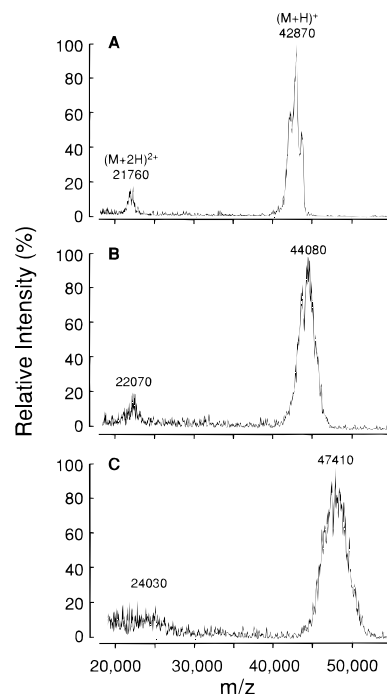


Figure 3. Mass spectral analysis of HRP and BE-HRP conjugates. Shown are the positive ion mass spectra obtained by the matrix-assisted laser desorption/ionization and time of flight mass analysis of HRP (A), benzoylecgonine hydrazide-HRP conjugates (B), and mono(*N*-2'-benzoylecgoninoyl)adipic hydrazide-HRP conjugates (C). The measured m/z values for $(M + H)^+$ and $(M + 2H)^{2+}$ ions are indicated.

amino groups of the peptide chain. However, in the absence of experimental evidence, we cannot exclude the possibility that benzoylecgonine is coupled directly to the polypeptide chain of HRP.

Identification of Benzoylecgonine Residues Bound to HRP. We analyzed the BE-HRP conjugates for their ability to bind a mouse mAb against benzoylecgonine (Figure 4). The HRP conjugates of the benzoylecgonine hydrazides and those prepared by coupling benzoylecgonine to the adipic dihydrazide-modified HRP were able to detect as little as 30–60 ng of the mAb. Similar results were obtained by dot blot analysis using a polyclonal rabbit anti-benzoylecgonine IgG (data not shown). These results demonstrate that benzoylecgonine is covalently coupled to HRP and that the HRP activity is retained.

CONCLUSION

Two convenient methods have been developed for the covalent attachment of benzoylecgonine to HRP through its oligosaccharide chains. First, to take advantage of the facile aldehyde-hydrazide condensation reactions at acidic pH to form hydrazones, we synthesized two hydrazide derivatives of benzoylecgonine. The hydrazides were purified, characterized, and then coupled to HRP through the glycoprotein's carbohydrate residues in a site specific manner.

Second, in a more simplistic approach, the terminal sugar residues in HRP were derivatized with adipic dihydrazide. A large molar excess of the adipic dihydrazide was used to ensure that only one end condenses with the aldehyde groups on the carbohydrate moieties of HRP. The hydrazide-derivatized HRP was then reacted with the carbodiimide-activated benzoylecgonine under acidic conditions to ensure that the hydrazide moieties rather than amino groups of the polypeptide chain react with benzoylecgonine. Therefore, it is likely that this approach also gives carbohydrate site-directed coupling.

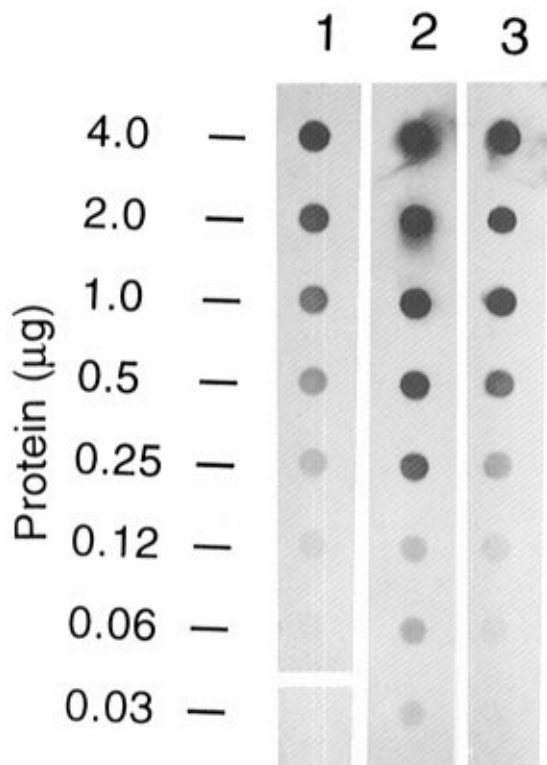


Figure 4. Dot blot analysis of benzoylecgonine-HRP conjugates. The indicated amounts of the benzoylecgonine monoclonal antibody were spotted onto PVDF membranes. After they were blocked with 1% BSA, the membranes were blotted with BE-HRP conjugates and then visualized with the 4-chloro-1-naphthol reagent as described in Experimental Procedures: lane 1, benzoylecgonine hydrazide-HRP conjugate; lane 2, mono(*N*-2'-benzoylecgoninoyl)adipic dihydrazide-HRP conjugate; lane 3, benzoylecgonine-adipic dihydrazide-derivatized HRP conjugate.

The presence of covalently bound benzoylecgonine in the BE-HRP conjugates was demonstrated by dot blot analysis using anti-benzoylecgonine antibodies. The stoichiometry of benzoylecgonine to HRP was determined reliably by MALDI-MS analysis.

The procedures described here provide convenient approaches for the preparation of BE-HRP conjugates. Approximately 4 and 10 benzoylecgonine residues could be coupled to 1 mol of HRP by using the two benzoylecgonine hydrazides described here. Further, it is possible to obtain BE-HRP conjugates containing the desired amounts of benzoylecgonine (in the range of 1–10 mol per mole of HRP) by controlling the degree of periodate oxidation and thus the number of sugar residues oxidized. We routinely use the BE-HRP conjugates that were prepared as above in a cocaine detection methodology. It is anticipated that the procedures described here will be valuable to other investigators for the preparation of BE-HRP conjugates of required stoichiometry and for the development of specific and sensitive immunological assays for cocaine detection.

ACKNOWLEDGMENT

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LITERATURE CITED

- (1) Fodor, G. (1970) Tropane alkaloids. In *Chemistry of Alkaloids* (S. W. Pelletier, Ed.) pp 444–468, Van Nostrand Reinhold Co., New York.
- (2) Faulstich, H., and Fiume, L. (1985) Protein conjugates of fungal toxins. *Methods Enzymol.* 112, 225–237.
- (3) Lonngren, J., and Goldstein, I. J. (1994) Coupling of aldobionic acids to proteins using water-soluble carbodiimides. *Methods Enzymol.* 252, 116–118.
- (4) O'Shannessy, D. J., and Quarles, R. H. (1987) Labeling of the oligosaccharide moieties of immunoglobulins. *J. Immunol. Methods* 99, 153–161.
- (5) O'Shannessy, D. J., and Wilchek, M. (1990) Immobilization of glycoconjugates by their oligosaccharides: Use of hydrazido-derivatized matrices. *Anal. Biochem.* 191, 1–8.
- (6) Rodwell, J. D., Alvarez, V. L., Lee, C., Lopes, A. D., Goers, J. W. F., King, H. D., Powsner, H. J., and McKearn, T. J. (1986) Site-specific covalent modification of monoclonal antibodies: *In vitro* and *in vivo* evaluations. *Proc. Natl. Acad. Sci. U.S.A.* 83, 2632–2636.
- (7) Burkot, T. R., Wirtz, R. A., and Lyon, J. (1985) Use of fluorodinitrobenzene to identify monoclonal antibodies which are suitable for conjugation to periodate-oxidized horseradish peroxidase. *J. Immunol. Methods* 84, 25–31.
- (8) Inman, J. K., and Dintzis, H. M. (1969) The derivatization of cross-linked polyacrylamide beads. Controlled introduction of functional groups for the preparation of special-purpose, biochemical adsorbents. *Biochemistry* 8, 4074–4082.
- (9) Hardy, M. R., and Townsend, R. E. (1994) High-pH anion-exchange chromatography of glycoprotein derived carbohydrates. *Methods Enzymol.* 230, 208–225.
- (10) Houen, G., and Leonardsen, L. (1992) A specific peroxidase-coupled activity stain for diamine oxidases. *Anal. Biochem.* 204, 296–299.
- (11) Hillenkamp, F., and Karas, M. (1990) Mass spectrometry of peptides and proteins by matrix-assisted ultraviolet laser desorption/ionization. *Methods Enzymol.* 193, 280–295.
- (12) Hirayama, K., Akashi, S., Furuya, M., and Fukuhara, K. (1990) Rapid confirmation and revision of the primary structure of bovine serum albumin by ES-MS and Frit-Fab LC/MS. *Biochem. Biophys. Res. Commun.* 173, 639–646.
- (13) Tufariello, J. J., Mullen, G. B., Tegeler, J. J., Trybulski, E. J., Wong, S. A., and Ali, S. A. (1979) Synthesis of the tropane class of alkaloids. Pseudotropine and dl-cocaine. *J. Am. Chem. Soc.* 101, 2435–2442.
- (14) Stecher, P. G., Windhold, M., Leahy, D. S., Bolton, D. M., and Eaton, L. G. (1968) In *The Merck Index: An Encyclopedia of Chemicals and Drugs*, 8th ed., pp 135–136, Merck & Co., Inc., Rathway, NJ.
- (15) Welinder, K. G. (1979) Amino acid sequence studies of horseradish peroxidase. Amino and carboxyl termini, cyanogen bromide and tryptic fragments, the complete sequence, and some structural characteristics of horseradish peroxidase C. *Eur. J. Biochem.* 96, 483–502.
- (16) Phleps, C., Forlani, L., and Antonini, E. (1971) The hydrogen ion equilibria of horseradish peroxidase and apoperoxidase. *Biochem. J.* 124, 605–614.
- (17) Hoffman, W. L., and O'Shannessy, D. J. (1988) Site-specific immobilization of antibodies by their oligosaccharide moieties to new hydrazide derivatized solid supports. *J. Immunol. Methods* 112, 113–120.
- (18) Zaborsky, O. R., and Ogletree, J. (1974) The immobilization of glucose oxidase via activation of its carbohydrate residues. *Biochem. Biophys. Res. Commun.* 61, 210–216.
- (19) Chua, M.-M., Fan, S.-T., and Karush, F. (1984) Attachment of immunoglobulin to liposomal membrane via protein carbohydrate. *Biochim. Biophys. Acta* 800, 291–300.
- (20) Thorpe, P. E., Detre, S. I., Foxwell, B. M., Brown, A. N., Skilleter, D. N., Wilson, G., Forrester, J. A., and Stirpe, F. (1985) Modification of the carbohydrate in ricin with meta-periodate-cyanoborohydride mixtures. Effects on toxicity and *in vivo* distribution. *Eur. J. Biochem.* 147, 197–206.

TECHNICAL NOTES

Synthesis of Norbiotinamine and Its Derivatives

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Synthesis of norbiotinamine (**II**) and its reactive derivatives, isothiocyanates (**III** and **IV**), iodoacetamide (**V**), and maleimide (**VI**), are described. In addition, *N*-norbiotinyl- γ -L-glutamylamide (**VII**) was prepared as a potential alternative to the fixable polar tracer biocytin (biotinoyllysine).

The strong ($K_d = 10^{-15} \text{ M}^{-1}$) specific binding of biotin¹ (**I**) to avidin has found widespread application in biochemical research, analyses, and separations. Many reagents are available for the coupling of biotin to different functional groups (1). All of these reagents are made by conversion of the carboxylic acid of biotin to an activated moiety, with the most widely used one being the *N*-hydroxysuccinimidyl ester. However, NHS esters have some limitations; for example, they do not react with aromatic amines. On the other hand, it is impractical to use biotinoyl chloride (even *in situ*), because the imidazole ring of biotin is acetylated. The goal of this work was to find alternatives to the above methods of biotinylation which would not have the limitation described. Since an isothiocyanate is more reactive than an NHS ester but less reactive than an acid chloride, we hypothesized that a biotin derivative with a terminal amino group in place of the carboxylic acid (compound **II**) could be used to prepare the isothiocyanate (**III**) and other reactive derivatives. Norbiotinamine (**II**) was obtained from biotin in 55% overall yield by modified Curtius rearrangement (2), using diphenylphosphoryl azide in *t*-BuOH, followed by hydrolysis of the intermediate *tert*-butoxycarbamate. Norbiotin isothiocyanate (**III**) was obtained in ca. 50% yield, after a reaction of the amine **II** with CS_2 , followed by oxidation of the thiouonium salt with $\text{Pb}(\text{NO}_3)_2$ (3). An alternative reaction using a less reactive TCDP substitute of thiophosgene (4, 5) gave the product **III** in 85% yield. We were also interested in making an extended version of the isothiocyanate. Thus, norbiotinamine (**II**) was treated with the NHS ester of Cbz-aminododecanoic (aminolauric) acid to form, after deprotection, *N*-norbiotinyl-12-aminododecanoamide (**VIII**). The reaction of the amine **VIII** with

TCDP yielded the isothiocyanate **IV**, containing a 13-atom aliphatic spacer.

The iodoacetyl (**V**) and maleimide (**VI**) derivatives of norbiotinamine (**II**) were prepared, since reagents of this type are commonly used for alkylation, especially of protein thiols. The iodoacetamide (**V**) was made from the amine (**II**) using succinimidyl iodoacetate. Attempts to prepare biotinylmaleimide using the Mitsunobu reaction (6) on biotinol (7) gave a mixture of many unidentified products which precluded the possibility of isolation of the maleimide in a significant yield. A maleimide derivative was eventually synthesized in 63% yield by reaction of norbiotinamine with succinimidyl β -maleimido-propionate to obtain *N*-norbiotinyl- β -maleimidopropionylamide (**VI**).

Norbiotinamine (**II**) is an interesting alternative to biotin. In order to couple biotin to amino acids, reaction has to be with an amino group. A popular example is biocytin (biotin lysine). However, norbiotinamine could be coupled with a carboxylic group of amino acids to give inverse peptides, having the amide linkage oriented in the opposite direction. As an example, norbiotinamine was coupled with the NHS ester of *N*-Boc-glutamic acid γ -benzyl ester. The resulting norbiotinylamide of γ -glutamic acid (**VII**), obtained after deprotection, is similar to biocytin, but it has a shorter aliphatic chain and an inverted amide bond.

The structures of all products were established by NMR analyses and derivatization. However, norbiotinamine (**II**), the title product and starting material for the reactive derivatives, had its purity confirmed by elemental analysis. This analysis showed that norbiotinamine (**II**) forms a stable carbonate in the presence of air (as seen with cadaverine). The reactions and purifications were easily monitored by thin layer chromatography (TLC) analyses. Usually components of even complex reaction mixtures were readily identified on a single fluorescent TLC plate by first checking under a UV lamp, next exposed to iodine vapors (I_2 on SiO_2), and, after evaporation of the iodine, dipped in a biotin sensitive reagent (or in ninhydrin solution) and heating.

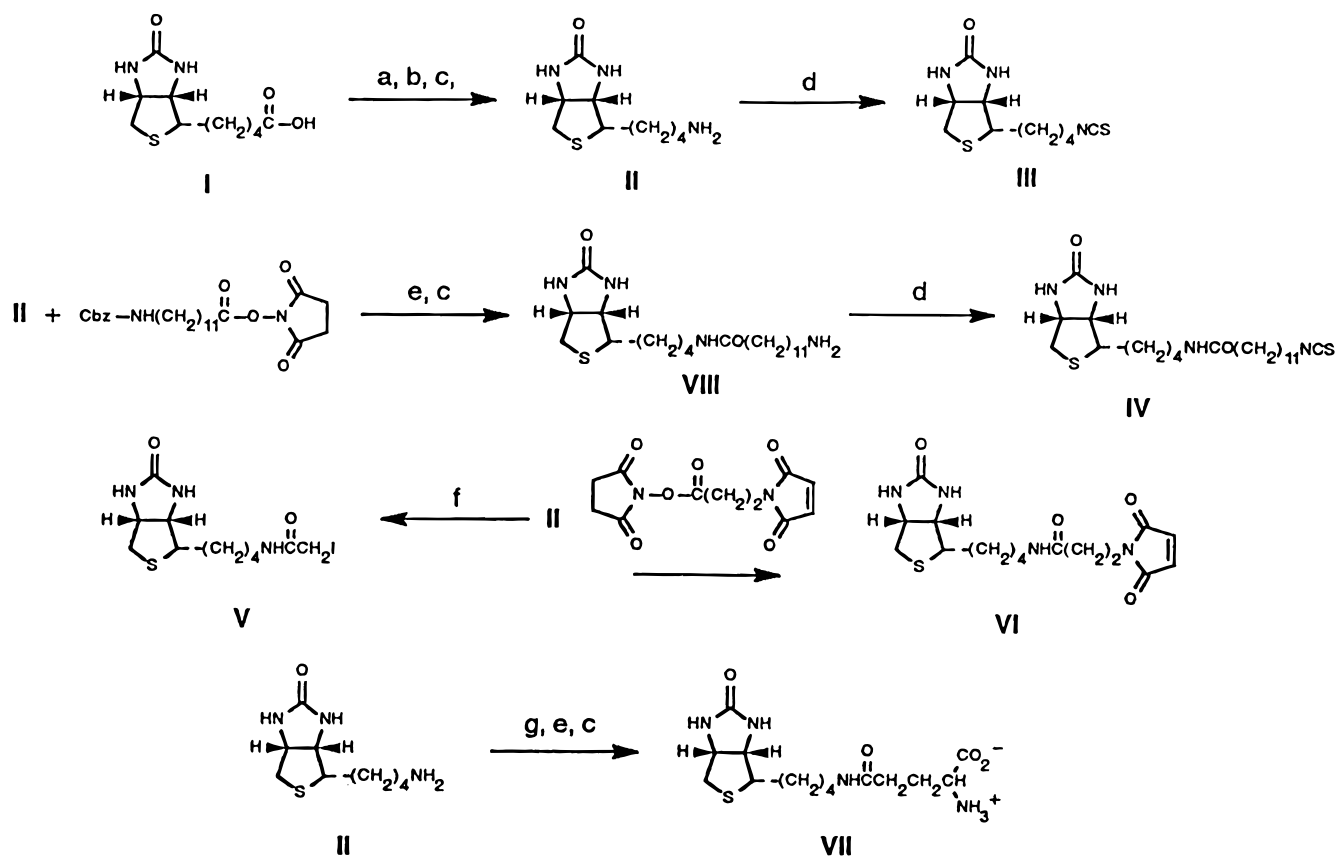
EXPERIMENTAL PROCEDURES

¹H NMR spectra were recorded on a 400 MHz Bruker FT NMR spectrometer. Chemical shifts are given in δ relative to the residual protons in a deuterated solvent. *J* values are in hertz. Melting points were not corrected. Elemental analysis was performed by Desert Analytics, Tucson, AZ. TLC utilized Merck 60 F-254, 0.2 mm precoated silica gel aluminum sheets. Biotin derivatives

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¹The Chemical Abstracts name for biotin (**I**) is 1*H*-thieno[3,4-*d*]imidazole-4-pentanoic acid, hexahydro-2-oxo-, [3 α S-(3 $\alpha\alpha$,4 β ,6 $\alpha\alpha$)]-. The following nomenclature was used in the paper: norbiotinamine (**II**) for the norbiotinylamine obtained from Curtius rearrangement of biotin, biotinoyl for the acyl residue of biotin, and biotinyl for the alkyl residue obtained by reduction of the carboxylic acid group of biotin. Other abbreviations: Boc, *tert*-butoxycarbonyl; Cbz, benzyloxycarbonyl; DPPA, diphenylphosphoryl azide; NHS, *N*-hydroxysuccinimide; DCC, dicyclohexylcarbodiimide; SIA, succinimidyl iodoacetate; TCDP, 1,1'-thiocarbonyldi-2,2'-pyridone; DMF, dimethylformamide.

Scheme 1^a

^a a, DPPA, NEt₃, *t*-BuOH; b, HCl, dioxane; c, NaOH; d, TCDP; e, HBr/AcOH; f, SIA; g, *N*-Boc-L-glutamic acid, α -Bz, γ -NHS ester.

were visualized by a dip consisting of a 0.4% solution of 4-(dimethylamino)cinnamaldehyde in 0.6 N HCl containing 90% 2-propanol. Primary amines were identified with a 0.3% solution of ninhydrin in 3% AcOH/BuOH. Column chromatography used silica gel 60, Merck "Geduran", 40–63 μ m. All operations involving iodoacetyl compounds were run under subdued light. Biotin and SIA were from Molecular Probes, Inc. DCC was from Fluka Chemical Corp. Diphenylphosphoryl azide was from Lancaster Synthesis, Inc. *N*-Boc-L-glutamic acid and α -Bz ester were from Sigma Chemical Corp. TCDP was from Aldrich Chemical Corp. Succinimidy esters were made using NHS and DCC.

Norbiotinamine (II). Molecular Formula, C₉H₁₇N₃OS. Formula Weight, 215.31 (277.34 for the carbonate). A mixture of biotin (I) (9.8 g, 40 mmol), diphenylphosphoryl azide (12.1 g, 44 mmol), and triethylamine (6 mL, 4.35 g, 43 mmol) in 200 mL of *t*-BuOH was heated to reflux for 18 h. Solvents were evaporated, and the residue was dissolved in 6 N HCl in 50% MeOH. After the solution was stirred overnight, the pH of the solution was adjusted to 12 using 50% NaOH. Inorganics were filtered off and washed with MeOH. The filtrates were evaporated, and the residue was then dissolved in water and poured on a column containing 300 mL of strongly acidic Dowex 50-X4 (200–400 mesh), H⁺ form. Impurities were removed with a 5% solution of triethylamine in 20% MeOH. The product II was eluted with 5% NaOH. A concentrated solution of product was purified on an LH-20 column. Lyophilization yielded 4.75 g (55%) of fluffy, white powder of II. The melting point of the amine is dependent on traces of CO₂ and H₂O; the melting point of the carbonate is 265–268 °C dec [TLC, concd NH₃/H₂O/PrOH (3:1:6), R_f 0.67]: ¹H NMR (D₂O) δ 4.62 (m, 1H), 4.45 (m, 1H), 3.37 (m, 1H), 3.02 (dd, 1H, *J* = 12.9, 4.9), 2.79 (d, 1H, *J* = 12.9), the triplet of CH₂-

NH₂ is at 2.55 for the "free" amine, 2.78 for the carbonate, or 2.88 for the hydrochloride, 1.7–1.2 (mm, 6H). Anal. Calcd for C₉H₁₇N₃OS + CO₂ + H₂O: C, 43.30; H, 6.90; N, 15.15. Found: C, 43.53; H, 6.47; N, 15.35.

Norbiotinyl Isothiocyanate (III). Molecular Formula, C₁₀H₁₅N₃OS. Formula Weight, 257.38. A solution of TCDP (465 mg, 2 mmol) in 20 mL of CHCl₃ was added to a stirred mixture of norbiotinamine (II) carbonate (554 mg, 2 mmol) in 100 mL of MeCN and 20 mL of CHCl₃. After 15 min, 1 drop of NEt₃ was added. The yellow-orange color of TCDP almost disappeared after an additional 15 min. The reaction mixture was flash chromatographed on 120 g of SiO₂, using 10% MeOH in CHCl₃ as eluent. The major fractions were evaporated. The residue was crystallized with EtOAc/hexanes to give 436 mg (85%) of light beige crystals of III: mp 185–186 °C; ¹H NMR (DMSO-*d*₆) δ 6.43 (s, 1H), 6.36 (s, 1H), 4.31 (m, 1H), 4.15 (m, 1H), 3.66 (t, 2H, *J* = 6.6, CH₂NCS), 3.13 (m, 1H), 2.84 (dd, 1H, *J* = 12.4, 5.1), 2.59 (d, 1H, *J* = 12.4), 1.7–1.3 (mm, 6H).

N-Norbiotinyl-12-aminododecanoamide (VIII). Succinimidy 12-Cbz-aminododecanoate (2.68 g, 6 mmol) was added to a mixture of norbiotinamine (II) carbonate (1.1 g, 4 mmol), 10 mL of DMF, and 200 mL of CHCl₃. After 18 h, excess NEt₃ (1.4 mL, 10 mmol) was added. After another 20 h, the reaction mixture was poured on a wide suction column with 200 g of dry SiO₂. The Cbz-amide was eluted with 5% MeOH/CHCl₃ and, after evaporation, deprotected by dissolving in 80 mL of 15% HBr/AcOH. After 6 h, the VIII hydrobromide was precipitated with ether and then flash chromatographed on 200 g of SiO₂, starting with 1:10:89 and finishing with 1:15:84 solution of AcOH/MeOH/CHCl₃. After evaporation, 1.55 g (80%) of a salt of VIII was obtained: ¹H NMR (DMSO-*d*₆) δ 7.73 (t, 1H, *J* = 5.4), 6.42 (s, 1H), 6.36 (s, 1H), 4.31 (m, 1H), 4.13 (m, 1H), 3.09 (m, 1H), 3.02 (m,

2H, CH_2NHCO), 2.82 (dd, 1H, $J = 12.4, 5.1$), 2.76 (t, 2H, $J = 7.5$, CH_2NH_2), 2.58 (d, 1H, $J = 12.4$), 2.03 (t, 2H, $J = 7.4$, CH_2CONH), 1.91 (s, <1H, acetate), 1.6–1.2 (mm, 24H). TLC of **II** and **VIII** in AcOH/MeOH/ CHCl_3 (1:7:12) gave R_f values of 0.08 and 0.45, respectively, and TLC in $\text{H}_2\text{O}/\text{AcOH}/\text{MeOH}/\text{CHCl}_3$ (1:1:8:10) gave R_f values of 0.25 and 0.80, respectively.

12-(Norbiotinylamino)carbonyldodecyl Isothiocyanate (IV). Molecular Formula, $\text{C}_{22}\text{H}_{38}\text{N}_4\text{O}_2\text{S}_2$. Formula Weight, 454.64. A solution of TCDP (493 mg, 2.12 mmol) was added to a mixture of **VIII** hydrobromide (750 mg, 1.52 mmol) and diisopropylethylamine (337 μL , 250 mg, 1.93 mmol) in 100 mL of MeCN. After being stirred for 30 min, the reaction mixture was chromatographed as in the case of **III** (above), using 5% MeOH in CHCl_3 . The major fractions still had to be extracted with water to remove some diisopropylethylamine. The CHCl_3 extract was dried with Na_2SO_4 and evaporated to give 192 mg (28%) of **IV**. An analytical sample was crystallized with CHCl_3 /hexanes: mp 158–159 °C; ^1H NMR ($\text{DMSO}-d_6$) δ 7.69 (m, 1H), 6.41 (s, 1H), 6.34 (s, 1H), 4.31 (m, 1H), 4.13 (m, 1H), 3.65 (t, 2H, $J = 6.4$, CH_2NCS), 3.09 (m, 1H), 3.01 (m, 2H, CH_2NHCO), 2.82 (dd, 1H, $J = 12.3, 4.9$), 2.58 (d, 1H, $J = 12.5$), 2.03 (t, 2H, $J = 7.3$, CH_2CONH), 1.7–1.2 (mm, 24H). TLC of **III** and **IV** in MeOH/ CHCl_3 (1:4) gave R_f values of 0.6 and 0.56, respectively, and TLC of ethanolamine-derivatized **III** and **IV** in MeOH/ CHCl_3 (1:4) gave R_f values of 0.3 and 0.4, respectively.

Norbiotinyl Iodoacetamide (V). Molecular Formula, $\text{C}_{11}\text{H}_{18}\text{N}_3\text{O}_2\text{S}$. Formula Weight, 383.26. SIA (0.85 mg, 3 mmol) was added to norbiotinamine (**II**) carbonate (554 mg, 2 mmol) in 40 mL of DMF and 40 mL of MeCN. After 1 h, the product was precipitated by addition of ether and then cooling to –5 °C. The solid was filtered and washed with ether to yield 450 mg (59%) of **V**: mp 156–158 °C. An excess of ether and hexanes gave the second crop, 315 mg (40%) [TLC, AcOH/MeOH/ CHCl_3 (1:5:14), R_f 0.37]: ^1H NMR ($\text{DMSO}-d_6$) δ 8.21 (m, 1H), 6.42 (s, 1H), 6.35 (s, 1H), 4.30 (m, 1H), 4.13 (m, 1H), 3.61 (s, 2H, COCH_2I), 3.09 (m, 1H), 3.02 (m, 4H, CH_2NHCO), 2.82 (dd, 1H, $J = 12.4, 5.0$), 2.58 (d, 1H, $J = 12.4$), 1.7–1.2 (mm, 6H).

N-Norbiotinyl- β -maleimidopropionylamide (VI). Molecular Formula, $\text{C}_{16}\text{H}_{22}\text{N}_4\text{O}_4\text{S}$. Formula Weight, 366.45. Succinimidyl β -maleimidopropionate (0.8 g, 3 mmol) was added to norbiotinamine (**II**) carbonate (0.55 g, 2 mmol) in 200 mL of CHCl_3 . The crude product was filtered after the solution was stirred for 18 h. Concentration of the filtrate gave the second crop. Both crops were combined and flash chromatographed on 200 g of SiO_2 with 10–15% MeOH/ CHCl_3 . Evaporation of the major fractions yielded 464 mg (63%) of **VI**: mp 186–188 °C; ^1H NMR ($\text{DMSO}-d_6$) δ 7.88 (m, 1H), 7.00 (s, 2H, $\text{CH}=\text{CH}$), 6.41 (s, 1H), 6.34 (s, 1H), 4.31 (m, 1H), 4.13 (m, 1H), 3.60 (t, 2H, $J = 7.3$, $\text{CH}_2\text{NCOCHCHCO}$), 3.08 (m, 1H), 2.87 (m, 2H, CH_2NH), 2.82 (dd, 1H, $J = 12.4, 5.0$), 2.58 (d, 1H, $J = 12.4$), 2.31 (t, 2H, $J = 7.1$, CH_2CONH), 1.7–1.2 (mm, 6H).

N-Norbiotinyl- γ -L-glutamylamide (VII). Molecular Formula, $\text{C}_{14}\text{H}_{24}\text{N}_4\text{O}_4\text{S}$. Formula Weight, 344.44. *N*-Boc-L-glutamic acid, α -Bz, and γ -NHS ester (2.6 g, 6 mmol) were added to a mixture of norbiotinamine (**II**) carbonate (1.1 g, 4 mmol), 10 mL of DMF, 50 mL of MeCN, and 200 mL of CHCl_3 . After 5 h, the product was precipitated by addition of ether and then cooling to –5 °C. The Cbz- and Bz-protected product was filtered and washed with ether and hexanes, to give 2.11 g (96%) of white powder: mp 153–155 °C [TLC, MeOH/ CHCl_3 (1:9), one spot, R_f 0.3]. The solid (1 g, 1.9 mmol) was dissolved in 75 mL of 30% HBr/AcOH. After 2 h, the hydrobromide of **VII** was precipitated by addition of ether and then cooling to –5 °C. The residue was dissolved in 40 mL of MeOH, and the product was again precipitated with ether. The solid was dissolved in 20 mL of water, and the pH was adjusted to 5 with diluted NaOH. The product was precipitated with acetone. The precipitate was washed with acetone, crystallized with 2 mL of water, filtered, and then washed with a couple drops of water to give 410 mg (66%) of white crystals of **VII**: mp 210–212 °C dec [TLC, $\text{H}_2\text{O}/\text{AcOH}/\text{MeOH}/\text{CHCl}_3$ (1:1:8:10), R_f 0.28]; ^1H NMR (D_2O) δ 4.66 (m, 1H), 4.48 (m, 1H), 3.80 (t, 1H, $J = 6.1$, $\text{CH}(\text{NH}_3^+)\text{CO}_2^-$), 3.39 (m, 1H), 3.25 (t, 2H, $J = 6.7$, CH_2NHCO), 3.05 (dd, 1H, $J = 13.0, 5.0$), 2.84 (d, 1H, $J = 13.0$), 2.44 (m, 2H), 2.19 (m, 2H), 1.85–1.4 (3m, 6H).

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LITERATURE CITED

- (1) Haugland, R. P. (1992) *Handbook of Fluorescent Probes and Research Chemicals*, pp 62–65, Molecular Probes, Inc., Eugene, OR 97402. The handbook is available free upon request from Molecular Probes, Inc.
- (2) Shioiri, T., Ninomiya, K., and Yamada, S. (1972) Diphenylphosphoryl Azide: A New Convenient Reagent for a Modified Curtius Reaction and for the Peptide Synthesis. *J. Am. Chem. Soc.* 94, 6203–6205.
- (3) Sandler, S. R., and Karo, W. (1983) *Organic Functional Group Preparations*, pp 371–372, Academic Press, New York.
- (4) Kim, S., and Yi, K. Y. (1985) Di-2-Pyridyl Thionocarbonate: A New Reagent for the Preparation of Isothiocyanates and Carbodiimides. *Tetrahedron Lett.* 26, 1661–1664.
- (5) Kim, S., and Yi, K. Y. (1986) 1,1'-Thiocarbonyldi-2,2'-pyridone: A New Useful Reagent for Functional Groups Conversion under Essentially Neutral Conditions. *J. Org. Chem.* 51, 2613–2615.
- (6) Walker, M. A. (1994) The Mitsunobu Reaction: A Novel Method for Synthesis of Bifunctional Maleimide Linkers. *Tetrahedron Lett.* 35, 665–668.
- (7) Guchhait, R. B., Polakis, S. E., Hollis, D., Fenslau, C., and Lane, M. D. (1974) Acetyl Coenzyme A Carboxylase System of *Escherichia coli*. *J. Biol. Chem.* 279, 6646–6656.

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A Convenient Method for the Preparation of Hapten Phosphoramidites

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Automated incorporation of haptens at the 3' or 5' end of oligonucleotides required the preparation of the corresponding hapten phosphoramidites. The requisite 1,3-diol framework was prepared in two steps from a carboxylic acid precursor first by a two-carbon homologation using Meldrum's acid to form the corresponding 3-oxo ester and then subsequent reduction to the diol. The primary alcohol was protected with a dimethoxytrityl group, while the secondary alcohol was converted to the reactive phosphoramidite.

INTRODUCTION

We required a flexible automated method of introduction of a variety of haptens into oligonucleotide probes for use in amplified hybridization assays (1). Incorporation of the hapten using the same phosphoramidite chemistry as used on the automated oligonucleotide synthesizer was imperative, as was using the same hapten phosphoramidite to label either the 3' or 5' end of the oligonucleotide. For 3' labeling, the use of a proprietary universal controlled pore glass (CPG) obviated the need for a solid support prelabeled with the individual hapten. Other universal solid supports have been described (2–4). Additionally, synthesis of the phosphoramidite had to be easily adapted to a diverse group of haptens. Conjugation of small molecules to oligonucleotides has been reviewed (5). In particular, the use of phosphoramidite chemistry for the incorporation of dyes, intercalators, and reporter molecules has been reported (6). While the strategies presented for incorporation of a variety of small ligands were extensive, none fully met our criteria. The synthesis of oligonucleotides bearing nucleotides modified with the small molecule itself required a rather involved preparation of the substrate, while synthesis with nucleotide or non-nucleotide phosphoramidites modified with a reactive functional group required nonautomated functionalization with the small molecule with less than perfect selectivity. Most of the reported ligand phosphoramidites were built on an amino monoalcohol scaffolding, thus precluding their use for labeling the 3' end of the oligonucleotide. Exceptions include a *trans*-4-hydroxyprolinol linker that was reported in the preparation of 3'-labeled oligonucleotides (7). In this case, the prelabeled CPG was required. A second exception employed a 2-(alkylamino)-1,3-propanediol backbone (8). This method generated hapten phosphoramidites in unacceptably low overall yield (5%). The approach taken in the present work was to incorporate primary and secondary alcohol moieties into the hapten, wherein the primary alcohol could be selectively protected with the standard 4,4'-dimethoxytrityl group and the secondary alcohol could be converted into the widely used 2-(cyanoethyl)-*N,N*-diisopropyl phosphoramidite.

EXPERIMENTAL PROCEDURES

General Comments. All reagents were purchased from Aldrich Chemical Co., Inc., Milwaukee, WI, and were used without further purification, except where noted. Solvents employed were of reagent or high-performance liquid chromatography (HPLC) grade and were used as received. ¹H NMR spectra were recorded at 300 MHz on a Varian Gemini 300 spectrometer in CDCl₃ with tetramethylsilane (TMS) as a standard unless otherwise noted. High-resolution mass spectra were recorded on a Kratos MS-50 double focusing mass spectrometer.

Preparation of 2-[(3-Carboxypropyl)oxy]carbazole (1b). (a) 2-Hydroxycarbazole (40 g, 218 mmol), 2-butanone (400 mL), ethyl 4-bromobutyrate (47 mL, 327 mmol), and potassium carbonate (45 g, 327 mmol) were mixed under dry nitrogen in a round-bottom flask equipped with a reflux condenser. The mixture was refluxed for 27 h and then filtered through a medium porosity, sintered glass Buchner funnel while still hot and the solid rinsed with 2-butanone (200 mL). The combined filtrate was removed *in vacuo*. The resulting solid was triturated with hexanes (500 mL) to remove unreacted ethyl 4-bromobutyrate and then isolated on a medium porosity, sintered glass Buchner funnel. Drying *in vacuo* for 18 h gave 2-[(3-carboethoxypropyl)oxy]carbazole (59 g, 91%) as a light tan solid. ¹H NMR (DMSO-*d*₆): δ 7.98–7.85 (2H, m), 7.39 (1H, d, *J* = 7.9 Hz), 7.3–7.2 (1H, m), 7.12–7.03 (1H, m), 6.93 (1H, s), 6.74 (1H, d, *J* = 7.3 Hz), 4.15–4.00 (3H, m), 3.3 (2H, bs), 2.5 (2H, bs), 2.1–1.95 (2H, m), 1.19 (3H, t, *J* = 7.2 Hz). MS: *m/z* at 298 (M + H)⁺, 315 (M + NH₄)⁺.

(b) 2-[(3-Carboethoxypropyl)oxy]carbazole (59 g, 198 mmol) was stirred with fresh glyme (800 mL). Lithium hydroxide monohydrate (16.6 g, 397 mmol) and distilled water (100 mL) were added. The reaction mixture was stirred at 15–30 °C for 30 h. Glyme was removed *in vacuo* to about 60% of the original volume. Distilled water (1000 mL) was added, and then the solution was brought to pH 1–2 with 6 N aqueous HCl with vigorous stirring. The resulting solid was isolated on a medium porosity, sintered glass Buchner funnel and washed with water until the pH of the wash was the same as that of the water or more basic. The solid was dried *in vacuo*, giving 2-[(3-carboxypropyl)oxy]carbazole **1b** as an off-white powder (53 g, 99%). ¹H NMR (DMSO-*d*₆): δ 8.0–7.88 (2H, m), 7.40 (1H, d, *J* = 7.6 Hz), 7.3–7.2 (1H, m), 7.15–7.05 (1H, m), 6.94 (1H, s), 6.76 (1H, d, *J* = 8.2 Hz), 4.06 (2H, t, *J* = 6.1 Hz), 3.45–3.20 (1H, bs), 2.43 (2H, t,

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$J = 6.7$ Hz), 2.04–1.95 (2H, m). MS: m/z at 287 ($M + \text{NH}_4$)⁺, 269 (M)⁺.

Preparation of 3-(4-Nitrophenyl)-*N*-(5-carboxypentyl)-1-adamantaneacetamide (1c). (a) 3-(4-Nitrophenyl)-1-adamantaneacetic acid (**7**) (10 g, 31.75 mmol) was suspended in methylene chloride (100 mL) and cooled to 0 °C in an ice bath under a dry nitrogen atmosphere. Oxalyl chloride (18.25 mL, 36.5 mmol, 2 M in methylene chloride) was added dropwise over 5 min. A catalytic amount of dimethylformamide (DMF) (1 drop) was added. After 1 h, the reaction mixture became homogeneous. The volatile components were removed *in vacuo*, and the residue of the acid chloride was redissolved in methylene chloride (200 mL) and cooled to 0 °C in an ice bath under a dry nitrogen atmosphere. The solution was treated with methyl 6-aminocaproate hydrochloride (5.8 g, 32 mmol) and triethylamine (10.1 mL, 73 mmol) and then stirred for 2 h at room temperature. The reaction mixture was then diluted with ethyl acetate (200 mL), washed with water (10 mL), citric acid (10% aqueous, 2 × 50 mL), saturated sodium bicarbonate (50 mL), and brine (50 mL), dried over sodium sulfate, filtered, and evaporated. The crude product was chromatographed on silica (200 g, eluted with ethyl acetate/cyclohexane, 1:1) and then recrystallized from benzene/diethyl ether to give 3-(4-nitrophenyl)-*N*-(5-carbomethoxypentyl)-1-adamantaneacetamide (10.8 g, 24.4 mmol, 77%). ¹H NMR: δ 8.16 (2H, d, $J = 8.95$ Hz), 7.50 (2H, d, $J = 8.95$ Hz), 5.6 (1H, b), 3.66 (3H, s), 3.25 (2H, q, $J = 6.79$ Hz), 2.29 (2H, t, $J = 7.39$ Hz), 2.22 (2H, s), 2.02 (2H, s), 1.87 (4H, s), 1.82 (2H, s), 1.71 (1H, s), 1.68 (4H, s), 1.35–1.72 (5H, m). ¹³C NMR: δ 174.03, 170.52, 158.09, 126.00, 123.42, 51.55, 51.18, 47.58, 42.13, 42.03, 41.58, 39.83, 37.83, 35.66, 33.86, 33.67, 29.38, 29.03, 26.43, 24.44. MS: m/z at 443 ($M + \text{H}$)⁺, 460 ($M + \text{NH}_4$)⁺.

(b) 3-(4-Nitrophenyl)-*N*-(5-carbomethoxypentyl)-1-adamantaneacetamide was dissolved in refluxing methanol (200 mL) containing sodium hydroxide (3.18 g, 79.6 mmol) and water (20 mL). Reflux was continued for 30 min, whereupon the reaction mixture was cooled to room temperature and the volatiles were removed *in vacuo*. The residue was diluted with water (20 mL) and acidified to pH 3 with 20% sulfuric acid. The aqueous solution was extracted with ethyl acetate (2 × 100 mL). The organic solution was dried over sodium sulfate, filtered, and evaporated under reduced pressure to give the crude product. Recrystallization from benzene afforded 3-(4-nitrophenyl)-*N*-(5-carboxypentyl)-1-adamantaneacetamide (**1c**) (8.3 g, 19.4 mmol, 97%). ¹H NMR: δ 8.16 (2H, d, $J = 8.95$ Hz), 7.50 (2H, d, $J = 8.95$ Hz), 5.7 (1H, b), 3.25 (2H, q, $J = 6.79$ Hz), 2.35 (2H, t, $J = 7.39$ Hz), 2.22 (2H, s), 2.02 (2H, s), 1.87 (4H, s), 1.82 (2H, s), 1.71 (1H, s), 1.68 (4H, s), 1.35–1.72 (5H, m). ¹³C NMR: δ 178.37, 171.00, 158.121, 145.98, 126.03, 123.43, 51.121, 47.54, 42.02, 41.58, 39.27, 37.85, 35.65, 33.88, 33.72, 29.31, 29.04, 26.97, 26.35, 24.23. MS: m/z at 429 ($M + \text{H}$)⁺, 446 ($M + \text{NH}_4$)⁺.

Preparation of Methyl 8-[*N*-(Benzyloxycarbonyl)amino]-3-oxooctanoate (2a). Into a reaction flask equipped with a magnetic stir bar and latex septum were added 6-[*N*-(benzyloxycarbonyl)amino]hexanoic acid (**1a**) (50 g, 0.188 mol), anhydrous methylene chloride (1 L), Meldrum's acid (27.15 g, 0.188 mol), and triethylamine (68.6 mL, 0.5 mol). While the mixture was stirred under nitrogen, diethyl cyanophosphonate (**9**) (30.8 mL, 0.188 mol) was added. After it was stirred for 18 h, the reaction mixture was transferred to a separatory funnel and washed with 3 N HCl (250 mL), distilled water (3 × 100 mL), and saturated sodium chloride solution (100 mL). The organic phase was dried over sodium sulfate, filtered,

and evaporated *in vacuo* to give the crude 6-[*N*-(benzyloxycarbonyl)amino]hexanoic acid–Meldrum's acid adduct (92.7 g). The adduct was transferred to a reaction flask equipped with a magnetic stir bar, reflux condenser, and gas inlet adapter and dissolved in anhydrous methanol (1 L). The reaction mixture was heated to reflux under nitrogen for 4 h. After it was cooled to room temperature, the solution was evaporated *in vacuo* to give crude methyl 8-[*N*-(benzyloxycarbonyl)amino]-3-oxooctanoate (91.3 g). The material was further purified by vacuum flash chromatography (10) on silica gel (1.4 kg), eluting with 20% ethyl acetate in cyclohexane at 250 mL/min to give methyl 8-[*N*-(benzyloxycarbonyl)amino]-3-oxooctanoate (**2a**) (52.5 g, 87%). ¹H NMR: δ 7.32 (5H, s), 5.06 (2H, s), 4.95 (1H, bs), 3.70 (3H, s), 3.41 (2H, s), 3.15 (2H, q, $J = 6.45$ Hz), 2.50 (2H, t, $J = 7.19$ Hz), 1.21–1.6 (6H, m). ¹³C NMR: δ 167.95, 156.52, 136.52, 128.51, 128.08, 66.56, 52.35, 48.99, 42.79, 40.81, 29.733, 26.03, 22.95. MS: m/z at 322 ($M + \text{H}$)⁺, 339 ($M + \text{NH}_4$)⁺.

Preparation of 2-[[4-Oxo-5-carbomethoxypentyl]oxy]carbazole (2b). 2-[(3-Carboxypropyl)oxy]carbazole **1b** (50 g, 186 mmol) was suspended in dry methylene chloride (800 mL) along with Meldrum's acid (28 g, 195 mmol), diethyl cyanophosphonate (30 mL, 195 mmol), and triethylamine (52 mL, 371 mmol). The reaction mixture was stirred at ambient temperature, under nitrogen for 25.5 h. The solvent was removed *in vacuo* and the residue taken up in ethyl acetate (1300 mL). To this solution was added aqueous phosphoric acid (0.5 M, 500 mL), followed by vigorous mixing for 30 min. The precipitated product was isolated by filtration through a medium porosity, sintered glass Buchner funnel, washed with water (200 mL), and then dried *in vacuo*. Additional material was isolated by separating the organic layer and washing with 5% aqueous sodium bicarbonate (500 mL). The combined aqueous washes were extracted with ethyl acetate (2 × 200 mL). Finally, the combined organic extracts were washed with saturated sodium chloride (300 mL), then dried over anhydrous sodium sulfate, filtered, and evaporated *in vacuo*. The resulting solid was combined with the initial precipitate in a round-bottom flask, to which was added a 1:1 mixture of dry tetrahydrofuran (THF)/methanol (1600 mL). Trifluoroacetic acid (60 mL) was added and the reaction mixture heated at reflux for 1 h. After the solvent was removed *in vacuo*, the residue was dissolved in ethyl acetate (4 L) and the solution was washed with 5% aqueous sodium bicarbonate (3 × 1500 mL) and then saturated aqueous sodium chloride (1500 mL). The solution was dried over anhydrous sodium sulfate and filtered and the solvent removed *in vacuo* to give **2b** (55.8 g, 92%) as a solid. ¹H NMR: δ 7.95 (2H, t, $J = 7.8$ Hz), 7.40 (1H, d, $J = 7.9$ Hz), 7.26 (1H, t, $J = 7.3$ Hz), 7.09 (1H, t, $J = 7.7$ Hz), 6.93 (1H, s), 6.74 (1H, d, $J = 8.3$ Hz), 4.02 (2H, t, $J = 6.1$ Hz), 3.66 (1H, s), 3.53 (3H, s), 3.31 (2H, s), 2.75 (2H, t, $J = 7.0$ Hz), 2.50–1.95 (2H, m). MS: m/z at 326 ($M + \text{H}$)⁺, 343 ($M + \text{NH}_4$)⁺.

N-(6-Oxo-7-carbomethoxyheptyl)-3-(4-nitrophenyl)-1-adamantaneacetamide (**2c**) was prepared following the procedure for compound **2a**. Thus, from 3-(4-nitrophenyl)-*N*-(5-carboxypentyl)-1-adamantaneacetamide (10.48 g, 24.5 mmol) was obtained the title compound (10.67 g, 22.0 mmol, 90%). ¹H NMR: δ 8.16 (2H, d, $J = 8.95$ Hz), 7.50 (2H, d, $J = 8.95$ Hz), 5.7 (1H, b), 3.72 (3H, s), 3.45 (2H, s), 3.25 (2H, q, $J = 6.79$ Hz), 2.55 (2H, t, $J = 7.39$ Hz), 2.22 (2H, s), 2.02 (2H, s), 1.87 (4H, s), 1.82 (2H, s), 1.71 (1H, s), 1.68 (4H, s), 1.35–1.72 (5H, m). MS: m/z at 485 ($M + \text{H}$)⁺, 502 ($M + \text{NH}_4$)⁺.

Preparation of 8-[*N*-(Benzyloxycarbonyl)amino]-1,3-octanediol (3a). Methyl 8-[*N*-(benzyloxycarbonyl)-

amino]-3-oxooctanoate (**2a**) (51.5 g, 0.16 mol) was dissolved in anhydrous tetrahydrofuran (350 mL) in a reaction flask equipped with a magnetic stir bar, reflux condenser, pressure-equalizing addition funnel, and nitrogen gas inlet. While the mixture was stirred under nitrogen, sodium borohydride (15.1 g, 0.4 mol) was cautiously added. The mixture was then heated to reflux, and methanol was added dropwise over 90 min. Stirring was continued at reflux temperature for 60 min after the addition was complete. After the solution was allowed to cool to ambient temperature, distilled water (78 mL) was added and 3 N HCl was added to pH 3. The mixture was evaporated *in vacuo* to remove the organic solvents, and solid potassium carbonate was added to adjust the pH back to 12. The mixture was then extracted with ethyl acetate (5 × 100 mL). The extract was washed with saturated sodium chloride (2 × 50 mL), dried over sodium sulfate, filtered, and evaporated *in vacuo*. The residue was dissolved in methanol (200 mL) and evaporated again. Evaporation from methanol was repeated a total of five times. The residue was crystallized from ether (700 mL), giving 8-[*N*-(benzyloxycarbonyl)amino]-1,3-octanediol (23 g). A second crop was collected upon addition of cyclohexane (300 mL) to the mother liquor (6.8 g) to increase the total yield (29.8 g, 0.1 mol, 63%). ¹H NMR: δ 7.34 (5H, s), 5.08 (2H, s), 4.97 (1H, bs), 3.76–3.88 (3H, m), 3.12–3.18 (2H, m), 2.8–3.2 (2H, bs), 1.33–1.67 (10H, m). ¹³C NMR: δ 156.52, 136.63, 128.55, 128.12, 66.66, 40.87, 38.33, 37.58, 29.93, 26.539, 25.03. MS: *m/z* at 296 (M + H)⁺, 313 (M + NH₄)⁺. Anal. Calcd for C₁₆H₂₅NO₄: C, 65.1%; H, 8.5%; N, 4.7%. Found: C, 65.22%; H, 8.74%; N, 4.71%.

Preparation of 2-[(4,6-Dihydroxyhexyl)oxy]carbazole (3b). 2-[(4-Oxo-5-carbomethoxypentyl)oxy]carbazole (**2b**) (35 g, 108 mmol) was dissolved in dry tetrahydrofuran (350 mL), and then lithium borohydride (2 M in THF, 175 mL, 355 mmol) was slowly added under nitrogen. The reaction mixture was stirred at ambient temperature for 23 h and then neutralized with 10% aqueous citric acid (500 mL). This neutralization must be done very slowly to avoid a dangerously fast release of gas. The reaction mixture was extracted with ethyl acetate (3 × 300 mL), and then the combined organic layers were washed with 5% sodium bicarbonate (200 mL) and saturated sodium chloride (200 mL). The solution was dried over anhydrous sodium sulfate and filtered and the solvent removed *in vacuo*. The product was dissolved in pyridine (300 mL) and methylene chloride (400 mL). To this solution was added silica gel (200 g, 230–400 mesh, flash chromatography grade), and then the solvent was removed *in vacuo*. This silica was added to the top of a column of silica gel (8 × 17 cm bed of flash silica gel) which had been slurry packed using ethyl acetate/hexanes/triethylamine (5:4:1). Elution was with ethyl acetate/hexanes/triethylamine (5:4:1) (4 L) and then ethyl acetate/hexanes/triethylamine (6:3:1) (2 L). The solvent system was changed to methanol/methylene chloride/triethylamine (5:85:10) for the remainder of the separation. The elution was followed by thin layer chromatography (TLC) [silica gel plates, methanol/methylene chloride/triethylamine (10:80:10)]. The appropriate fractions were combined, and the solvent was removed *in vacuo*. Residual triethylamine was removed by coevaporation with toluene (3 × 500 mL) and residual toluene removed by coevaporation with methylene chloride (3 × 500 mL) to give the diol **3b** (23.3 g, 72%). ¹H NMR (DMSO-*d*₆): δ 7.92–7.88 (2H, m), 7.39 (1H, d, *J* = 7.8 Hz), 7.26 (1H, t, *J* = 7.7 Hz), 7.08 (1H, t, *J* = 7.4 Hz), 6.93 (1H, s), 6.74 (1H, d, *J* = 8.5 Hz), 4.41 (1H, s), 4.33 (1H, s), 4.04 (2H, t, *J* = 5.9 Hz), 3.65–3.51 (3H, m), 3.34

(1H, s), 1.95–1.63 (2H, m), 1.63–1.45 (4H, m). MS: *m/z* at 300 (M + H)⁺, 317 (M + NH₄)⁺.

N-(6,8-Dihydroxyoctyl)-3-(4-nitrophenyl)-1-adamantaneacetamide (**3c**) was prepared following the procedure for compound **3a**. Thus, from **2c** (6.7 g, 13.8 mmol) was obtained **3c** (5.1 g, 11.1 mmol) as a crisp foam after purification by chromatography on silica gel. ¹H NMR: δ 8.14 (2H, d, *J* = 8.81 Hz), 7.50 (2H, d, *J* = 8.82), 5.5 (1H, bs), 3.7–4.1 (3H, m), 3.16–3.4 (2H, m), 2.21 (2H, bs), 2.00 (2H, s), 1.85 (4H, s), 1.80 (2H, s), 1.69 (1H, s), 1.66 (4H, s), 1.26–1.49 (10H, m). MS: *m/z* at 459 (M + H)⁺.

Preparation of 8-[*N*-(Benzyloxycarbonyl)amino]-3-hydroxy-1-[(4,4'-dimethoxytrityl)oxy]octane (4a). 8-[*N*-(Benzyloxycarbonyl)amino]-1,3-octanediol (**3a**) (25 g, 85 mmol) was dissolved in anhydrous pyridine (200 mL), evaporated *in vacuo*, and redissolved in anhydrous pyridine (200 mL). Diisopropylethylamine (38.1 mL, 88 mmol) and 4-(dimethylamino)pyridine (170 mg) were added. The mixture was stirred under nitrogen and cooled in an ice bath while 4,4'-dimethoxytrityl chloride (30 g, 88 mmol) in tetrahydrofuran (200 mL) was added dropwise over 1.5 h. Stirring was continued for 15 min after the addition was complete. The mixture was evaporated *in vacuo* and purified by vacuum flash chromatography on silica gel (1.6 kg), eluting with 20:80:1 ethyl acetate/cyclohexane/triethylamine (8.5 L), then 50:50:1 ethyl acetate/cyclohexane/triethylamine (4 L), and finally 100:1 ethyl acetate/triethylamine (2.5 L) at 100–200 mL/min. Evaporation of the appropriate fractions gave 8-[*N*-(benzyloxycarbonyl)amino]-3-hydroxy-1-[(4,4'-dimethoxytrityl)oxy]octane (**4a**) (44 g, 0.073 mol, 73%) as a crisp foam. ¹H NMR: δ 7.21–7.44 (14H, m), 6.84 (4H, d, *J* = 8.9 Hz), 5.099 (2H, s), 4.85 (1H, bs), 3.78 (6H, s), 3.1–3.45 (6H, m), 1.33–1.72 (10H, m). MS: *m/z* at 636 (M + K)⁺.

Preparation of 2-[[4-Hydroxy-6-[(4,4'-dimethoxytrityl)oxy]hexyl]oxy]carbazole (4b). 2-[(4,6-Dihydroxyhexyl)oxy]carbazole (**3b**) (19 g, 63 mmol) was dissolved in dry pyridine (100 mL) and dry tetrahydrofuran (100 mL). To this solution were added diisopropylethylamine (14.4 mL, 82 mmol) and 4-(dimethylamino)pyridine (0.8 g, 6.3 mmol). The solution, under nitrogen, was cooled in an ice bath. A solution of 4,4'-dimethoxytrityl chloride (23.7 g, 70 mmol) in THF (100 mL) was added dropwise over 1 h, and then the ice bath was removed and stirring continued for 18 h at ambient temperature. The solvent was removed *in vacuo* and the residue taken up in methylene chloride (400 mL) and triethylamine (40 mL) and then added to silica gel (200 g, 230–400 mesh, in 300 mL of 1:9 triethylamine/dichloromethane). The solvent was removed *in vacuo* and this silica gel added to the top of a column of silica [8 × 30 cm silica gel bed, slurry packed with triethylamine/hexanes (1:9)]. Elution was with triethylamine/hexanes (1:9, 1 L), then with triethylamine/hexanes/ethyl acetate (1:5:4, 1 L), and finally with triethylamine/hexanes/ethyl acetate (1:4:5). The elution was monitored by TLC [silica gel plates, triethylamine/hexanes/ethyl acetate (1:4:5)], and the appropriate fractions were combined. The solvent was removed *in vacuo* and residual triethylamine coevaporated with toluene (3 × 1 L). Residual toluene was removed by coevaporation with methylene chloride (4 × 1 L). The glass was made into a foam *in vacuo*, giving **4b** (34.9 g, 91.4%). ¹H NMR: δ 7.98–7.90 (3H, m), 7.44–7.18 (12H, m), 6.88–6.82 (5H, m), 4.10–4.05 (2H, m), 3.95–3.85 (1H, m), 3.70 (6H, s), 3.43–3.39 (1H, m), 3.30–3.25 (1H, m), 2.05–1.52 (8H, m). MS (FAB): *m/z* at 601 (M⁺).

N-[6-Hydroxy-8-[(4,4'-dimethoxytrityl)oxy]octyl]-3-(4-

nitrophenyl)-1-adamantaneacetamide (**4c**) was prepared following the procedure for compound **4a**. Thus, from compound **3c** (4.1 g, 8.95 mmol) was obtained **4c** (4.9 g, 6.5 mmol, 73%). ¹H NMR: δ 8.14 (2H, d, J = 8.81 Hz), 7.49 (2H, d, J = 8.82), 7.41 (2H, d, J = 7.39 Hz), 7.31 (4H, d, J = 8.75), 7.26 (2H, d, J = 2.3 Hz), 7.21 (1H, d, J = 7.19), 6.83 (4H, d, J = 8.68 Hz), 5.5 (1H, bs), 3.78 (6H, s), 3.16–3.4 (6H, m), 2.21 (2H, bs), 2.00 (1H, s), 1.85 (4H, s), 1.80 (1H, s), 1.69 (2H, s), 1.66 (4H, s), 1.26–1.49 (10H, m). ¹³C NMR: δ 170.81, 158.84, 158.40, 145.09, 136.38, 130.31, 128.364, 128.27, 127.18, 126.34, 123.75, 113.54, 87.15, 71.81, 63.04, 55.58, 51.54, 47.91, 42.36, 41.88, 39.69, 38.16, 37.57, 37.05, 35.99, 33.99, 30.03, 29.35, 27.31, 25.58. MS: m/z at 799 (M + K)⁺. HPLC [column, μ Bondapak C18, 3.9 $\times$ 300 mm; eluent, 75:25 acetonitrile/0.1 mM triethylammonium acetate buffer (pH 8); 2 mL/min, 254 nm]: retention time, 4.36 min; 96% purity.

Alternatively, compound **4c** was prepared as shown in Scheme 2. (a) 8-[*N*-(Benzyloxycarbonyl)amino]-3-hydroxy-1-[(4,4'-dimethoxytrityl)oxy]octane (**4a**) (28.4 g, 47.6 mmol) was dissolved in methanol (200 mL) and hydrogenated over 10% palladium on carbon (2.84 g) at 45 psi hydrogen for 2.5 h. After filtration and evaporation *in vacuo* there remained 8-amino-3-hydroxy-1-[(4,4'-dimethoxytrityl)oxy]octane (**6**) (21.5 g, 46.9 mmol, 98%). ¹H NMR: δ 7.26–7.42 (9H, m), 6.84 (4H, d, J = 8.9 Hz), 3.78 (6H, s), 3.2–3.41 (3H, m), 2.68 (2H, t, J = 3.9 Hz), 2.43 (3H, bs), 1.33–1.72 (10H, m). MS: m/z at 502 (M + K)⁺.

(b) 3-(4-Nitrophenyl)-1-adamantaneacetic acid (**7**) (10 g, 31.7 mmol), 8-amino-3-hydroxy-1-[(4,4'-dimethoxytrityl)oxy]octane (**6**) (21.5 g, 46.9 mmol), *N*-hydroxybenzotriazole (6.48 g, 48 mmol), and triethylamine (13 mL, 96 mmol) were dissolved in anhydrous methylene chloride (250 mL). 1-Ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride (9.2 g, 48 mmol) was added, and the mixture was stirred under nitrogen for 4 h. The mixture was purified by vacuum flash chromatography on silica gel (1 kg), eluting with 50:50:1 ethyl acetate/cyclohexane/triethylamine (6 L) and then 80:20:1 ethyl acetate/cyclohexane/triethylamine (6 L) to give *N*-[5-hydroxy-8-[(4,4'-dimethoxytrityl)oxy]octyl]-3-(4-nitrophenyl)-1-adamantaneacetamide (**4c**) (19.4 g, 25.5 mmol, 80.5%).

Preparation of *N*-[6-Hydroxy-8-[(4,4'-dimethoxytrityl)oxy]octyl]dansylsulfonamide (4d**).** Compound **6** (7.8 g, 16.8 mmol) was dissolved in THF/water (4:3, 175 mL) containing sodium carbonate (7.2 g, 68 mmol). Dansyl chloride (4.5 g, 16.8 mmol) was added as a solid with vigorous stirring followed by THF (100 mL). After 30 min, aqueous sodium chloride (25% w/v, 200 mL) was added and the mixture was extracted with ethyl acetate (200 mL). The organic layer was washed with aqueous sodium chloride (25% w/v, 100 mL) and then dried over anhydrous sodium sulfate. After filtration and removal of the solvent *in vacuo*, the residue was taken up in triethylamine/dichloromethane (1:5, 60 mL). This solution was applied to a flash chromatography column [9 cm diameter $\times$ 22.5 cm length silica gel bed, slurry packed with ethyl acetate/hexanes/triethylamine (30:68:2)]. The column was eluted with ethyl acetate/hexanes/triethylamine (30:68:2, 1 L; 40:58:4, 2 L; and finally 50:48:2). Appropriate fractions were combined, and the solvent was removed *in vacuo*. Residual triethylamine was removed by coevaporation with toluene (6 $\times$ 400 mL) and then residual toluene coevaporated with dichloromethane (6 $\times$ 400 mL), giving a greenish-yellow, glassy foam product (9.0 g, 77% yield). ¹H NMR (acetonitrile-*d*₃): δ 8.68 (1H, d, J = 8.5 Hz), 8.44 (1H, d, J = 8.7 Hz), 8.33 (1H, q, J = 1.3 Hz), 7.73 (2H, q, J = 7.7 Hz), 7.59 (2H, d, J = 1.5 Hz), 7.56–7.37 (8H, m), 7.04–7.00 (4H, m), 5.90 (1H, m), 3.92 (6H, s), 3.70–3.60 (1H, m), 3.29–

3.21 (2H, m), 3.03–2.94 (8H, m), 2.30 (1H, s), 2.12–2.09 (6H, m), 1.74–1.65 (2H, m), 1.45–1.35 (2H, m), 1.30–1.15 (6H, m). MS: m/z at 696 (M⁺), 735 (M + K)⁺. Anal. Calcd for C₄₁H₄₈N₂O₆S: C, 70.7%; H, 6.9%; N, 4.0%; S, 4.6%. Found: C, 70.88%; H, 7.01%; N, 3.96%; S, 4.58%.

Preparation of 3-(4-Nitrophenyl)-1-adamantaneacetic Acid (7**).** (a) Methyl 3-bromoadamantaneacetate (80 g, 0.28 mol) was dissolved in benzene (1.6 L) in a 3 L, three-neck round-bottom flask equipped with a magnetic stirrer, thermometer, and nitrogen inlet. Aluminum chloride (39.6 g, 0.3 mol) was added as a solid in six equal portions over 2 h to the stirred solution. The reaction was then immediately quenched with phosphoric acid (0.5 M, 1.6 L), the layers were separated, and the organic layer was dried over sodium sulfate. After filtration, the solution was evaporated *in vacuo* to give methyl 3-phenyl-1-adamantaneacetate (80 g, 100%) as an oil. ¹H NMR: δ 7.17–7.34 (5H, m, Ph), 3.64 (3H, s, OCH₃), 2.17 (4H, s), 1.86 (4H, s), 1.75 (2H, s), 1.69 (2H, s), 1.65 (4H, s). ¹³C NMR: δ 172.1 (C=O), 150.4 (C¹ phenyl), 128.22 (C³, C⁵ phenyl), 125.76 (C⁴ phenyl), 124.93 (C², C⁶ phenyl), 51.20 (OCH₃), 48.55, 48.05, 42.33, 41.56, 37.06, 35.91, 33.75, 29.27. MS: m/z at 302 (M + NH₄)⁺. GC (column, 1.5% OV-17 + 1.9% OV-202 on Chrom WHP, 6 $\times$ 4 mm inside diameter; N₂ gas flow, 35 mL/min; injection temperature, 275 °C; FID temperature, 275 °C; temperature gradient, 150 °C at 10 min and then increase at 20 °C/min to 250 °C): retention time, 18.52 min; 96% purity.

(b) Methyl 3-phenyl-1-adamantaneacetate (78.8 g, 0.277 mol) was dissolved in anhydrous acetonitrile (800 mL) in a 3 L, three-neck round-bottom flask equipped with an overhead stirrer, thermometer, and latex septum. The solution was cooled in an ice bath while being stirred under a nitrogen atmosphere. Nitronium tetrafluoroborate (40.88 g, 0.26 mol, 94 mol %) was dissolved in anhydrous acetonitrile (800 mL) in a 1 L flask under nitrogen and added *via* canula to the solution of methyl 3-phenyl-1-adamantaneacetate over 45 min so as to maintain the temperature below 10 °C. After the mixture was stirred for another hour, the reaction was quenched by the addition of crushed ice (985 g). While the mixture was being vigorously stirred, the product precipitated and was collected by filtration. The precipitate was washed with water (200 mL) and then dried *in vacuo* for 14 h to give methyl 3-(4-nitrophenyl)-1-adamantaneacetate (55.9 g, 0.17 mol, 65%). ¹H NMR: δ 8.15 (2H, d, J = 8.95 Hz), 7.50 (2H, d, J = 8.95 Hz), 3.65 (3H, s), 2.2 (4H, m), 1.86 (4H, s), 1.77 (2H, s), 1.71 (2H, s), 1.66 (4H, s). ¹³C NMR: δ 171.91, 157.95, 125.97, 123.43, 51.27, 48.22, 47.36, 37.81, 35.56, 28.98. MS: m/z at 347 (M + NH₄)⁺, 364 (M + NH₃ + NH₄)⁺. HPLC (column, μ Bondapak C18, 3.9 $\times$ 300 mm; eluent, 80:20 methanol/water; 2 mL/min; 254 nm): retention time, 5.74 min; 96% purity.

(c) Methyl 3-(4-nitrophenyl)-1-adamantaneacetate (55.9 g, 0.17 mol) was dissolved in methanol (790 mL) and water (7.9 mL). Sodium hydroxide (25.3 g, 0.633 mol) was added and the solution heated at reflux for 3 h. The solution was allowed to cool to ambient temperature and then evaporated *in vacuo* to a pasty solid. The solid was suspended in water (790 mL), and the pH was adjusted to <3 with 20% aqueous sulfuric acid. The precipitated product was collected by filtration and air dried to give 3-(4-nitrophenyl)-1-adamantaneacetate (**7**) (51.6 g, 0.163 mol, 98%). ¹H NMR: δ 8.16 (2H, d, J = 8.95 Hz), 7.50 (2H, d, J = 8.95 Hz), 2.22 (4H, s), 1.88 (4H, s), 1.82 (4H, s), 1.72 (2H, s), 1.71 (6H, s). ¹³C NMR: δ 177.82, 157.82, 125.94, 123.49, 48.19, 47.28, 41.97, 41.20, 37.82, 35.54,

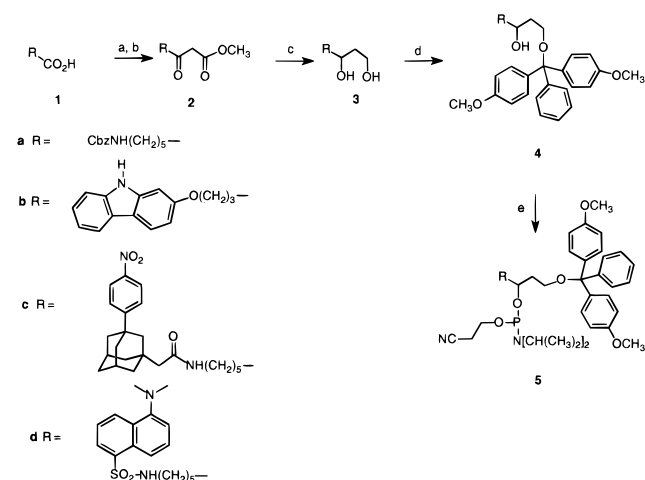
33.55, 28.96. MS: m/z at 333 ($M + \text{NH}_4$)⁺, 350 ($M + \text{NH}_3 + \text{NH}_4$)⁺.

Preparation of 2-[[4-O-[O-(2-Cyanoethyl)-N,N-diisopropylphosphatidyl]oxy]-6-[[4,4'-dimethoxytrityl]oxy]hexyl]oxy]carbazole (5b). 2-[[4-Hydroxy-6-[[4,4'-dimethoxytrityl]oxy]hexyl]oxy]carbazole (**4b**) (34 g, 56 mmol) was dissolved in dry methylene chloride (400 mL, distilled from calcium hydride) under a dry, inert atmosphere. To this solution were added diisopropylethylamine (39 mL, 226 mmol) and then 2-cyanoethyl-N,N-diisopropylchlorophosphoramidite (19 mL, 85 mmol). The reaction mixture was protected from light and kept at ambient temperature for 1 h. Dry methanol (3 mL) was added, and after 10 min, triethylamine (200 mL) and ethyl acetate (1 L) were also added. The solution was washed with 10% sodium carbonate (3 × 500 mL) and saturated sodium chloride (2 × 500 mL) and then dried over anhydrous sodium sulfate. The solution was filtered and the solvent removed *in vacuo*. The residue was dissolved in methylene chloride (200 mL) and triethylamine (50 mL), and then silica gel (200 g, 230–400 mesh) was added slowly. Enough solvent was removed *in vacuo* to allow the removal of the silica gel from the round-bottom flask. This silica was added to the top of a column of silica gel (12 × 23 cm bed, 230–400 mesh flash chromatography silica) which had been slurry packed with triethylamine/hexanes (1:9). Elution was with triethylamine/hexanes (1:9, 1.5 L), triethylamine/ethyl acetate/hexanes (1:2.5:6.5, 3 L), and then triethylamine/ethyl acetate/hexanes (1:3:6). The elution was monitored by TLC [silica gel plates, triethylamine/ethyl acetate/hexanes (1:5:4)], and the appropriate fractions were combined. The solvent was removed *in vacuo* and residual triethylamine coevaporated with toluene (3 × 500 mL). The residual toluene was coevaporated with methylene chloride (3 × 500 mL) and the product made into a glassy foam *in vacuo* to give phosphoramidite **5b** (30.5 g, 67%). ¹H NMR (acetonitrile-*d*₃): 9.18 (1H, bs), 7.99–7.89 (2H, m), 7.47–7.42 (3H, m), 7.36–7.12 (10H, m), 6.99–6.93 (1H, m), 6.87–6.72 (5H, m), 4.11–4.02 (3H, m), 3.77–3.71 (6H, m), 3.69–3.48 (4H, m), 3.21–3.1 (2H, m), 2.54–2.60 (1H, m), 2.51–2.45 (1H, m), 1.99–1.76 (6H, m), 1.74–1.65 (2H, m), 1.16–1.02 (12H, m). ¹³C NMR (acetonitrile-*d*₃): δ 96.3, 86.8, 72.8, 69.0, 61.3, 59.1, 58.9, 55.8, 43.9, 43.7, 37.2, 33.3, 25.7, 24.9, 24.8, 2.1, 1.9, 1.6, 1.3, 1.0, 0.8, 0.5. ³¹P NMR (acetonitrile-*d*₃): δ 148.262 (s). MS: m/z at 840 ($M + \text{K}$)⁺. HPLC [column, Dynamax 300A C18; eluent, 90:10 acetonitrile/0.1 mM triethylammonium acetate buffer (pH 8); 1.5 mL/min; 254 nm]: retention time, 5.20, 5.59 min; 99.5% purity. Anal. Calcd for C₄₈H₅₆N₃O₆P: C, 71.9%; H, 7.0%; N, 5.2%; P, 3.9%. Found: C, 72.17%; H, 7.03%; N, 5.32%; P, 3.63%.

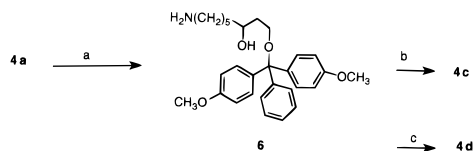
Preparation of N-[[6-O-[O-(2-Cyanoethyl)-N,N-diisopropylphosphatidyl]oxy]-8-[[4,4'-dimethoxytrityl]oxy]octyl]-3-(4-nitrophenyl)-1-adamantaneacetamide (5c). N-[6-Hydroxy-8-[[4,4'-dimethoxytrityl]oxy]octyl]-3-(4-nitrophenyl)-1-adamantaneacetamide (**4c**) (19.4 g, 25.5 mmol) and diisopropylethylamine (21.3 mL) were dissolved in anhydrous methylene chloride (300 mL). (2-Cyanoethyl)-N,N-diisopropylchlorophosphoramidite (8.5 mL, 38.1 mmol) was added and the mixture stirred under nitrogen for 45 min. Methanol (1.8 mL) was added, and stirring was continued for 20 min to quench the reaction. The reaction mixture was transferred to a separatory funnel and diluted with 15% triethylamine in ethyl acetate (1 L). The solution was washed with 10% aqueous sodium carbonate (425 mL × 2) and saturated sodium chloride (425 mL), dried over sodium sulfate, filtered, and evaporated *in vacuo*. The residue was dissolved in 10% triethylamine in methylene

chloride (600 mL) and chromatographed on silica gel (900 g), eluting with 10% triethylamine in hexane (600 mL), followed by 7:2:1 hexane/ethyl acetate/triethylamine (3 L), and finally by 5:4:1 hexane/ethyl acetate/triethylamine (12 L). The appropriate fractions were combined and evaporated *in vacuo*. The residue was thrice dissolved in toluene (600 mL) and evaporated *in vacuo* and, finally, thrice dissolved in methylene chloride (600 mL) and evaporated *in vacuo* to give N-[[6-O-[O-(2-cyanoethyl)-N,N-diisopropylphosphatidyl]oxy]-8-[[4,4'-dimethoxytrityl]oxy]octyl]-3-(4-nitrophenyl)-1-adamantaneacetamide (**5c**) (18.2 g, 19 mmol, 74%) as a crisp foam. ¹H NMR (acetonitrile-*d*₃): δ 8.31 (2H, d, *J* = 8.88 Hz), 7.71 (2H, d, *J* = 8.75), 7.56–7.76 (2H, m), 7.35–7.47 (6H, m), 6.98–7.02 (5H, m), 6.5 (1H, b), 4.05 (1H, b), 3.91 (6H, s), 3.67–3.9 (2H, m), 3.24–3.29 (2H, m), 2.74 (1H, t, *J* = 4.66 Hz), 2.64 (1H, t, *J* = 4.66 Hz), 2.31 (2H, bs), 1.7–2.19 (11H, m), 1.26–1.65 (10H, m), 1.15–1.28 (12H, m). ¹³C NMR (acetonitrile-*d*₃): δ 170.819, 159.008, 146.095, 136.687, 130.409, 130.377, 128.492, 128.418, 128.231, 127.137, 126.611, 123.748, 117.784, 113.435, 86.158, 73.510, 61.048, 55.366, 50.822, 47.918, 43.950, 42.167, 41.716, 39.120, 38.172, 37.575, 37.058, 35.831, 33.794, 29.598, 27.191, 24.464, 24.376, 24.343, 24.308. ³¹P NMR: δ 146 (H₃PO₄ reference). MS: m/z at 999 ($M + \text{K}$)⁺. HPLC [column, μBondapak C18, 3.9 × 300 mm; eluent, 90:10 acetonitrile/0.1 mM triethylammonium acetate buffer (pH 8); 2 mL/min; 254 nm]: retention time, 3.84, 4.02 min; 92% purity. Anal. Calcd for C₅₆H₇₃N₄O₈P: C, 70.0%; H, 7.7%; N, 5.8%; P, 3.2%. Found: C, 69.99%; H, 7.70%; N, 5.81%; P, 3.13%.

Preparation of N-[[6-O-[O-(2-cyanoethyl)-N,N-diisopropylphosphatidyl]oxy]-8-[[4,4'-dimethoxytrityl]oxy]octyl]dansylsulfonamide (5d). Compound **4d** (9.0 g, 12.9 mmol) was dissolved in dry pyridine (100 mL) and then evaporated *in vacuo*. The residue was dissolved in dry dichloromethane (100 mL) and then treated with dry diisopropylethylamine (9.0 mL, 51.7 mmol) and (2-cyanoethyl)-N,N-diisopropylchlorophosphoramidite (4.0 mL, 18 mmol). After 1 h at ambient temperature, dry methanol (0.523 mL, 12.9 mmol) was added. After 30 min, triethylamine (60 mL) and ethyl acetate (200 mL) were added and the solution was washed with 10% sodium carbonate (2 × 200 mL) and 25% aqueous sodium chloride (2 × 200 mL). The organic layer was dried over anhydrous sodium sulfate, filtered, and evaporated *in vacuo*. The residue was dissolved in dichloromethane (200 mL) and triethylamine (10 mL) and added to silica gel (100 g, 230–400 mesh). The solvent was removed *in vacuo* to give a free-flowing powder. This silica was added to the top of a slurry-packed flash column [9 × 31 cm silica gel bed, packed with ethyl acetate/hexanes triethylamine (20:78:2)], and elution was with ethyl acetate/hexanes/triethylamine (30:68:2, 2 L; then 35:63:2). Appropriate fractions were combined, and the solvent was removed *in vacuo*. Residual triethylamine was removed by coevaporation with toluene (6 × 300 mL), and then residual toluene was removed by coevaporation with dichloromethane (6 × 300 mL) to give **5d** as a glassy foam (7.1 g, 61% yield). ¹H NMR (acetonitrile-*d*₃): δ 8.69 (1H, d, *J* = 8.6 Hz), 8.44 (1H, d, *J* = 8.7 Hz), 8.33 (1H, d, *J* = 8.6 Hz), 7.71 (2H, q, *J* = 7.7 Hz), 7.60–7.55 (2H, m), 7.49–7.32 (8H, m), 7.05–6.98 (4H, m), 5.93–5.88 (0.5H, m), 4.05–3.50 (6H, m), 3.30–3.19 (2H, m), 3.06–2.90 (4H, m), 2.70 (1H, t, *J* = 6.0 Hz), 2.62 (1H, t, *J* = 6.0 Hz), 2.15–2.09 (2H, m), 2.0–1.8 (2H, m), 1.5–1.1 (12H, m). ³¹P NMR (acetonitrile-*d*₃): δ 150.25. MS: m/z at 935 ($M + \text{K}$)⁺. HPLC [column, μBondapak C18, 3.9 × 300 mm; eluent, 85:15 acetonitrile/100 mM triethylammonium acetate (pH 8); 1 mL/min;

Scheme 1^a

^a (a) Meldrum's acid, diethyl cyanophosphonate, triethylamine; (b) methanol, reflux; (c) NaBH₄, THF, methanol, reflux; (d) 4,4'-dimethoxytrityl chloride, pyridine, DMAP, diisopropylethylamine; (e) (2-cyanoethyl)-N,N-diisopropylchlorophosphoramidite, diisopropylethylamine.

Scheme 2^a

^a (a) H₂, Pd/C, MeOH; (b) HOBt, EDAC, 3-(4-nitrophenyl)-adamantaneacetic acid **7**; (c) dansyl chloride, Et₃N.

254 nm]: retention time, 10.7, 11.5 min; 99% purity. Anal. Calcd for C₅₀H₆₅N₄O₇PS: C, 66.9%; H, 7.3%; N, 6.2%; P, 3.5%; S, 3.6%. Found: C, 66.91%; H, 7.33%; N, 6.15%; P, 3.74%; S, 3.65%.

RESULTS

As shown in Scheme 1, carboxylic acids **1a–c** were converted into the 3-oxo esters **2a–c** via the acylation of Meldrum's acid followed by methanolysis in excellent yield (85–90%). Reduction of the 3-oxo esters **2a–c** proceeded either with sodium borohydride in refluxing methanol/THF or with lithium borohydride in THF to give the diols **3a–c** in 63–94% yield after chromatography or recrystallization. The dimethoxytrityl protecting group was introduced on the primary alcohol in **3a–c** by the standard method in 73–92% yield. Compounds **4b,c** were converted directly to the phosphoramidites **5b,c** in 67 and 75% yield, respectively. The benzyloxycarbonyl protecting group of derivative **4a** was removed by hydrogenolysis in methanol to give **6** in quantitative yield (Scheme 2). Conjugation with 3-(4-nitrophenyl)-adamantaneacetic acid gave **4c** in 90–95% yield, whereas reaction with dansyl chloride gave **4d** in 77% yield. Compound **4d** was likewise converted to the phosphoramidite **5d** in 61% yield.

DISCUSSION

Commercially available amino diols are rather limited, consisting mostly of 1,2-diols. In our earliest investigations into preparing hapten phosphoramidites using 1-amino-2,3-propanediol as the scaffolding, we found differentially protecting the three functional groups to be problematic. Additionally, while preparation of sulfonamide-containing phosphoramidites was possible, they could not be incorporated into oligonucleotides. We hypothesized that the problem was the steric hindrance

associated with the DMT-protected vicinal diol or the intramolecular reaction of the sulfonamide nitrogen with the phosphoramidite. Recent success using an *N*-methylsulfonamide phosphoramidite (**11**) suggests that the latter reasoning was correct. We found that constructing similar scaffolding with amino 1,3-diols obviated these problems, allowing the efficient preparation and use of even *N*-unsubstituted sulfonamide-containing phosphoramidites.

A straightforward approach to such 1,3-diols was the reduction of 3-oxo esters. A number of methods have been reported for the preparation of 3-oxo esters (12, 13). Of these, the route that employed Meldrum's acid (**14**) offered the advantages of being widely applicable, using commercially available starting materials, and being amenable to a 20–25 g scale.

Our initial attempts to prepare amino-protected 3-oxo esters by acylation of Meldrum's acid with (*tert*-butyloxycarbonyl)aminobutyric or (*tert*-butyloxycarbonyl)aminovaleric acid failed at the methanolysis stage, giving instead the dihydropyrrole and/or tetrahydropyridine, respectively, from attack of the carbamate nitrogen on the newly formed ketone. However, we found that with the linker **1a** this side reaction was not a problem. Compound **1a**, along with haptens, such as **1b,c**, which contain a carboxylic acid, was efficiently transformed to the desired 3-oxo esters (85–90% yield). For **1b**, a small amount of the corresponding methyl enol ether was also formed but was easily removed by chromatography at this or a later step. In the case of **1c**, the product was easily purified by crystallization. It was also noted that traces of triethylamine in the initial Meldrum's acid adduct effectively prevented the methanolysis from proceeding to completion. Conscientious removal of base in the workup or subsequent addition of an acid (e.g. trifluoroacetic acid) restored efficient conversion. Reduction of the 3-oxo ester to the 1,3-diol was straightforward. Decomposition of the borate esters formed with the 1,3-diol was somewhat more difficult than with normal alcohols but was readily accomplished by repeated evaporation from methanol. Protection of the primary alcohol with the DMT group was carried out in good yield. Selectivity for the primary alcohol was enhanced by slow addition of the DMT-Cl to a solution of the diol. Conversion to the phosphoramidite was also uneventful. Unlike most nucleoside phosphoramidites, the hapten phosphoramidites prepared could not be easily purified by precipitation. Chromatography on silica with an eluent containing triethylamine was sufficient to produce the compounds as a crisp foam, which could then be aliquoted and stored under argon at –20 °C until it was necessary to reconstitute the material for use on the automated synthesizer. The phosphoramidites made and stored in this manner have been stable for over 2 years.

While hapten phosphoramidites **5b,c** were prepared in a sequential manner, the availability of the protected amino diol linker **4a** allowed for a convergent approach also. This was exemplified by the preparation of hapten phosphoramidites of Scheme 2. Removal of the benzyloxycarbonyl protecting group of **4a** by hydrogenolysis proceeded without effect on the DMT group. Subsequent coupling to the adamantaneacetic acid derivative **7** gave **4c** in superior overall yield.

In conclusion, we have shown that hapten phosphoramidites can be conveniently prepared sequentially or convergently from carboxylic acid-containing haptens or linkers through the intermediacy of a Meldrum's acid-derived 3-oxo ester. Hapten phosphoramidites were routinely produced on the 20–25 g scale with this procedure and were suitable for use on an automated

DNA synthesizer for labeling either the 3' or 5' end of oligonucleotide probes.

LITERATURE CITED

- (1) Laffler, T. G., Carrino, J. J., and Marshall, R. L. (1993) The ligase chain reaction in DNA-based diagnosis. *Ann. Biol. Clin.* 51, 821–826.
- (2) Gupta, K. C., Sharma, P., Sathyanarayana, S., and Kumar, P. (1990) A universal solid support for the synthesis of 3'-thiol group containing oligonucleotides. *Tetrahedron Lett.* 31, 2471–2474.
- (3) Asseline, U., and Thuong, N. T. (1989) Solid-phase synthesis of modified oligodeoxyribonucleotides with an acridine derivative or a thiophosphate group at their 3' end. *Tetrahedron Lett.* 30, 2521–2524.
- (4) Gottikh, M., Asseline, U., and Thuong, N. T. (1990) Synthesis of oligonucleotides containing a carboxyl group at either their 5' end or their 3' end and their subsequent derivatization by an intercalating agent. *Tetrahedron Lett.* 31, 6657–6660.
- (5) Goodchild, J. (1990) Conjugates of oligonucleotides and modified oligonucleotides: a review of the synthesis and properties. *Bioconjugate Chem.* 1, 165–191.
- (6) Beaucage, S. L., and Iyer, R. P. (1993) The functionalization of oligonucleotides via phosphoramidite derivatives. *Tetrahedron* 49, 1925–1963.
- (7) Reed, M. W., Adams, A. D., Nelson, J. S., and Meyer, R. B., Jr. (1991) Acridine- and cholesterol-derivatized solid supports for improved synthesis of 3'-modified oligonucleotides. *Bioconjugate Chem.* 2, 217–225.
- (8) Nelson, P. S., Kent, M., and Muthini, S. (1992) Oligonucleotide labeling methods. 3. Direct labeling of oligonucleotides employing a novel, non-nucleosidic, 2-aminobutyl-1,3-propanediol backbone. *Nucleic Acids Res.* 20, 6253–6259.
- (9) Shioiri, T., and Hamada, Y. (1978) New methods and reagents in organic synthesis. 3. Diethyl phosphorocyanidate: a new reagent for C-alkylation. *J. Org. Chem.* 43, 3631–3632.
- (10) Yau, E. K., and Coward, J. K. (1988) Filtering column chromatography—a fast convenient chromatographic method. *Aldrichimica Acta* 21, 106.
- (11) Burmeister, J., Azzawi, A., and Kiedrowski, G. v. (1995) Synthesis of novel phosphoramidite derivatives bearing pyrenyl and dansyl groups. *Tetrahedron Lett.* 36, 3667–3668.
- (12) Clay, R. J., Collum, T. A., Karrick, G. L., and Wemple, J. (1993) A safe, economical method for the preparation of β -oxo esters. *Synthesis*, 290–292.
- (13) Wang, X., Monte, W. T., Napier, J. J., and Ghannam, A. (1994) A process for the synthesis of β -keto esters using in-situ generated trimethylsilyl malonates. *Tetrahedron Lett.* 35, 9323–9326.
- (14) Oikawa, Y., Sugano, K., and Yonemitsu, O. (1978) Meldrum's acid in organic synthesis. 2. A general and versatile synthesis of β -keto esters. *J. Org. Chem.* 43, 2087–2088.

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CORRECTIONS

Volume 7, Number 1, January/February 1996.

Robert Plourde,* Alison T. Phillips, Catherine H. Wu, Richard M. Hays, Jayanta Roy Chowdhury, Namita Roy Chowdhury, and George Y. Wu

A HEPATOCYTE-TARGETED CONJUGATE
CAPABLE OF DELIVERING BIOLOGICALLY
ACTIVE COLCHICINE IN VITRO

Page 133. In the legend to Figure 1, compound **5**
should be compound **8**.

BC950187X

COMMUNICATIONS

Incorporation of an Artificial Protease and Nuclease at the HIV-1 Tat Binding Site of Trans-activation Responsive RNA

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The synthesis of a C-5 modified uridine phosphoramidite which contains a primary amino group protected with Fmoc is described. During cleavage and deprotection of chemically synthesized RNA, the Fmoc protecting group is removed to yield a free amino group at a predetermined position in the RNA sequence that can be covalently modified with any reporter group, small structural probes, and biological molecules. This modified uridine phosphoramidite was used to incorporate a reactive primary amino group at position 24 in the HIV-1 Tat binding site of a trans-activation responsive (TAR) RNA sequence during chemical syntheses. Modified RNA phosphoramidite was incorporated into RNA oligomers with more than 97% coupling efficiencies. RNA containing modified uridine was cleaved from the support, deprotected, and desalted according to standard procedures. After deprotection and gel purification, nuclease digestion and HPLC analysis were performed to confirm the incorporation of C-5-aminouridine into the RNA sequence. The effect of modified uridine on TAR RNA structure was analyzed by CD spectroscopy and protein binding assays. Site-specific incorporation of EDTA was accomplished by treating primary amine bearing TAR RNA with an isothiocyanato derivative of nitrobenzyl-EDTA.

INTRODUCTION

RNA molecules fold into discrete tertiary structures for their biological and chemical functions (1, 2). Folded structures of RNA contain regions of double-stranded duplex, hairpin, internal loop, bulged base, and pseudoknotted structures (3, 4). Due to the complexity of RNA structure, the rules governing RNA folding and sequence-specific RNA-protein recognition are not well

understood. Knowledge of the three-dimensional structure and general rules for RNA folding will be valuable to infer a more detailed mechanism of RNA function.

RNA-protein interactions are vital for many regulatory processes, especially, in gene regulation where proteins specifically interact with binding sites found within RNA transcripts. One example of such interactions is the mechanism of trans-activation in the human immunodeficiency virus type 1 (HIV-1). The promoter of the HIV-1, located in the U3 region of the viral long terminal repeat (LTR), is an inducible promoter which can be stimulated by the trans-activator protein, Tat (5). As in other lentiviruses, Tat protein is essential for trans-activation of viral gene expression (6–9). In the absence

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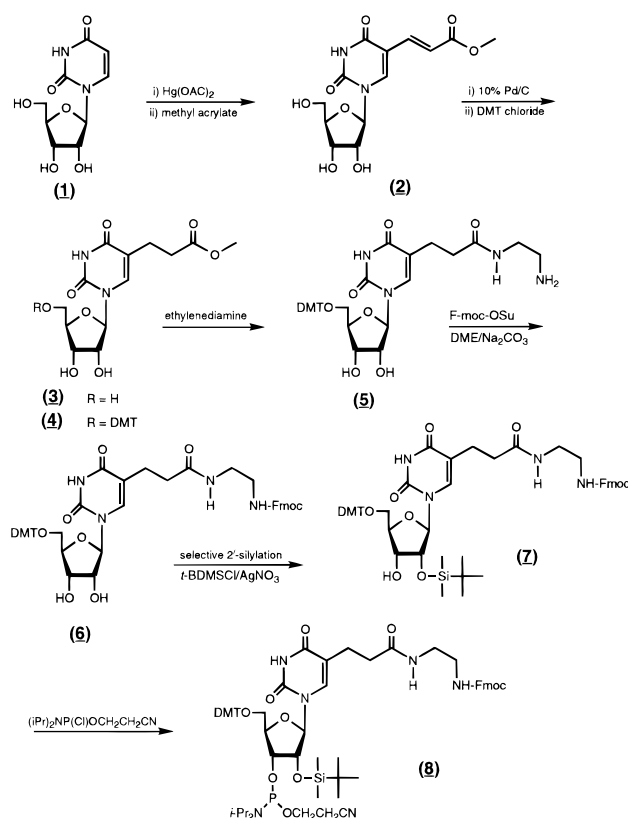
[®] Abstract published in *Advance ACS Abstracts*, April 15, 1996.

of Tat, most of the viral transcripts terminate prematurely, producing short RNA molecules ranging in size from 60 to 80 nucleotides. The Tat protein is a small, cysteine-rich nuclear protein containing 86 amino acids and comprised of three important functional regions: core (32–48), basic (49–60), and Gln (61–72). HIV-1 Tat protein acts by binding to the TAR (trans-activation responsive) RNA element, a 59-base stem-loop structure located at the 5' ends of all nascent HIV-1 transcripts (10). Upon binding to the TAR RNA sequence, Tat causes a substantial increase in transcript levels (11–15). The increased efficiency in transcription may result from preventing premature termination of the transcriptional elongation complex (16, 17). TAR RNA was originally localized to nucleotides +1 to +80 within the viral long terminal repeat (LTR) (18). Subsequent deletion studies have established that the region from +19 to +42 incorporates the minimal domain that is both necessary and sufficient for Tat responsiveness *in vivo* (19–21). TAR RNA contains a six-nucleotide loop and a three-nucleotide pyrimidine bulge which separates two helical stem regions (10, 13, 18, 19). The trinucleotide bulge is essential for high affinity and specific binding of the Tat protein (22, 23). The loop region is required for *in vivo* trans-activation but is not involved in tat binding (23–26). Since Tat can bind duplex TAR RNA (without the loop residues) with approximately one-half the affinity for wild-type TAR RNA, chemically synthesized duplex TAR RNAs without loop residues have been used to study the role of various functional groups in Tat recognition (26–28).

The Tat protein is absolutely essential for viral replication, making it an important potential target for the development of new drug therapies against HIV infections. Understanding the principles of Tat–TAR interactions is a crucial step for drug design. However, there is little structural information available for this RNA–protein complex. In the absence of high-resolution crystallographic and nuclear magnetic resonance data, new methods are needed to determine the topology of RNA–protein complexes under physiological conditions. Metal-catalyzed cleavage of proteins and nucleic acids is an important new approach to study nucleic acid and their complexes with proteins under physiological conditions (29–38). Proximity-dependent cleavage of proteins has been achieved by introducing Fe-EDTA analogues within the protein sequences (39–44). Recently, this Fe-EDTA-catalyzed protein cleavage method has been applied to complex biological systems such as membrane proteins (45). There has been a recent interest in utilizing Fe-EDTA to cleave RNA (38, 46–51).

We planned to use proximity-dependent nucleic acid and protein cleavage properties of Fe-EDTA in our studies of Tat–TAR interactions. A specifically incorporated Fe-EDTA moiety in the TAR RNA sequence can provide structural information about a Tat–TAR complex in two ways: (i) RNA folding in the presence and absence of Tat can be followed by its nuclease properties; (ii) regions of protein close to the site of Fe-EDTA can be mapped by its proteolytic activities. To achieve this goal, we synthesized a modified uridine phosphoramidite (compound **8**, Scheme 1). This reagent **8** contains a primary amino group protected with Fmoc and can be used on any commercially available DNA/RNA synthesizers to synthesize RNA. During cleavage and deprotection of chemically synthesized RNA, the Fmoc protecting group will be removed to yield a free amino group at a predetermined position in the RNA sequence. This amino group can be covalently modified with any reporter group, small structural probes, and biological molecules.

Scheme 1



Here, we report the synthesis of a modified uridine phosphoramidite (**8**, Scheme 1) and incorporation into TAR RNA. After deprotection, the presence of modified uridine was confirmed by nuclease digest and HPLC analysis. The effect of modified uridine on TAR RNA structure was analyzed by CD spectroscopy and protein binding capabilities of the modified RNA. Site-specific labeling of EDTA was achieved by treating primary amine bearing TAR RNA with an isothiocyanato chelate (52–54).

RESULTS AND DISCUSSION

We planned to site-specifically label TAR RNA with an Fe-EDTA analogue at the C24 position in the trinucleotide region. Chemical synthesis of long oligoribonucleotides, such as 29-base TAR RNA, is still not very efficient. To circumvent this problem, two short oligoribonucleotides were synthesized and annealed together to form a duplex RNA that contained the trinucleotide bulge to create a structure similar to the Tat binding site in TAR RNA (26–28). The duplex TAR sequence contained wild-type residues from C17 to C31. Flanking residues were added to achieve efficient annealing (Figure 1). Strand 1 in our model duplex contained only one amino-modified uridine in the RNA sequence. Site-specific incorporation of modified uridine at position 24 was carried out by chemical synthesis of RNA using reagent **8** (Scheme 1).

Synthesis of Fmoc aminouridine phosphoramidite **8** is delineated in Scheme 1. Nucleoside **5** was synthesized according to the procedure of Dreyer and Dervan (55). C-5 substitution on uridine was achieved by mercuration followed by a Pd(II)-mediated coupling reaction carried out in the presence of methyl acrylate to yield nucleoside **2** in good yield. Catalytic hydrogenation yielded nucleoside **3** quantitatively. Protection of the 5' OH was carried out in the presence of DMTCl (dimethoxytrityl chloride) in dry pyridine to yield nucleoside **4**. Aminouridine

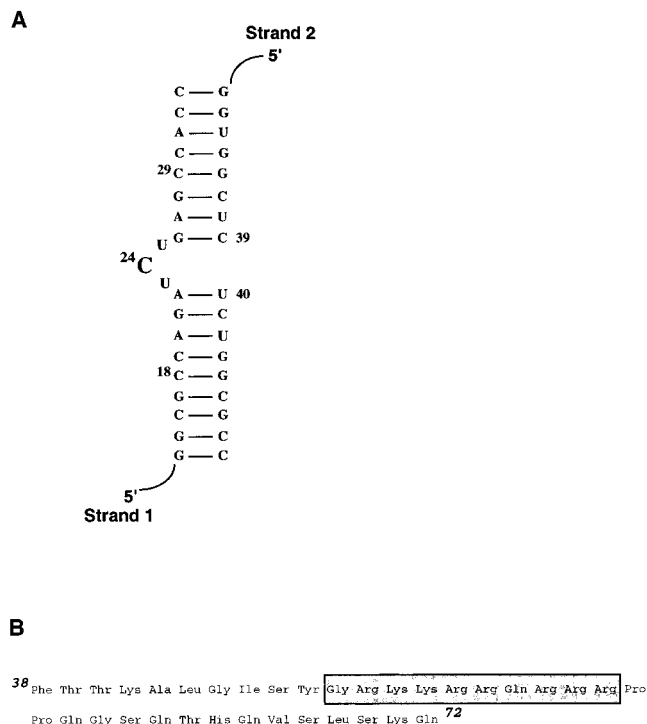


Figure 1. (A) Secondary structure of the designed duplex TAR RNA containing the minimal sequences that are required for *in vitro* Tat binding (27). Sequences containing the trinucleotide bulge and the complementary sequence are labeled as strand 1 and strand 2, respectively. C24 was substituted with amino-uridine during chemical synthesis of RNA, and strand 2 was synthesized by T7 polymerase transcription reactions. (B) The 36-residue peptide, amino acids 38–72, contains the RNA-binding domain of Tat. The arginine-rich region of Tat, corresponding to the Tat(48–57) peptide, is highlighted.

5 was obtained by the treatment of compound **4** with ethylenediamine for 2 days at room temperature. Fmoc group protection of amino-uridine **5** provided nucleoside **6**. Nucleoside **7** was obtained by selective 2' silylation in the presence of pyridine and silver nitrate (56). Final compound **8** was obtained by phosphoramidite formation of 3' OH using 2-cyanoethyl *N,N*-diisopropylchlorophosphoramidite.

Modified uridine phosphoramidite **8** was used to incorporate a reactive primary amino group at position 24 (strand 1) in the TAR RNA sequence during chemical syntheses. Modified RNA phosphoramidite (**8**, Scheme 1) was incorporated into RNA oligomers with more than 97% coupling efficiencies. RNA containing modified uridine was cleaved from the support, deprotected, and desalted according to standard procedures. After deprotection and gel purification, nuclease digestion and HPLC analysis were performed to confirm the incorporation of amino-uridine into the RNA sequence.

To characterize and evaluate the effect of amino-uridine incorporation on the TAR RNA structure, we determined the dissociation constants of a Tat fragment for wild-type and amino-modified duplex TAR RNA. For these experiments, we used a Tat fragment (amino acids 38–72) which binds TAR RNA with high specificity and includes the basic and part of the core regions of Tat (57–59). Equilibrium dissociation constants of the Tat(38–72): TAR complexes were measured using direct and competition electrophoretic mobility assays (22, 23, 57, 60). For direct mobility shift assays, the fractional saturation of 0.5 μ M 5'-³²P end-labeled TAR RNA was measured as a function of Tat(38–72). A typical gel of these experiments is shown in Figure 2. The dissociation constant (K_d) of Tat(38–72) for the modified TAR RNA duplex was

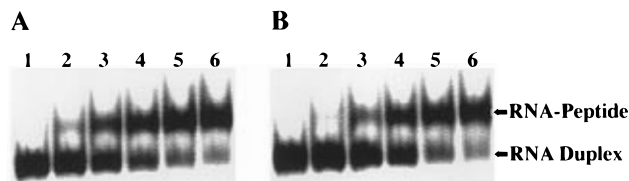


Figure 2. Binding of Tat(38–72) to wild-type TAR RNA duplex (A) or TAR RNA duplex containing amino-uridine at position 24 (B). Binding reactions contained 0.5 μ M of 5'-³²P end-labeled TAR RNA (strand 1) and 0.1, 0.2, 0.3, 0.4, 0.5 μ M of Tat(38–72), lanes 2–6, respectively. Lane 1 was a control lane without the peptide. Complex formation was performed in the binding buffer and incubation at room temperature for 10 min. Complexes were separated from unbound RNA by electrophoresis in nondenaturing 8% polyacrylamide gels containing 0.1% Triton X-100. Gels were run in a cold room at 260 V for 2. The relative amounts of free and bound RNA were determined by phosphor imaging.

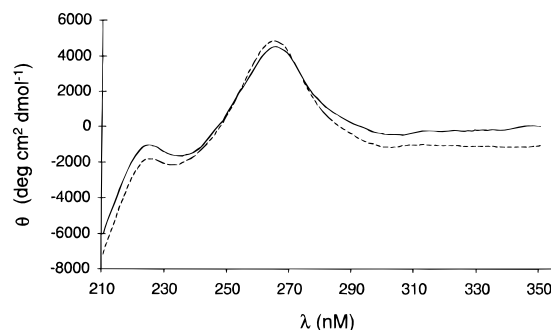


Figure 3. CD spectra of wild-type (solid line) and amino-uridine containing (broken line) duplex TAR RNA. CD spectra were measured using an Aviv model 60DS spectropolarimeter. Samples were prepared in 10 mM K_2HPO_4 (pH 7.5) and 70 mM KF (62). Two strands of RNA were annealed by heating to 90 °C and then cooling to room temperature. Samples were maintained at 25 °C. RNA concentrations were 40–60 μ g/mL. Spectra were recorded from 350 to 210 nm using a 1 cm path length cuvette, and the signal was averaged for 3 s at each wavelength.

calculated to be 0.37 μ M, while for the wild-type TAR RNA duplex, it was 0.33 μ M. From the results of gel shift experiments, we conclude that incorporation of an amino-uridine at position 24 in TAR RNA did not significantly alter the structure of RNA, thus preserving the Tat binding affinities of the RNA.

Further characterization of the secondary structure of modified TAR RNA duplex was carried out by CD (circular dichroism) spectroscopy. Previous CD studies showed that TAR RNA has a spectrum which is typical for the A conformation of a polynucleotide helix (61–63). The CD spectrum of TAR RNA has a maximum near 265 nm and is highly sensitive to the conformation of the helix (63). For example, binding of a peptide or arginine ligands alters the conformation of the TAR RNA structure, which can be observed by a change in the CD spectrum (62). We performed CD experiments on wild-type and modified TAR RNA duplexes as described by Tan and Frankel (62). As shown in Figure 3, CD spectra of both duplex RNAs were similar with a maximum near 265 nm and a minimum near 210 nm. These results show that the secondary structure of modified TAR RNA is similar to the wild-type RNA.

A bifunctional chelating agent, chelate isothiocyanate (CITC), was used for site-specific EDTA labeling of an amino-modified TAR RNA duplex. The experimental outline and the structure of CITC are shown in Scheme 2. Bifunctional chelating agents are compounds that comprise both a strong metal-chelating group and a chemically reactive functional group. Bifunctional chelating agents have been synthesized and used to provide

Scheme 2

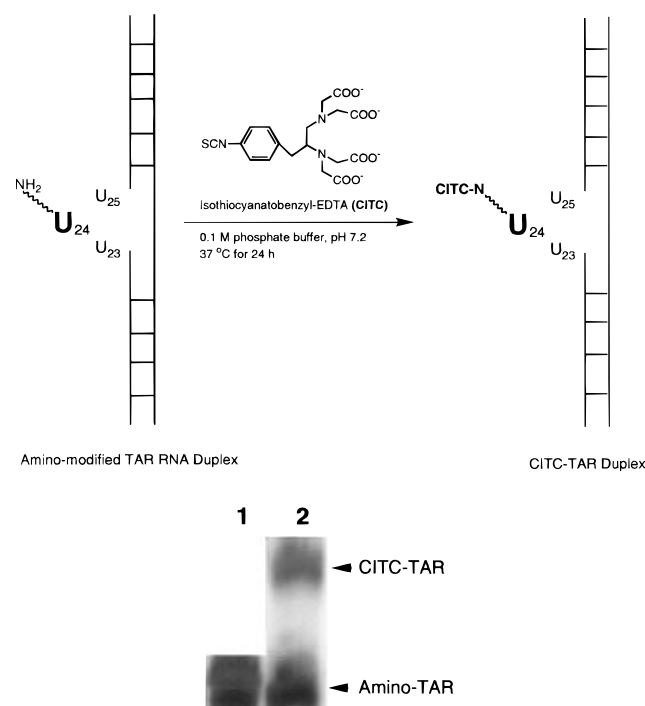


Figure 4. Conjugation reaction of CITC and amino-modified TAR RNA. The reaction mixture contained 4 μ L of 0.25 nM amino-modified strand 1 of TAR RNA (Figure 1), 10 μ L of 15 mM CITC, and 20 μ L of 0.1 M phosphate buffer (pH 7.2) and was incubated at 37 °C for 24 h. The RNA sequence was labeled at its 5' end with ^{32} P before the conjugation reaction with CITC. Labeling reactions were carried out at room temperature for 30 min in a mixture containing 10 μ M RNA, 10 mM MgCl_2 , 0.5 μ M [γ - ^{32} P]ATP (6000 Ci/mmol), and 4 units of T4 polynucleotide kinase in 50 mM Tris-HCl (pH 7.4). The 5'-end-labeled RNA was purified by denaturing gel, ethanol precipitated, and dissolved in phosphate buffer for the conjugation reaction. The conjugation reaction was quenched by the addition of sample loading buffer, and products were resolved on 20% polyacrylamide-8 M urea gels and visualized by autoradiography. Lane 1: aminouridine containing strand 1 of TAR RNA. Lane 2: conjugation reaction mixture. Unmodified aminouridine containing RNA and CITC-RNA conjugates are labeled as amino-TAR and CITC-TAR, respectively.

biological molecules with the nuclear, physical, or chemical properties of chelated metal ions (52, 53, 64). Synthesis of CITC was accomplished as described by Meares *et al.* (53). Conjugation reaction of CITC and amino-modified RNA was carried out at pH 7.2 at 37 °C for 24 h and analyzed on 8 M urea-20% polyacrylamide gel. As shown in Figure 4, the CITC-TAR conjugate migrates with lower electrophoretic mobility on denaturing polyacrylamide gel. A similar observation has been recently reported describing that oligonucleotide-EDTA conjugates show reduced electrophoretic mobility on 20% polyacrylamide-8 M urea gels (38, 49). The yields of CITC-TAR conjugation reactions were $\approx$ 20–30% because the reactions were performed at relatively lower pH (7.2) conditions which were required for TAR RNA stability. A control reaction between wild-type TAR and CITC gave no slow migrating products (data not shown). Experiments to map the cleavage sites in TAR RNA and Tat protein are in progress.

MATERIALS AND METHODS

Uridine, dimethoxytrityl chloride, *tert*-butyldimethylsilyl chloride, 2-cyanoethyl *N,N*-diisopropylchlorophosphoramidite, mercuric acetate, potassium tetrachloropalladate(II), triethylamine, dry THF, pyridine, DME,

diaminoethane, dioxane, 10% Pd on charcoal, and methyl acrylate were purchased from Aldrich. (9-Fluorenylmethyl)succinimidyl carbonate was obtained from Bachem.

^1H NMR spectra were recorded at 200 MHz on a Gemini 200 spectrometer (Varian). All spectra were taken in either CDCl_3 or D_2O . Mass spectra were recorded using fast atom bombardment (FAB) ionization. TLC was performed with precoated 0.2 mm silica gel 60 F-254 TLC plates (EM Reagents, Darmstadt, FRG). Plates were visualized under long-wave UV light and with iodine vapors. Dimethoxytrityl-containing compounds were visualized by exposing the TLC plate to concentrated HCl vapors. Column chromatography was performed with silica gel (70–230 mesh, 60 Å) purchased from Aldrich. Reagent grade chemicals were used without purification unless otherwise stated.

Buffers. All buffer pH values refer to measurements at room temperature. *TK buffer*: 50 mM Tris-HCl (pH 7.4), 20 mM KCl, 0.1% Triton X-100. *Transcription buffer*: 40 mM Tris-HCl (pH 8.1), 1 mM spermidine, 0.01% Triton X-100, 5 mM DTT. *TBE buffer*: 45 mM Tris-borate, pH, 8.0, 1 mM EDTA. *Sample loading buffer*: 9 M urea, 1 mM EDTA, and 0.1% bromophenol blue in 1X TBE buffer. *Binding buffer*: 25 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1 mM MgCl_2 , 0.1% Triton X-100.

Synthesis of Amino-Modified Uridine Phosphoramidite. *Methyl Uridine-5-acrylate (2)*. To a stirred solution of uridine (2 g, 8.19 mmol) in water (10 mL) was added $\text{Hg}(\text{OAc})_2$ (2.74 g, 8.6 mmol), and stirring was continued for 16 h at 50 °C. The reaction was stopped by the addition of NaCl (1.23 g, 21 mmol) in water (8.2 mL). After 5 min the reaction mixture was evaporated to dryness under reduced pressure. The residue was filtered off, rinsed with methanol (5×10 mL), and dried to yield 5-(chloromercurio)uridine quantitatively. To a stirred mixture of 5-(chloromercurio)uridine (3.91 g, 8.19 mmol), methyl acrylate (3.07 mL, 41 mmol), and methanol (82 mL) was added a solution of K_2PdCl_4 (2.66 g, 8.19 mmol) in water (30 mL), and stirring was continued for 4 h. The reaction mixture was treated with H_2S for 2 min, followed by filtration through a pad of Celite. The solvent was evaporated under reduced pressure, and the crude product obtained was purified by column chromatography (methanol:ethyl acetate 2:8) to yield compound **2** as white solid (1.69 g, 5.15 mmol, 63%). ^1H NMR (D_2O) (ppm): δ 8.31 (1H, s, H_6), 7.34 (1H, d, olefinic, $J = 16$ Hz), 6.69 (1H, d, olefinic, $J = 16$ Hz), 5.85 (1H, d, $\text{H}_{1'}$, $J = 3.42$ Hz), 4.27 (1H, m, H_2), 4.19 (1H, m, H_3), 4.10 (1H, m, H_4), 3.91 (1H, dd, H_5), 3.80 (1H, dd, H_5'), 3.72 (3H, s, OCH_3). MS ($\text{CH}_4 + \text{NH}_3$): m/e 329 ($\text{M} + 1$) $^+$. TLC (15% methanol:ethyl acetate): R_f 0.58.

Methyl Uridine-5-propanoate (3). A solution of nucleoside **2** (500 mg, 1.5 mmol) and 10% Pd on charcoal (200 mg) in methanol (75 mL) was stirred under H_2 atmosphere for 4 h. The reaction mixture was filtered, and the filtrate was evaporated under reduced pressure to yield pure nucleoside **3** (498 mg, 99%). ^1H NMR (D_2O) (ppm): δ 7.72 (1H, s, H_6), 5.86 (1H, d, $\text{H}_{1'}$, $J = 4.4$ Hz), 4.30–4.16 (2H, m, H_2' and H_3), 4.09 (1H, m, H_4), 3.88 (1H, dd, H_5), 3.68 (1H, dd, H_5'), 3.63 (3H, s, OCH_3), 2.58 (4H, br.s, 2CH_2). MS (MNBA + NaI): m/e 353 ($\text{M} + \text{Na}$) $^+$. TLC (10% methanol:ethyl acetate): R_f 0.23.

Methyl 5'-O-(4,4'-Dimethoxytrityl)uridine-5-propanoate (4). A solution of nucleoside **3** (330 mg, 1 mmol) and (DMT)Cl (373 mg, 1.1 mmol) in dry pyridine (5 mL) was stirred for 6 h at room temperature under N_2 atmosphere. Methanol (1 mL) was added, and after 15 min of additional stirring, the reaction mixture was concentrated to dryness, the residue was dissolved in ethyl acetate (25 mL) and washed with 5% NaHCO_3 (10 mL), and the

organic layer was dried and evaporated. Column purification (ethyl acetate) yielded compound **4** as a white solid (458 mg, 72%). ^1H NMR (CDCl_3) (ppm): δ 7.67 (1H, s, H_6), 7.42–7.23 (9H, m, aromatic), 6.83 (4H, d, aromatic, $J = 8.92$), 5.90 (1H, d, $\text{H}_{1'}$, $J = 3.30$ Hz), 4.35 (2H, m, $\text{H}_{2'}$ and $\text{H}_{3'}$), 4.22 (1H, m, $\text{H}_{4'}$), 3.77 (6H, s, OCH_3), 3.56 (3H, s, OCH_3), 3.50–3.38 (2H, m, $\text{H}_{5'}$ and $\text{H}_{5''}$), 2.40–2.15 (4H, m, CH_2). MS (MNBA + NaI): m/e 655 ($\text{M} + \text{Na}$) $^+$. TLC (ethyl acetate): R_f 0.28.

5'-O-(4,4'-Dimethoxytrityl)uridine-5-(N-2-aminoethyl)propionamide (5). A solution of compound **4** (450 mg, 0.71 mmol) and freshly distilled ethylenediamine (2 mL) was stirred for 2 days at room temperature. Solvent was removed under reduced pressure, and the residue obtained was lyophilized using dioxane. The resulting white solid was used as such for the next reaction.

5'-O-(4,4'-Dimethoxytrityl)uridine-5-(N-Fmoc-aminoethyl)propionamide (6). To a solution of Fmoc-OSu (417 mg, 1.24 mmol) in DME (10 mL) was added dropwise a solution of aminouridine **5** (410 mg, 0.62 mmol) in 10% Na_2CO_3 (10 mL). The reaction mixture was stirred overnight at room temperature. Solvent was evaporated under reduced pressure, and the residue was extracted with ethyl acetate (3×20 mL). The organic layer was dried and evaporated to yield the crude product which was purified by column chromatography (ethyl acetate: benzene, 8:2): yield 256 mg, 0.29 mmol, 47%. ^1H NMR (CDCl_3) (ppm): δ 7.69–7.66 (2H, m, H_6 and aromatic), 7.54–7.13 (16H, m, aromatic), 6.82–6.72 (4H, m, aromatic), 5.93 (1H, d, $\text{H}_{1'}$, $J = 4.84$ Hz), 4.37–4.27 (3H, m, $\text{H}_{2'}$, $\text{H}_{3'}$, and $\text{H}_{4'}$), 4.18–4.07 (3H, CH_2 and CH), 3.71 (6H, s, OCH_3), 3.50–3.25 (2H, m, $\text{H}_{5'}$ and $\text{H}_{5''}$), 3.25–3.02 (4H, m, CH_2), 2.40–1.96 (4H, m, 2CH_2). MS (NaI + MNBA): m/e 905 ($\text{M} + \text{Na}$) $^+$. TLC (5% methanol:ethyl acetate): R_f 0.73.

5'-O-(4,4'-Dimethoxytrityl)-2'-O-(tert-butyldimethylsilyl)uridine-5-(N-Fmoc-aminoethyl)propionamide (7). To a solution of nucleoside **6** (200 mg, 0.22 mmol) in dry THF (5 mL) were added dry pyridine (0.08 mL, 1 mmol) and silver nitrate (45 mg, 0.26 mmol). The reaction mixture was stirred for 15 min, followed by the addition of *tert*-butyldimethylsilyl chloride (43 mg, 0.28 mmol). Stirring was continued with additional 3 h, followed by filtration into 5% NaHCO_3 solution (10 mL). The aqueous layer was extracted with ethyl acetate (3×10 mL), dried over molecular sieves, and evaporated under reduced pressure. Column purification (ethyl acetate:benzene 6:4) provided the 2'-silyl nucleoside as a white solid (121 mg, 0.13 mmol, 55%). ^1H NMR (CDCl_3) (ppm): δ 7.74–7.68 (2H, m, H_6 and aromatic), 7.59–7.55 (3H, m, aromatic), 7.36–7.22 (13H, m, aromatic), 6.83 (4H, d, aromatic, $J = 8.80$ Hz), 6.02 (1H, d, $\text{H}_{1'}$, $J = 5.18$ Hz), 4.45–4.38 (3H, m, $\text{H}_{2'}$, $\text{H}_{3'}$, and $\text{H}_{4'}$), 4.25–4.14 (3H, m, CH_2 and CH), 3.76 (6H, s, OCH_3), 3.56 (1H, d, H_5), 3.36 (1H, d, $\text{H}_{5''}$), 3.16 (4H, m, 2CH_2), 2.30–1.92 (4H, m, CH_2), 0.90 (9H, s, *tert*-butyl), 0.10 (6H, s, CH_3). MS ($\text{CH}_4 + \text{NH}_3$): m/e 997 ($\text{M} + 1$) $^+$. TLC (ethyl acetate): R_f 0.60.

5'-O-(4,4'-Dimethoxytrityl)-2'-O-(tert-butyldimethylsilyl)uridine-5-(N-Fmoc-aminoethyl)propionamide 3'-O-[2-cyanoethyl (N,N-diisopropylamino)phosphoramidite] (8). To an ice-cooled solution of nucleoside **7** (50 mg, 0.06 mmol) in dry THF (1 mL) was added dry pyridine (0.02 mL, 0.25 mmol), followed by dropwise addition of 2-cyanoethyl *N,N*-diisopropylchlorophosphoramidite (0.02 mL, 0.09 mmol) in dry THF (1 mL). Stirring was continued for 2 h at room temperature. The reaction mixture was worked up by diluting it with ethyl acetate (25 mL) and washing the organic phase with 5% NaHCO_3 solution (5 mL) and brine (5 mL). Evaporation followed by column purification (ethyl acetate:benzene (8:2), 0.05% Et_3N)

yielded phosphoramidite **8** as a white solid (34 mg, 0.028 mmol, 57%). ^1H NMR (CDCl_3) (ppm): δ 7.78–7.65 (2H, m, H_6 and aromatic), 7.59–7.45 (3H, m, aromatic), 7.36–7.23 (13 H, m, aromatic), 6.85–6.68 (4H, m, aromatic), 6.15–5.80 (1H, m, $\text{H}_{1'}$), 4.49–4.10 (8H, m, 2OCH_2 , CH , $\text{H}_{2'}$, $\text{H}_{3'}$, and $\text{H}_{4'}$), 3.76 and 3.69 (6H, 2s, OCH_3), 3.82–3.05 (8H, m, 2CH_2 , 2CH , $\text{H}_{5'}$ and $\text{H}_{5''}$), 2.75 (2H, t, $\text{CH}_2\text{-CN}$), 2.50–2.10 (4H, m, 2CH_2), 1.29–1.24 (12H, m, isopropyl), 0.91–0.71 (9H, m, *tert*-butyl), 0.19–0.12 (6H, m, CH_3). MS (NaI + 2-hydroxyethyl disulfide): m/e 1106 ($\text{M} + \text{Na} - t\text{-BDMS}$) $^+$. TLC (ethyl acetate): R_f 0.55.

RNA Synthesis and Deprotection. All RNA syntheses were performed on ABI synthesizer Model 392 using standard protocols. All the monomers of 2-cyanoethyl phosphoramidites were obtained from Glen Research. Cleavage from the support and deprotection were carried out with 2 M methanolic ammonia (1 mL) for 16 h at room temperature. Product was filtered and purified by a Sep Pak C_{18} cartridge by standard methods. Purified and 2'-protected RNA was dried and further treated with 0.5 mL of TBAF (tetra-*n*-butylammonium fluoride) (1.0 M solution in THF) for 24 h at room temperature. Reaction was quenched with 1.0 mL of 0.1 M TEAA (pH 7.0) and dried to a total volume of 1.0 mL. Desalting was achieved by Sep Pak C_{18} cartridge purification. RNA was purified on 20% acrylamide 8 M urea denaturing gels and stored in DEPC (diethyl pyrocarbonate) water at -20°C .

Strand 2 of TAR RNA was prepared by *in vitro* transcription (65). The template strand of DNA was annealed to an equimolar amount of top-strand DNA, and transcriptions were carried out in transcription buffer and 4.0 mM NTPs at 37°C for 2–4 h. For reactions (20 μL) containing 8.0 pmol of template DNA, 40–60 units of T7 polymerase (Promega) was used. Transcription reactions were stopped by adding an equal volume of sample loading buffer. RNA was purified on 20% acrylamide–8 M urea denaturing gels.

Enzymatic Digestion of Oligonucleotides. Enzymatic digestion was carried out by incubating a 0.2 A_{260} unit of purified oligonucleotide at 37°C overnight with 6 μL (12 μg) of snake venom phosphodiesterase and 2 μL (2 units) of alkaline phosphatase in a total volume of 78.2 μL of 32 mM Tris (pH 7.5) and 15 mM MgCl_2 (66). Nucleosides were recovered by adding 10 μL of 3 M NaOAc (pH 5.2) and 234 μL of 95% ethanol, mixing, and chilling to -80°C for 30 min. After centrifugation at 13 000 rpm for 20 min at 4°C , the supernatant was taken and dried in a Speed-Vac (Savant) and redissolved in 200 μL of water for HPLC analysis.

HPLC Analysis of Oligonucleotides. Nucleosides were chromatographed on a C_{18} reverse phase column (Beckman 5 μm , 4.6 mm $\times$ 25 cm, ultrasphere). Solvent A: 97.5% 0.01 M KH_2PO_4 (pH 5), 2.5% methanol. Solvent B: 80% 0.01 M KH_2PO_4 (pH 5.1), 20% methanol. Gradient: at 0 min, 0% B; at 7 min, start ramp to 10% B over 5 min; at 12 min, start ramp to 25% B over 3 min; at 15 min, start ramp to 60% B over 5 min; at 20 min, start ramp to 62% B in 2.5 min; at 22.5 min, start ramp to 100% B over 6 min; at 28.5 min, continued 100% B for 10 min. UV absorbance of nucleosides was monitored at 254 nm. Retention times for ribonucleosides (in min) were as follows: C, 12.37; U, 17.57; amino-U, 28.72; G, 29.95; A, 36.21. Quantitation of HPLC data was performed by comparison to authentic nucleoside standards as described by Eadie *et al.* (67).

Peptide Synthesis. All Fmoc-amino acids, piperidine, 4-(dimethylamino)pyridine, dichloromethane, *N,N*-dimethylformamide, 1-hydroxybenzotriazole (HOBt), 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluoro-

rophosphate (HBTU), diisopropylethylamine, and HMP-linked polystyrene resin were obtained from Applied Biosystems Division, Perkin-Elmer. Trifluoroacetic acid, 1,2-ethanedithiol, phenol, and thioanisole were from Sigma. Tat-derived peptide (from amino acids 38–72) was synthesized on an Applied Biosystems 431A peptide synthesizer using standard FastMoc protocols. Cleavage and deprotection of the peptide were carried out in 2 mL of reagent K for 6 h at room temperature. Reagent K contained 1.75 mL of TFA, 100 μ L of thioanisole, 100 μ L of water, and 50 μ L of ethanedithiol (68). After cleavage from the resin, the peptide was purified by HPLC on a Zorbax 300 SB-C₈ column. The mass of fully deprotected and purified peptides was confirmed by FAB mass spectrometry; mass for Tat(38–72) C₁₇₅H₂₉₈N₆₄O₄₃ calcd 4082.7, found 4083.7 (M + H).

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LITERATURE CITED

- (1) Cech, T. R. (1990) Self-splicing of Group I Intron. *Annu. Rev. Biochem.* 59, 543–68.
- (2) Draper, D. E. (1992) The RNA-folding problem. *Acc. Chem. Res.* 25, 201–207.
- (3) Tinoco, I., Jr., Puglisi, J. D., and Wyatt, J. R. (1990) RNA folding. *Nucleic Acids Mol. Biol.* 4, 205–226.
- (4) Wyatt, J. R., Puglisi, J. D., and Tinoco, I., Jr. (1989) RNA folding: Pseudoknots, loops and bulges. *Bioassays* 11, 100–106.
- (5) Jones, K. A., and Peterlin, B. M. (1994) Control of RNA Initiation and Elongation at The HIV-1 Promoter. *Annu. Rev. Biochem.* 63, 717–43.
- (6) Cullen, B. R. (1992) Mechanism of action of regulatory proteins encoded by complex retroviruses. *Microbiol. Rev.* 56, 375–394.
- (7) Gaynor, R. (1992) *AIDS* 6, 374–63.
- (8) Fisher, A. G., et al. (1986) The *trans*-activator gene of HTLV-III is essential for virus replication. *Nature* 320, 367–371.
- (9) Dayton, A. I., Sodroski, J. G., Rosen, C. A., Goh, W. C., and Haseltine, W. A. (1986) The *trans*-activator gene of human T cell lymphotropic virus type III is required for replication. *Cell* 44, 941–947.
- (10) Berkhout, B., Silverman, R. H., and Jeang, K. T. (1989) Tat *trans*-activates the Human Immunodeficiency Virus Through a Nascent RNA Target. *Cell* 59, 273–82.
- (11) Cullen, B. R. (1986) *Trans*-activation of human immunodeficiency virus occurs via a bimodal mechanism. *Cell* 46, 973–982.
- (12) Peterlin, B. M., Luciw, P. A., Barr, P. J., and Walker, M. D. (1986) Elevated levels of mRNA can account for the *trans*-activation of human immunodeficiency virus. *Proc. Natl. Acad. Sci. U.S.A.* 83, 9734–9738.
- (13) Muesing, M. A., Smith, D. H., and Capon, D. A. (1987) Regulation of mRNA by a Human Immunodeficiency Virus *trans*-activator protein. *Cell* 48, 691–701.
- (14) Rice, A. P., and Mathews, M. B. (1988) Transcriptional but not translational regulation of HIV-1 by the tat gene product. *Nature* 332, 551–553.
- (15) Laspias, M. F., Rice, A. P., and Mathews, M. B. (1989) HIV-1 tat protein increases transcriptional initiation and stabilises elongation. *Cell* 59, 283–292.
- (16) Kao, S.-Y., Calman, A. F., Luciw, P. A., and Peterlin, B. M. (1987) Antitermination of transcription within the long terminal repeat of HIV-1 tat gene product. *Nature* 330, 489–493.
- (17) Zhou, Q., and Sharp, P. A. (1995) Novel mechanism and factor for regulation by HIV-1 Tat. *EMBO J.* 14, 321–328.
- (18) Rosen, C. A., Sodroski, J. G., and Haseltine, W. A. (1985) Location of Cis-acting Regulatory Sequences in the Human T cell Lymphotropic Virus type III (HTLV-III/LAV) Long Terminal Repeat. *Cell* 41, 813–823.
- (19) Jakobovits, A., Smith, D. H., Jakobovits, E. B., and Capon, D. J. (1988) A Discrete Element 3' of Human Immunodeficiency Virus 1 (HIV-1) and HIV-2 mRNA Initiation Sites Mediates Transcriptional Activation by an HIV Trans Activator. *Mol. Cell. Biol.* 8, 2555–2561.
- (20) Selby, M. J., Bain, E. S., Luciw, P. A., and Peterlin, B. M. (1989) Structure, Sequence, and Position of the Stem-loop in Tar Determine Transcriptional Elongation by Tat through the HIV-1 Long Terminal Repeat. *Genes Dev.* 3, 547–558.
- (21) Garcia, J. A., et al. (1989) Human Immunodeficiency Virus Type 1 LTR TATA and TAR Region Sequences Required for Transcriptional Regulation. *EMBO J.* 8, 765–778.
- (22) Dingwall, C., et al. (1989) Human Immunodeficiency Virus Tat protein Binds Trans-activation-responsive Region (TAR) RNA in vitro. *Proc. Natl. Acad. Sci. U.S.A.* 86, 6925–6929.
- (23) Dingwall, C., et al. (1990) HIV-1 Tat Protein Stimulates Transcription by Binding to the Stem of the TAR RNA Structure. *EMBO J.* 9, 4145–4153.
- (24) Cordingley, M. G., et al. (1990) Sequence-specific Interaction of Tat protein and Tat peptides with the Transactivation-responsive Sequence Element of Human Immunodeficiency Virus Type 1 in vitro. *Proc. Natl. Acad. Sci. U.S.A.* 87, 8985–8989.
- (25) Feng, S., and Holland, E. C. (1988) HIV-1 tat *trans*-activation requires the loop sequence within TAR. *Nature* 334, 165–168.
- (26) Sumner-Smith, M., et al. (1991) Critical chemical features in *trans*-acting-responsive RNA are required for interaction with human immunodeficiency virus type 1 tat protein. *J. Virol.* 65, 5196–5202.
- (27) Hamy, F., et al. (1993) Hydrogen-bonding Contacts in the Major Groove are Required for Human Immunodeficiency Virus Type-1 tat Protein Recognition of TAR RNA. *J. Mol. Biol.* 230, 111–123.
- (28) Pritchard, C. E., et al. (1994) Methylphosphonate mapping of phosphate contacts critical for RNA recognition by the human immunodeficiency virus tat and rev proteins. *Nucleic Acids Res.* 22, 2592–2600.
- (29) Chen, C.-H. B., and Sigman, D. S. (1986) Nuclease Activity of 1, 10-phenanthroline-copper: Sequence-Specific Targeting. *Proc. Natl. Acad. Sci. U.S.A.* 83, 7147–7151.
- (30) Dervan, P. B. (1986) Design of Sequence-Specific DNA-Binding Molecules. *Science* 232, 464–471.
- (31) Moser, H. E., and Dervan, P. B. (1987) Sequence-Specific Cleavage of Double Helical DNA by Triple Helix Formation. *Science* 238, 645–650.
- (32) Sluka, J. P., Horvath, S. J., Bruist, M. F., Simon, M. I., and Dervan, P. B. (1987) Synthesis of a sequence-specific DNA-cleaving Peptide. *Science* 238, 1129–1132.
- (33) Chen, C.-H. B., and Sigman, D. S. (1987) Chemical conversion of a DNA-binding protein into a site-specific nuclease. *Science* 237, 1197–1201.
- (34) Ebright, R. H., Ebright, Y. W., Pendergrast, P. S., and Gunasekera, A. (1990) Conversion of a helix-turn-helix motif sequence-specific DNA binding protein into a site-specific DNA cleavage agent. *Proc. Natl. Acad. Sci. U.S.A.* 87, 2882–6.
- (35) Sigman, D. S., Kuwabara, M. D., Chen, C.-H. B., and Bruce, T. C. (1991) Nuclease activity of 1,10-phenanthroline,copper in study of protein-DNA interactions. *Methods Enzymol.* 208, 414–433.
- (36) Bruce, T. W., Wise, J. G., Rosser, D. S. E., and Sigman, D. S. (1991) Conversion of λ phage Cro operator-specific nuclease. *J. Am. Chem. Soc.* 113, 5446–5447.
- (37) Ebright, Y. W., Chen, Y., Ludescher, R. D., and Ebright, R. H. (1993) N-(iodoacetyl)-p-phenylenediamine-EDTA: a reagent for high-efficiency incorporation of an EDTA-metal complex at a rationally selected site within a protein. *Bioconjugate Chem.* 4, 219–25.
- (38) Han, H., and Dervan, P. B. (1994) Visualization of RNA tertiary structure by RNA-EDTA Fe(II) autocleavage: analysis of tRNA^{Phe} with uridine-EDTA Fe(II) at position 47. *Proc. Natl. Acad. Sci. U.S.A.* 91, 4955–4959.

- (39) Rana, T. M., and Meares, C. F. (1990) Specific Cleavage of a Protein by an Attached Iron Chelate. *J. Am. Chem. Soc.* 112, 2457–2458.
- (40) Rana, T. M., and Meares, C. F. (1991) Transfer of Oxygen from an Artificial Protease to Peptide Carbon During Proteolysis. *Proc. Natl. Acad. Sci. U.S.A.* 88, 10578–10582.
- (41) Rana, T. M., and Meares, C. F. (1991) Iron Chelate-Mediated Proteolysis: Protein Structure Dependence. *J. Am. Chem. Soc.* 113, 1859–1861.
- (42) Ermacora, M. R., Delfino, J. M., Cuenoud, B., Schepartz, A., and Fox, R. O. (1992) Conformation-dependent cleavage of staphylococcal nuclease with a disulfide-linked iron chelate. *Proc. Natl. Acad. Sci. U.S.A.* 89, 6383–6387.
- (43) Schepartz, A., and Cuenoud, B. (1990) Site-specific cleavage of the protein calmodulin using a trifluoperazine-based affinity reagent. *J. Am. Chem. Soc.* 112, 3247–3249.
- (44) Ermacora, M. R., Ledman, D. W., Hellings, H. W., Hsu, G. W., and Fox, R. O. (1994) Mapping staphylococcal nuclease conformation using an EDTA-Fe derivative attached to genetically engineered cysteine residue. *Biochemistry* 33, 13625–13641.
- (45) Ghaim, J. B., Greiner, D. P., Meares, C. F., and Gennis, R. B. (1995) Proximity mapping the surface of a membrane protein using an artificial protease: demonstration that the quinone-binding domain of subunit I is near the N-terminal region of subunit II of cytochrome *bd*. *Biochemistry* 34, 11311–11315.
- (46) Latham, J. A., and Cech, T. R. (1989) Defining the Inside and Outside of a Catalytic RNA Molecule. *Science* 245, 276–282.
- (47) Celander, D. W., and Cech, T. R. (1991) Visualizing the higher order folding of a catalytic RNA molecule. *Science* 251, 401–407.
- (48) Wang, J.-F., and Cech, T. R. (1992) Tertiary structure around the guanosine-binding site of the *Tetrahymena* ribozyme. *Science* 256, 526–529.
- (49) Han, H., and Dervan, P. B. (1994) Different conformational families of pyrimidine · purine · pyrimidine triple helices depending on backbone composition. *Nucleic Acids Res.* 22, 2837–2844.
- (50) Han, H., Schepartz, A., Pellegrini, M., and Dervan, P. B. (1994) Mapping RNA regions in eukaryotic ribosomes that are accessible to methidiumpropyl-EDTA-Fe(II) and EDTA-Fe(II). *Biochemistry* 33, 9831–9844.
- (51) Richardson, P. L., Gross, M. L., Light-Wahl, K. J., Smith, R. D., and Schepartz, A. (1994) A uniquely modified RNA: Introduction of a single RNA cleavage agent into the M1 ribozyme. *Biorg. Med. Chem. Lett.* 4, 2133–2138.
- (52) Meares, C. F., and Goodwin, D. A. (1984) Linking Radiometals to Proteins with Bifunctional Chelating Agents. *J. Protein Chem.* 3, 215–227.
- (53) Meares, C. F., et al. (1984) Conjugation of Antibodies with Bifunctional Chelating Agents: Isothiocyanate and Bromoacetamide Reagent, Method of Analysis, and Subsequent Analysis of Metal Ions. *Anal. Biochem.* 142, 68–78.
- (54) Rana, T. M., and Meares, C. F. (1990) N-terminal Modification of Immunoglobulin Polypeptide Chains Tagged with Isothiocyanato Chelate. *Bioconjugate Chem.* 1, 357–362.
- (55) Dreyer, G. B., Dervan, P. B. (1985) Sequence-specific Cleavage of Single-stranded DNA: Oligodeoxynucleotide-EDTA. Fe(II). *Proc. Natl. Acad. Sci. U.S.A.* 82, 968–972.
- (56) Hakimelahi, G. H., Proba, Z. A., and Ogilvie, K. (1982) New Catalysts and Procedures for the Dimethoxytritylation and Selective Silylation of Ribonucleosides. *Can. J. Chem.* 60, 1106–1113.
- (57) Churcher, M. J., et al. (1993) High Affinity Binding of TAR RNA by the Human Immunodeficiency Virus Type-1 *tat* Protein Requires Base-pairs in the RNA Stem and Amino Acid Residues Flanking the Base Region. *J. Mol. Biol.* 230, 90–110.
- (58) Wang, Z., and Rana, T. M. (1995) Chemical Conversion of a TAR RNA-Binding Fragment of HIV-1 Tat Protein into a Site-Specific Crosslinking Agent. *J. Am. Chem. Soc.* 117, 5438–5444.
- (59) Neenhold, H. R., and Rana, T. M. (1995) Major Groove Opening at the HIV-1 Tat Binding Site of TAR RNA Evidenced by a Rhodium Probe. *Biochemistry* 34, 6303–6309.
- (60) Fried, M., and Crothers, D. M. (1981) Equilibria and kinetics of lac repressor-operator interactions by polyacrylamide gel electrophoresis. *Nucleic Acids Res.* 9, 6505.
- (61) Calnan, B. J., Biancalana, S., Hudson, D., and Frankel, A. D. (1991) Analysis of Arginine-Rich Peptides from the HIV Tat Protein Reveals Unusual Features of RNA Protein Recognition. *Genes Dev.* 5, 201–210.
- (62) Tan, R., and Frankel, A. D. (1992) Circular dichroism studies suggest that TAR RNA changes conformation upon specific binding of arginine or guanidine. *Biochemistry* 31, 10288–10294.
- (63) Loret, E. P., Georgel, P., Johnson, W. C., and Ho, S. P. (1992) Circular dichroism and molecular modeling yield a structure for the complex of human immunodeficiency virus type 1 trans-activation response RNA and the binding region of Tat, the trans-acting transcriptional activator. *Proc. Natl. Acad. Sci. U.S.A.* 89, 9734–9738.
- (64) Meares, C. F., and Wensel, T. G. (1984) Metal Chelates as Probes for Biological Systems. *Acc. Chem. Res.* 17, 202–209.
- (65) Milligan, J. F., Groebe, D. R., Witherell, G. W., and Uhlenbeck, O. C. (1987) Oligoribonucleotide synthesis using T7 RNA polymerase and synthetic DNA templates. *Nucleic Acids Res.* 15, 8783–8798.
- (66) Shah, K., Wu, H., and Rana, T. M. (1994) Synthesis of uridine phosphoramidite analogues: Reagents for site-specific incorporation of photoreactive sites into RNA sequences. *Bioconjugate Chem.* 5, 508–512.
- (67) Eadie, J. S., McBride, L. J., Efcavitch J. W., Hoff, L. B., and Cathcart, R. (1987) High Performance Liquid Chromatographic Analysis of Oligodeoxynucleotides Base Composition. *Anal. Biochem.* 165, 442–447.
- (68) King, D. S., Fields, C. G., and Fields, G. B. (1990) A cleavage method which minimizes side reactions following Fmoc solid phase peptide synthesis. *Int. J. Pept. Protein Res.* 36, 255–266.

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ARTICLES

Thiolations, ^{99m}Tc Labelings, and Animal *In Vivo* Biodistributions of Divalent Monoclonal Antibody Fragments[†]

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A number of divalent monoclonal antibody fragments have been derivatized with a bifunctional reagent containing a latent thiol group, and the conjugates were thiol-deprotected by exposure to aqueous hydroxylamine. This two-step procedure enabled convenient ^{99m}Tc labeling of thiolated MAb divalent fragments, using either preformed ^{99m}Tc -glucoheptonate or [^{99m}Tc]pertechnetate and single-vial lyophilized formulations of the conjugate and tin. Nonspecific incorporation of radiolabel was negligible, if any, when using nonthiolated antibodies. In animal biodistributions, in nude mice bearing LS174T human colon carcinoma xenografts, the ^{99m}Tc -labeled MAb divalent fragments studied were found to result in a 3-fold reduction in the kidney uptake of radioactivity at 24 h, compared to a ^{99m}Tc -Fab, and also to lead to improvements in other tumor:nontumor ratios.

INTRODUCTION

Radioimmunodetection (RAID¹) using γ -emitting radioisotopes is emerging as a clinical diagnostic tool as a result of many advances made in antibody technology and radiolabeling chemistry (1–4). Of the various radioisotopes available for RAID, technetium-99m is most attractive due to its ready availability via a $^{99}\text{Mo}/^{99m}\text{Tc}$ generator system, ideal photon energy, and economy (4).

The 6-h physical half-life of the technetium-99m radioisotope necessitates image acquisition within 24 h, a time frame which is not ideal when using whole antibodies. Intact IgG gives useful tumor accretion but has significant blood retention at 24-h postadministration. This is one reason considerable attention has been devoted to the study of antibody fragments, with promising results (1, 5, 6). The smaller size of the fragments results in faster penetration into tumor and quicker clearance from circulation, both of which are suited to the decay characteristics of the ^{99m}Tc isotope. Another advantage to using the fragments of murine antibodies is the lack of an Fc region, which more often elicits human anti-mouse antibody response in patients. In a

clinical diagnostic study, Behr et al. (7) compared a ^{99m}Tc -Fab' with the corresponding ^{99m}Tc -IgG in colorectal patients and found the Fab' fragment more sensitive, and better suited to early detection of lesions, than the IgG. The Fab' fragment has, however, a lower affinity for tumor antigen than F(ab')₂ fragment or intact IgG (8). This, coupled with rapid whole-body clearance kinetics, probably contributes to the low absolute uptake of Fab' in tumor. Behr et al. (7) point out that neither an IgG nor a Fab' fragment is ideally suited to the important application of liver metastases detection and suggest that a technetium-99m-labeled F(ab')₂ fragment would be a better alternative for immunoscintigraphy.

The divalent fragment has antigen affinity similar to that of IgG but clears faster from circulation and also has lower nonspecific liver uptake than an IgG (9). Also, the longer circulatory half-life of the divalent fragment relative to Fab', due to larger size, contributes to greater tumor accretion relative to Fab'. Higher absolute uptake in tumor of radiolabeled F(ab')₂, compared to Fab', will also render image acquisition easier. Most importantly, high kidney uptake and retention of radiometal, which is a problem when using radiometalated antibody fragments, will be somewhat less pronounced with a F(ab')₂ than with a Fab'.

This report describes a simple method for thiolating antibody divalent fragments, and subsequently labeling with reduced ^{99m}Tc , producing a conjugate with favorable pharmacokinetic properties.

EXPERIMENTAL PROCEDURES

General Procedures. Antibody fragments were obtained from Immunomedics' antibody production laboratories. In the case of MN-14, a monoclonal antibody against CEA (10), the divalent fragment was obtained by a papain digest, since a pepsin digest, as with certain

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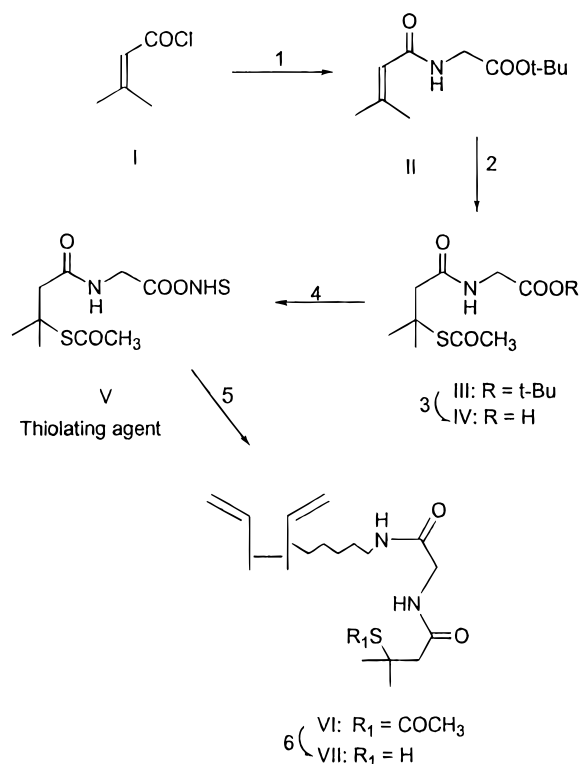
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¹ Abbreviations: ABS, acetate-buffered 150 mM sodium chloride (pH 5.3); CEA, carcinoembryonic antigen; CDCl₃, deuteriochloroform; DCC, dicyclohexylcarbodiimide; DMF, dimethylformamide; DTNB, 2,2'-dithiobis(5-nitrobenzoic acid); GH, glucoheptonate; ¹H NMR, proton nuclear magnetic resonance; MAb, monoclonal antibody; ITLC, instant thin layer chromatography; NHS, N-hydroxysuccinimide; RAID, radioimmunodetection; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SEC, size-exclusion chromatography.

Scheme 1



Reagents: (1) $\text{H}_2\text{N-CH}_2\text{-COOt-Bu}$, Et_3N , CH_2Cl_2 (2) CH_3COSH

(3) CF_3COOH (4) NHS , DCC (5) MAb F(ab')_2 , pH 7.5 (6) NH_2OH

other MABs, leads to overdigestion of MN-14.² All chemicals and solvents, of high-purity grade, were obtained from commercial sources and used without further purification. ^1H NMR spectra for synthetic intermediates were obtained on a Varian VXR-400S instrument in CDCl_3 using tetramethylsilane as an internal standard. IR spectra were obtained using a Beckman Acculab spectrophotometer. Mass spectrum of **V** was obtained from Mass Consortium Corp., San Diego, CA.

Succinimidyl N-[3-(Acetylthio)-3-methylbutyryl]-glycinate (V, Scheme 1). By adapting a procedure for the synthesis of an antibody-toxin cross-linker (**11**), analogous reagent **V** was prepared by starting with glycine *tert*-butyl ester and dimethylacryloyl chloride, in a 11.3% unoptimized isolated yield. **V**: Off-white solid. IR (Nujol): λ 3260, 1810, 1780, 1730, 1680, 1640 cm^{-1} . ^1H NMR: δ 4.40 (d, 2H, $J = 5.6$), 2.86 (s, 2H), 2.84 (s, 4H), 2.25 (s, 3H), 1.52 (s, 6H). Electrospray mass spectrometry: m/e 331 ($\text{M}^+ + \text{H}$); exact mass calcd 353.0783 ($\text{M}^+ + \text{Na}$), found 353.0776.

Conjugation of V to MAB Fragments (Scheme 1). A conjugation of MN-14 F(ab')_2 is illustrative: A solution of the antibody fragment (885 μL ; 6.26 mg) in 0.1 M phosphate buffer (pH 7.5) was mixed with a 4-fold molar excess of a DMF solution of **V**, and the clear solution was left at room temperature for 2 h. The conjugate was purified by centrifuged-SEC (12) on Sephadex G-50/80 in 0.1 M sodium phosphate (pH 7). Thiol deprotection was carried out by incubating with 20% v/v of 1 M

hydroxylamine pH 8 for 10 min at room temperature in an argon atmosphere. The product was purified on a centrifuged-SEC (Sephadex G-50/80, 50 mM ABS buffer (pH 5.3)). The eluate was analyzed for concentration by absorbance at 280 nm and for thiol content by Ellman's assay using 10 mM DTNB (13).

SDS-PAGE Analysis. Each unlabeled conjugate (5 μg) was applied to a lane of precast 4–20% polyacrylamide gradient gel containing 0.1% sodium dodecyl sulfate (Daiichi Pure Chemicals Co., Tokyo, Japan) and electrophoresed at 30 mA per gel at room temperature (nonreducing conditions). Gels were fixed and stained with Coomassie brilliant blue R-250 in methanol–acetic acid–water (5:1:4) mixture. Thiol groups in samples were blocked with iodoacetamide (1.6 mM) for 0.5 h at 37 $^\circ\text{C}$, prior to electrophoresis. Unmodified F(ab')_2 's and molecular weight standards (Bio-Rad Laboratories, Hercules, CA) were controls.

^{99m}Tc Labelings. Method 1. A Glucoscan vial (DuPont-Merck Pharmaceutical Co., Billerica, MA) of tin glucoheptonate, containing 200 mg of sodium gluceptate and 60–140 μg of stannous chloride, was reconstituted with 2 mL of degassed saline. This glucoheptonate–tin mixture (20 μL ; tin content: 1.4 μg ; GH:tin ratio $\approx$ 1000) was dispensed into a 0.5-mL Eppendorf tube. To this was added 30 μL of [^{99m}Tc]pertechnetate, which was either obtained by saline elution of $^{99}\text{Mo}/^{99m}\text{Tc}$ generator (New England Nuclear, Wilmington, DE) or purchased as unit doses (Syncor, Fairfield, NJ). This mixture was vortexed and allowed to incubate at room temperature for 15 min. Freshly deprotected F(ab')_2 conjugate (200 μg), at 4–7 mg/mL (pH 5.3), was dispensed into an argon-purged Eppendorf tube and was mixed with one-tenth its volume of 0.5 M sodium acetate (pH 6.0). The conjugate was mixed with 1 mCi of ^{99m}Tc -GH (tin content: 0.2–4 μg), incubated at room temperature for 15–60 min, and then diluted to 1 mL with degassed saline. An aliquot was appropriately diluted and analyzed by HPLC (0.5–1 μCi injected). A control experiment was done by subjecting 200 μg of unmodified MN-14 F(ab')_2 to ^{99m}Tc -GH transchelation conditions and analyzing for nonspecific incorporation by HPLC.

Method 2. The conjugate (1 mg) in a 2-mL vial was mixed with 2 M sucrose such that the final concentration of sucrose was 100–300 mM, lyophilized, and stored under vacuum at 4 $^\circ\text{C}$. Separately, a lyophilized formulation of tin glucoheptonate, containing 25 μg of tin at a GH:Sn ratio of 250, was prepared. This formulation (pH 5.43) was a mixture of glucoheptonate (200 mM)–sodium acetate (21 mM) (pH 5.3, 5.08 mL), 2.5 mg/mL tin solution (pH 5.3, 0.2 mL), 50 mM ABS (0.42 mL), and 1 M sodium acetate (pH 6, 0.3 mL), aliquoted in 0.3-mL lots in 2-mL vials, lyophilized, and septum-sealed under vacuum. Other formulations, with GH:Sn ratios in the 100–300 range, were also tested. ^{99m}Tc labelings were carried out by first adding [^{99m}Tc]pertechnetate into the tin glucoheptonate vial, incubating for 10 min, and transferring the ^{99m}Tc -GH so formed into the vial containing the lyophilized formulation of the conjugate. The labeling was analyzed by HPLC after a 30–60-min incubation. A control experiment involved labeling 1 mg of unmodified MN-14 F(ab')_2 [buffer-exchanged with 50 mM ABS (pH 5.3)] with preformed ^{99m}Tc -GH. The latter was prepared using a lyophilized formulation of stannous glucoheptonate containing 25 μg of tin at GH:Sn ratio of 250.

Method 3. Lyophilized Formulation for Single-Vial ^{99m}Tc Labeling. Because of its availability in quantity, only murine MN-14 F(ab')_2 was examined under a variety of single-vial formulation conditions. A stan-

² The divalent fragment of murine MN-14 antibody is therefore F(ab')_2 , not F(ab')_2 . The corresponding monovalent fragment is likewise identified as Fab. Except when specifically referred to fragments of MN-14, divalent and monovalent MAB fragments are generally designated as F(ab')_2 and Fab', respectively.

ard tartrate formulation (6) with 25 μg of tin (Sn) and a tartrate:tin ratio of 6.5 was first tested. Freshly thiol-deprotected MN-14 F(ab)₂ conjugate (200 μg or 1 mg) was mixed with a solution of 25 μg of tin(II) in 150 μL of tartrate (9.2 mM)–acetate (50 mM)–sodium chloride (150 mM) buffer, 1 M sucrose was added to a final concentration of 0.1 M, and the mixture was lyophilized in a 2-mL glass vial. In addition, various formulation conditions were tested which used glucoheptonate, in lieu of tartrate, in various GH:Sn ratios in order to simulate the labeling obtained in the two-step procedure using ^{99m}Tc-GH described above. For a GH:Sn ratio of 250 with 25 μg of tin, 200 μg (or 1 mg) of freshly thiol-deprotected MN-14 F(ab)₂ was mixed with a solution of 25 μg of tin(II) in 269 μL of glucoheptonate–acetate (200/21 mM) buffer, the solution was made 0.1 M in sucrose, and the mixture was lyophilized. For other GH:Sn ratios (17 $\times$ to 500 $\times$) and tin amounts (5–66 μg), glucoheptonate and tin were varied appropriately. ^{99m}Tc radiolabeling was performed by adding to the lyophilized vial 1 mL of saline-diluted [^{99m}Tc]pertechnetate generator–eluate. A specific activity of 5 mCi/mg was typically employed. The labeling was usually performed on the formulated material after the lyophilization step. A control experiment involved preparation of a lyophilized formulation of 200 μg of unmodified MN-14 F(ab)₂ with 25 μg of tin (“tartrate” formulation) and subsequent analysis for any nonspecific ^{99m}Tc incorporation.

Analyses of Radiolabelings. Radiolabeled preparations were routinely analyzed by HPLC on an analytical Bio-Sil 250 size-exclusion column, in series with a guard column (both from Bio-Rad Laboratories, Hercules, CA), using 0.2 M phosphate buffer (pH 6.8) (1 mL/min) as the mobile phase, with in-line UV (280 nm) and/or radioactivity detection. In each case, the retention time of the labeled antibody was found to correspond to that of the corresponding unlabeled divalent fragment (9.5–11 min). The HPLC system employed clearly separated the labeled protein from any aggregate, ^{99m}Tc-Fab’ which eluted about 1 min later than ^{99m}Tc-F(ab’)₂, ^{99m}Tc-glucoheptonate (or ^{99m}Tc-tartrate) which eluted at 13–14 min, and [^{99m}Tc]pertechnetate eluting at 16 min.

Colloidal precipitation was determined by ITLC analyses on silica gel impregnated glass fiber strips (Gelman Sciences, Ann Arbor, MI). The labeled material was co-spotted with human serum albumin (HSA) and developed with a 5:2:1 water–ethanol–ammonia mixture. In this system all but colloidal radioactivity moved to the eluent front.

Immunoreactivity (IMR). IMR for ^{99m}Tc-MN-14 F(ab)₂ or ^{99m}Tc-hMN-14 F(ab)₂ was performed by mixing 0.5 μCi of labeled conjugate with an 80-fold molar excess of CEA (Scripps Clinic, La Jolla, CA) in 1% HSA, incubating for 10 min at room temperature, and injecting onto the HPLC.

In Vitro Stability. Serum stability was performed using a ^{99m}Tc-MN-14 F(ab)₂ preparation at a concentration of 0.2 mg/mL. This study was performed in duplicate by adding 33 μL of the labeled conjugate to 0.5 mL of fresh human serum, incubating at 37 °C, and examining aliquots by HPLC analysis at various times. The concentration of the conjugate in serum was 123.8 nM. ^{99m}Tc-labeled MN-14 F(ab)₂, prepared by method 2, using lyophilized formulations of the conjugate (1 mg) and of stannous glucoheptonate (25 μg of tin, GH:Sn = 250), was used for three other stability studies as described below. The integrity of the labeled conjugate, contained in the labeling vial under argon or partial vacuum, was determined 4.5 and 24 h post-labeling. A cysteine challenge was performed by adding to ^{99m}Tc-labeled material a 1

mg/mL stock solution of cysteine in 0.1 M phosphate buffer (pH 7.5) such that the final concentration of cysteine was 1 mM and cysteine was in a 500-fold molar excess. The solution was incubated at room temperature and analyzed at 30 min and 1 h by HPLC. Stability to HSA was determined by adding 100 μL of the labeled material to 400 μL of 5% HSA in an argon-purged vial and analyzing after 18 h.

Animal Biodistributions. Outbred, female, athymic nude mice (ν/ν), purchased at 3–4 weeks of age (Harlan, Madison, WI), were used in these studies after 1 week of quarantine. The LS174T cell tumor xenograft nude mouse model (14) was used for animal experiments. Tumor cells were grown in cell culture, prior to subcutaneous or intramuscular injection of 5×10^6 cells into nude mice. Conjugates were labeled using preformed ^{99m}Tc-GH (method 1) and injected into mice for biodistribution studies. For each experiment, 200 μg of freshly deprotected F(ab)₂/F(ab’)₂ conjugate was labeled with 2 mCi of ^{99m}Tc-glucoheptonate in a final volume of 1 mL. ^{99m}Tc-MN-14 Fab was prepared by labeling a lyophilized preparation (200 μg of Fab’ 25 μg of tin, tartrate:tin ratio of 6.7), in one step, using 2 mCi of [^{99m}Tc]pertechnetate in a volume of 1 mL. Each radiolabeled preparation (50 μL ; 10 μg) was injected intravenously into each of 10 animals; five animals were sacrificed at 4 h, and five at 24 h. The animals were anesthetized, bled by cardiac puncture, and then killed by cervical dislocation. Organs were excised, weighed, and counted for radioactivity in a γ -counter. The percentage of the injected dose per gram of tissue (%ID/g) and tumor-to-tissue ratio were then calculated for each organ.

RESULTS

The thiolating agent **V** was prepared in four steps as shown in (Scheme 1). Briefly, the sequence involves acylation of glycine *tert*-butyl ester with 3,3-dimethylacryloyl chloride, followed by 1,4-addition of thiolacetic acid, removal of *tert*-butyl ester group, and activation of carboxylic acid as succinimide ester. Reagent **V** was stored in an argon-flushed vial in a freezer. No decomposition has been observed in over 18 months to date.

A number of MAb F(ab’)₂ fragments were thiolated using **V** to demonstrate wide applicability. Conjugates were prepared of the divalent fragments of murine CEA antibody MN-14 (10), its humanized version hMN-14 (15), CSAP antibody Mu-9 (16), pan-carcinoma antibody RS-7 (17), non Hodgkin’s lymphoma antibody LL2 (18), and infectious disease imaging antibody MN-3 (19).

For the two-step thiolation, the antibody concentration in the range of 5.5–15.0 mg/mL, 4–6 molar excess of **V**, a final DMF concentration of about 5%, and a pH of 7.5–7.7 were typically employed. Thiol deprotection was carried out using aqueous hydroxylamine (20). Antibody recovery in the two-step procedure, after two sets of two successive purifications on centrifuged SEC columns, was in the range of 65–75% (>90% recovery after each purification). The number of thiol groups introduced into different F(ab’)₂ fragments was in the 1.1–2.6 range (not shown).

On nonreducing SDS–PAGE, the patterns for protected and deprotected conjugates were quite similar to those for the unprotected conjugates (Figure 1). It should also be noted that, when lyophilized vials of murine antibody (MN-14 F(ab)₂, 300 $\times$ GH:Sn, 25 and 35 μg of Sn) were resuspended in 0.1 M sodium phosphate (pH 7.0) and blocked with iodoacetamide, these showed no evidence of Fab being generated by the amounts of tin used. The only occasion when Fab’ formation was observed was when a particularly impure lot of hMN-14

Table 1. Representative ^{99m}Tc Labelings of Thiolated Antibody Divalent Fragments^a

conjugate		labeling information			% ^{99m}Tc incorporations (HPLC)			
F(ab) ₂ /F(ab') ₂	SH/conj	concn (mg/mL)	method no. ^b	SA (mCi/mg)	bound		unbound	
					agg	F(ab') ₂	GH	TcO ₄ ⁻
MN-14	1.0	0.2	1	5.2		91.4	1.7	6.9
	2.0	1.0	2	2.5	0.6	91.5	6.0	1.9
	2.0	1.0	2	7.4	2.1	88.0	6.2	3.1
	1.7	1.0	3	5.0		91.5		5.7
	2.0	0.2	3	5.0		90.0	8.0	2.0
	2.0	0.2	3	5.0		89.0	11.0	0
MN-3	1.8	1.0	2	8.8	3.4	91.0	4.3	1.3
	2.1	1.0	2	5.9	1.4	93.0	4.9	0.5
Mu-9	2.6	1.0	2	10.0	4.8	89.0	3.9	2.3
hMN-14	2.2	3.7	1	5.0	5.0	89.0	1.0	5.0
RS-7	1.8	1.8	1	5.0	3.0	88.8	5.3	3.0
control ^c	0	3.3	1	5.0		0	27.4	72.6
control ^{c,d}	0	1.0	3	5.0		0.9	71.7	27.2
control ^c	0	1.0	2	1.8		1.5	96.0	2.5

^a Data obtained 15–60 min post-labelings. ^b No. 1: labeled 0.2 mg with preformed ^{99m}Tc -glucoheptonate (Tc-GH), tin content was 1–4 μg . No. 2: lyophilized formulations of Sn-GH (25 μg of tin at GH:Sn = 250) and conjugate (1 mg) used for two-step labeling. No. 3: reconstituted single-vial lyophilized formulation (1 mg of conjugate, 25 μg of tin, GH:Sn = 100–300) with 1 mL of [^{99m}Tc]pertechnetate. ^c MN-14 F(ab)₂. ^d Tartrate as transfer ligand in lieu of GH.

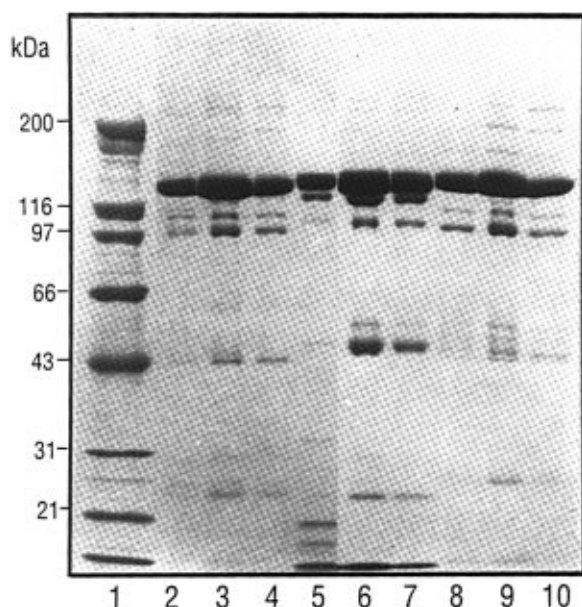


Figure 1. Nonreducing SDS-PAGE of thiolated F(ab)₂ fragments on a 4–20% polyacrylamide gel, with lane descriptors as follows: molecular weight standards (lane 1), unmodified and thiol-protected and thiol-deprotected conjugates of MN-14 F(ab)₂ (lanes 2–4), hMN-14 F(ab)₂ (lanes 5–7), and Mu-9 F(ab)₂ (lanes 8–10).

F(ab')₂, which already contained some lower molecular weight chains, was used.

^{99m}Tc labelings were performed using three different formats. Details of labelings and incorporations are given in Table 1. Method 1, involving transchelation with ^{99m}Tc -glucoheptonate, at a specific activity of 5 mCi/mg, produced typically over 90% incorporation into the antibody, with the remainder distributed among reduced ^{99m}Tc (Tc-GH) and [^{99m}Tc]pertechnetate. A control experiment gave no ^{99m}Tc incorporation into unmodified F(ab)₂, with HPLC analysis showing only the presence of ^{99m}Tc -GH at 13.9 min, along with the pertechnetate peak at 16.2 min (Table 1).

Method 2 is a modification of the procedure described above, which enabled the use of lyophilized formulations in place of freshly prepared conjugates. The control experiment in this category gave incorporations of 1.5% into the antibody, 96% as ^{99m}Tc -GH, and 2.5% as [^{99m}Tc]pertechnetate.

Method 3 involved ^{99m}Tc labelings using one-vial lyophilized formulations of the MN-14 F(ab)₂ conjugate and stannous glucoheptonate. The best ^{99m}Tc labeling was obtained with one-vial formulations of the conjugate with 25–35 μg of tin at a GH:Sn ratio of 100–300. These conditions reproducibly gave about 90% incorporation, the rest being predominantly reduced ^{99m}Tc -GH with a little aggregation in some instances. Figure 2 shows HPLCs of ^{99m}Tc labelings of MN-14 F(ab)₂ conjugate labeled by method 3 (top), and of MN-14 Fab labeled by direct method (bottom). A difference of about 1 min in the retention times, observed for this pair, is also noted with F(ab')₂ and Fab' derived from other antibodies. A control experiment involving standard tartrate/tin formulation of unmodified MN-14 F(ab)₂ gave 0.9% of ^{99m}Tc incorporation.

A small percentage of aggregate (usually <5%) is observed in the radiolabelings which eluted from the HPLC column about 1 min earlier than ^{99m}Tc -F(ab)₂. No Fab' formation was observed during labeling, except when the antibody preparation itself contained a small percentage of the monovalent fragment as contaminant. Analytical HPLC conditions, using radioactivity detection, clearly separated higher MW species, ^{99m}Tc -F(ab')₂ fragment, ^{99m}Tc -Fab', ^{99m}Tc -GH, and [^{99m}Tc]pertechnetate, thereby enabling quantifications.

Colloidal precipitation was less than 1% in radiolabelings. In some initial experiments, the assay employed tended to give a streaking pattern on ITLC and overestimate the percent colloid (by as much as 5%), if insufficient HSA was used. This problem was subsequently obviated.

Immunoreactivity, determined for ^{99m}Tc -mMN14 F(ab)₂ and ^{99m}Tc -hMN14 F(ab')₂ by a CEA complexation assay, was 90–95%, as noted by the shift of the radioactivity peak on HPLC from the retention time of the ^{99m}Tc -F(ab')₂ to near the void volume of the column, signifying the formation of an antibody:antigen complex.

Stability studies were performed on ^{99m}Tc -MN-14 F(ab)₂. The latter maintained its integrity in the labeling vial, with only 6.4% reoxidation to pertechnetate seen at 24 h post-labeling. ^{99m}Tc -MN-14 Fab formation was not observed at least out to 4.5 h post-labeling, but at 24 h, about 20% of the same was indicated by HPLC analysis. In HSA, more than 80% of ^{99m}Tc -F(ab)₂ could be complexed with CEA after 18 h of incubation, as judged by HPLC analyses. In fresh human serum, reoxidation to pertechnetate was not seen, but loss of 56.2%, 52.7%, and

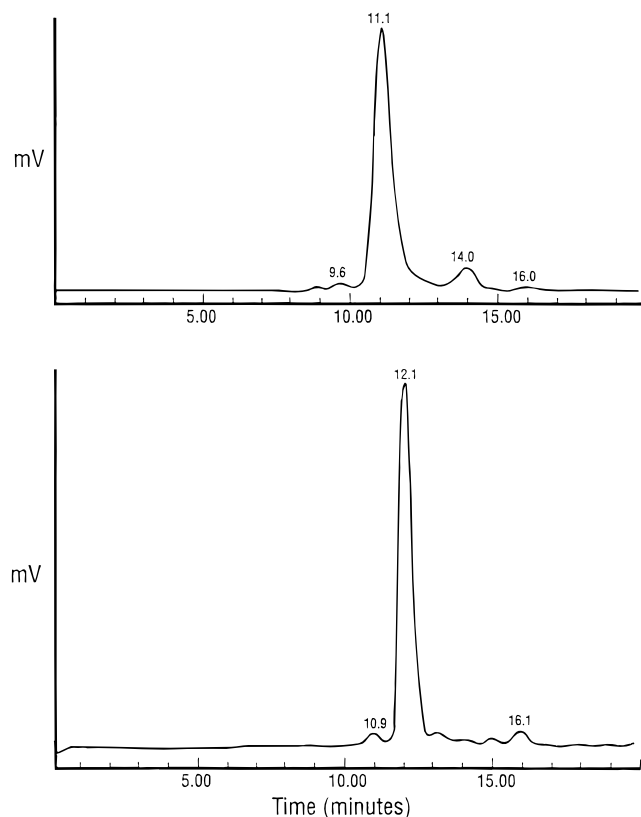


Figure 2. (top) HPLC analysis (radioactivity trace) of labeling of a single-vial formulation, containing 1 mg of thiolated MN-14 F(ab)₂ and 25 μ g of tin, which was reconstituted with 1 mL of [^{99m}Tc]pertechnetate. A 91% ^{99m}Tc incorporation for F(ab)₂ (11.1 min) is seen, the remainder being mainly glucoheptonate (14.0 min) with negligible aggregate (9.6 min) and pertechnetate (16.0 min). (bottom) HPLC analysis of ^{99m}Tc labeling of MN-14 Fab, showing the main peak due to ^{99m}Tc Fab (12.1 min). ^{99m}Tc-F(ab)₂ (10.9 min) and [^{99m}Tc]pertechnetate (16.1 min) are the other two peaks identified.

53.6% of ^{99m}Tc to low molecular weight components was observed at 1, 4, and 24 h post-incubation, respectively. In challenge with 1 mM cysteine, loss of label to cysteine was determined to be 38% at 30 min and 39% at 1 h. Under HPLC conditions used, ^{99m}Tc-cysteine appeared as a peak at 15 min.

In a baseline experiment (not shown), biodistributions of ^{99m}Tc-MN-14 F(ab)₂ conjugate and ^{99m}Tc-MN-14 Fab in normal BALB/c mice were examined. At 24 h, 10.4% ID/g of ^{99m}Tc-F(ab)₂ and 27.9% ID/g of ^{99m}Tc-Fab, respectively, localized to the kidney.

In the second experiment, three ^{99m}Tc-labeled F(ab)₂/F(ab')₂ conjugates, MN-14, hMN-14, and Mu-9, were compared with ^{99m}Tc-MN-14 Fab. Animal data are set forth in Tables 2 and 3. Biodistributions and tumor:nontumor ratios were found to be similar to those in Table 2 when a preparation of ^{99m}Tc-MN-14 F(ab)₂ using a lyophilized formulation of 1 mg of thiolated antibody and stannous glucoheptonate (25 μ g of tin, GH:Sn = 100) was examined in LS174T nude mouse model (data not shown).

DISCUSSION

We have been interested in developing a simple method to produce ^{99m}Tc-labeled MAb divalent fragments which are capable of generating useful target:nontarget discrimination in *in vivo* applications. Other workers have shown that indirect labeling through thiol-based bifunctional chelating agents gives well-defined products but necessitates a multistep strategy (21). When used with

MAb fragments, N₃S-type or, alternatively, the hydrazinopyridine chelating agents have required the incorporation of special cleavable linkers between chelate and protein in order to reduce nontarget accumulation of radioactivity (22, 23). Derivatization of antibodies with metallothionein (a ^{99m}Tc-chelating entity) has been reported to lead to aggregation, requiring careful preparative purification (24). Direct labeling methods, applicable to the ^{99m}Tc labeling of an IgG or a Fab' fragment, are problematic with F(ab')₂ fragments in that substantial formation of ^{99m}Tc-Fab' occurs under labeling conditions (25–27). A conceptually new direct labeling, by Griffiths et al. (28), involved thiol-reducing an IgG and pepsin-digesting the resultant product to a sulfhydryl-containing F(ab')₂ that was used for ^{99m}Tc labeling.

We have developed a new thiolation approach to label divalent antibody fragments with ^{99m}Tc. Use of 2-iminothiolane (2-IT) for protein thiolation can lead to up to 50% of aggregate formation, unless the thiol group generated in the reaction is further derivatized *in situ* (29). Therefore, we investigated a different approach involving controlled introduction of thiol groups onto the divalent fragments. Modeled on a cross-linker used for preparing an antibody–toxin conjugate (11), an analogous thiolating agent **V** was examined for the present study. The 1,5-relationship of the *tert*-thiol and the amide nitrogen enables a six-membered chelate ring formation when complexed with reduced ^{99m}Tc, with an additional five-membered chelate ring formation feasible with the antibody amino group involved in the prior conjugation step.

MAb divalent fragments were derivatized with **V** using conditions under which the *tertiary* thiolacetate group was not affected. Thiol deprotection in a subsequent step enabled facile postlabeling of F(ab')₂ fragments with reduced ^{99m}Tc. The preparation of the thiolating agent has been a one-time effort, as numerous thiolations have been carried out using the same batch of **V**. The use of thiol-protected thiolation reagent essentially eliminates aggregate formation at the thiolation stage, as evidenced by HPLC analyses (not shown) as well as by SDS–PAGE analyses. Also, SDS–PAGE analyses did not show evidence of fragmentation of F(ab')₂ to Fab' during thiolation of fragments, thiol deprotection, or formulations with stannous chloride.

^{99m}Tc labeling has been achieved typically with about 90% incorporation into the fragment, with an additional few percent incorporation as aggregate. The aggregated protein was also immunoreactive, as judged by the HPLC analysis of the antigen:conjugate complex. Total ^{99m}Tc incorporation into the antibody, therefore, was usually greater than 90%. The small amount of aggregate formation at the radiolabeling stage, which is likely to be a dimeric species based on the HPLC retention time, did not result in a marked increase in hepatic uptake in animal studies. Control experiments in both single-vial and two-vial formats of radiolabeling demonstrated negligible nonspecific ^{99m}Tc incorporation into the antibody.

The specific activities examined during this study were in the 2–10 mCi/mg range. Recently, Larsen et al. (30) have found a pronounced improvement of achievable specific activity in the ^{99m}Tc labeling of a hydrazinonitricotinate (SHNH)–IgG conjugate (previously labeled only in low specific activity) by changing the ^{99m}Tc transfer ligand from glucoheptonate (GH) to tricine. At a specific activity of 1.5 mCi/mg, they found that ^{99m}Tc-GH gave $\approx$ 90% labeling of SHNH–IgG in 1 h, but ^{99m}Tc-tricine yielded near quantitative incorporation in about 10 min. Also, using tricine as the transfer ligand, greater than

Table 2. Biodistributions of ^{99m}Tc -MN-14 F(ab) $_2$ and ^{99m}Tc -MN-14 Fab, in Nude Mice Bearing LS174T Human Colon Carcinoma Xenografts, at 4 and 24 h Post-injection (%ID/g $\pm$ SD; $n = 5$)

tissue	time (h)	^{99m}Tc -MN-14 F(ab) $_2$		^{99m}Tc -MN-14 Fab	
		%ID/g $\pm$ SD	T/NT ratio	%ID/g $\pm$ SD	T/NT ratio
LS174T tumor	4	3.57 $\pm$ 0.67		3.03 $\pm$ 0.07	
	24	6.25 $\pm$ 1.31		2.90 $\pm$ 0.80	
liver	4	2.62 $\pm$ 0.41	1.39 $\pm$ 0.37	2.06 $\pm$ 0.30	1.50 $\pm$ 0.25
	24	1.21 $\pm$ 0.17	5.13 $\pm$ 0.60	1.50 $\pm$ 0.26	2.09 $\pm$ 0.76
spleen	4	1.23 $\pm$ 0.35	3.12 $\pm$ 1.10	1.92 $\pm$ 0.51	1.70 $\pm$ 0.60
	24	0.80 $\pm$ 0.14	7.76 $\pm$ 0.48	1.67 $\pm$ 0.36	1.90 $\pm$ 0.68
kidney	4	15.46 $\pm$ 1.92	0.23 $\pm$ 0.06	46.65 $\pm$ 6.22	0.07 $\pm$ 0.01
	24	9.22 $\pm$ 0.64	0.68 $\pm$ 0.14	27.53 $\pm$ 3.90	0.11 $\pm$ 0.02
lungs	4	5.04 $\pm$ 0.92	0.74 $\pm$ 0.25	10.20 $\pm$ 7.28	0.65 $\pm$ 0.62
	24	1.68 $\pm$ 0.66	4.35 $\pm$ 2.15	3.23 $\pm$ 2.60	3.11 $\pm$ 3.08
blood	4	4.83 $\pm$ 0.76	0.75 $\pm$ 0.19	1.37 $\pm$ 0.32	2.31 $\pm$ 0.59
	24	0.97 $\pm$ 0.14	6.44 $\pm$ 1.18	0.39 $\pm$ 0.09	7.99 $\pm$ 3.26
stomach	4	10.13 $\pm$ 3.96	0.39 $\pm$ 0.17	1.06 $\pm$ 0.61	3.85 $\pm$ 2.28
	24	2.25 $\pm$ 0.75	3.00 $\pm$ 1.02	0.54 $\pm$ 0.20	7.19 $\pm$ 6.15
sm int	4	2.51 $\pm$ 0.63	1.48 $\pm$ 0.43	0.75 $\pm$ 0.19	4.32 $\pm$ 1.37
	24	0.50 $\pm$ 0.09	12.48 $\pm$ 1.77	0.24 $\pm$ 0.04	13.19 $\pm$ 5.37
lg int	4	4.49 $\pm$ 1.05	0.81 $\pm$ 0.18	1.97 $\pm$ 0.48	1.61 $\pm$ 0.41
	24	2.57 $\pm$ 0.39	2.45 $\pm$ 0.51	0.94 $\pm$ 0.37	3.43 $\pm$ 1.34
bone	4	0.80 $\pm$ 0.08	4.51 $\pm$ 0.99	0.64 $\pm$ 0.09	4.83 $\pm$ 0.86
	24	0.66 $\pm$ 0.10	9.60 $\pm$ 2.03	0.80 $\pm$ 0.13	3.76 $\pm$ 0.71

Table 3. Biodistributions of ^{99m}Tc -hMN-14 F(ab') $_2$ and ^{99m}Tc -Mu-9 (Fab') $_2$, in Nude Mice Bearing LS174T Human Colon Carcinoma Xenografts, at 4 and 24 h Post-injection (%ID/g $\pm$ SD; $n = 5$)

tissue	time (h)	^{99m}Tc -hMN-14 F(ab') $_2$		^{99m}Tc -Mu-9 (Fab') $_2$	
		%ID/g $\pm$ SD	T/NT ratio	%ID/g $\pm$ SD	T/NT ratio
LS174T tumor	4	2.33 $\pm$ 0.60		1.94 $\pm$ 0.35	
	24	2.94 $\pm$ 1.20		3.92 $\pm$ 1.99	
liver	4	2.99 $\pm$ 0.52	0.78 $\pm$ 0.14	1.34 $\pm$ 0.15	1.44 $\pm$ 0.12
	24	1.62 $\pm$ 0.40	1.78 $\pm$ 0.38	1.31 $\pm$ 0.49	3.85 $\pm$ 3.15
spleen	4	3.13 $\pm$ 1.30	0.79 $\pm$ 0.19	0.75 $\pm$ 0.11	2.61 $\pm$ 0.40
	24	2.42 $\pm$ 0.62	1.21 $\pm$ 0.33	2.32 $\pm$ 1.42	3.43 $\pm$ 3.62
kidney	4	20.66 $\pm$ 3.40	0.11 $\pm$ 0.02	15.87 $\pm$ 1.94	0.12 $\pm$ 0.01
	24	8.63 $\pm$ 0.81	0.33 $\pm$ 0.11	9.29 $\pm$ 1.17	0.42 $\pm$ 0.22
lungs	4	1.49 $\pm$ 0.18	1.56 $\pm$ 0.31	1.26 $\pm$ 0.34	1.71 $\pm$ 0.92
	24	0.53 $\pm$ 0.10	5.37 $\pm$ 1.32	0.60 $\pm$ 0.12	6.32 $\pm$ 2.19
blood	4	2.31 $\pm$ 0.31	1.01 $\pm$ 0.22	2.65 $\pm$ 0.36	0.73 $\pm$ 0.10
	24	0.21 $\pm$ 0.01	13.88 $\pm$ 5.09	0.41 $\pm$ 0.29	11.30 $\pm$ 3.70
stomach	4	2.51 $\pm$ 0.66	0.99 $\pm$ 0.36	2.55 $\pm$ 0.83	0.80 $\pm$ 0.18
	24	0.35 $\pm$ 0.18	8.84 $\pm$ 2.13	0.67 $\pm$ 0.25	5.94 $\pm$ 2.43
sm int	4	1.76 $\pm$ 0.37	1.36 $\pm$ 0.39	0.96 $\pm$ 0.18	2.06 $\pm$ 0.44
	24	0.51 $\pm$ 0.13	5.59 $\pm$ 1.16	0.45 $\pm$ 0.14	9.75 $\pm$ 6.21
lg int	4	1.76 $\pm$ 0.22	1.34 $\pm$ 0.38	3.22 $\pm$ 0.51	0.61 $\pm$ 0.11
	24	0.96 $\pm$ 0.37	3.05 $\pm$ 0.52	1.34 $\pm$ 0.58	3.27 $\pm$ 1.63
bone	4	1.48 $\pm$ 0.39	1.63 $\pm$ 0.44	0.48 $\pm$ 0.05	4.12 $\pm$ 0.86
	24	1.55 $\pm$ 0.30	1.88 $\pm$ 0.65	1.28 $\pm$ 0.55	4.19 $\pm$ 4.22

90% incorporation in 1 h was observed at a specific activity of up to 140 mCi/mg. These findings inspire confidence for achieving similar higher specific activity radiolabelings of our thiolated antibodies.

The ^{99m}Tc -F(ab) $_2$ preparation was found to be stable to reoxidation when contained under argon or partial vacuum in the labeling vial and was practically inert to HSA. Some ^{99m}Tc -Fab' formation was observed, *in vitro* at 24 h, when 25 $\mu\text{g/mL}$ of tin was used in labeling (method 3). This should be inconsequential since the labeled material would be expected to be utilized for *in vivo* applications within a few hours of postlabeling. *In vitro* incubation of the labeled material in fresh human serum showed that the label was successfully challenged by low-MW serum components such as cysteine, although about 50% of the label was still associated with the antibody after 24 h. When using frozen serum from stock, however, loss to lower MW serum components ranged from a few percent to none (data not shown), which may be attributable to oxidative loss of serum cysteine and glutathione over extended serum storage. Serum age is obviously an important factor in this type of study. Loss of 39% at 1 h to a 500-fold molar excess of cysteine, adapting a known assay (31), is similar to

that observed with 2-IT-modified ^{99m}Tc -labeled proteins (32, 33), as might be expected.

For RAID, what is most relevant is the relative *in vivo* retention of radioactivity in tumor versus nontarget organs, the tumor:nontumor ratios. A ^{99m}Tc -labeled 2-IT-modified IgG was reported to lose 50% of label in 4 h to *in vitro* cysteine challenge (33), but a kit similar to 2-IT-derivatized IgG was used in one of the early clinical trials of ^{99m}Tc -IgG with encouraging results (34). Indeed, it has been pointed out that moderate *in vivo* instability can be an advantage for imaging purposes, if the target:nontarget ratios are improved thereby (35, 36). The animal data reported here with the new agents lend support to this hypothesis.

The 24-h kidney uptake (%ID/g), in tumor-bearing mice, of ^{99m}Tc -labeled MAb Fab' fragment is in the 30–40% range, based on historical data on a number of MAb Fab' fragments from our laboratory. The data obtained with ^{99m}Tc -MN-14 Fab, in normal and tumor bearing mice, in the present study are consistent with this. For each of the three ^{99m}Tc -labeled MAb divalent fragments examined, the 24-h kidney uptake has been consistently about 10% ID/g. That is, a 3-fold reduction in the kidney uptake of ^{99m}Tc -F(ab) $_2$ versus ^{99m}Tc -Fab has been achieved

in *in vivo* studies with normal and tumor-bearing mice. Tumor:kidney ratios obtained with this $^{99m}\text{Tc-F(ab')}_2$ are comparable to those reported for a cleavable-linker-incorporated Fab' (22).

Reduction in the kidney uptake of radioactivity has occurred without a concomitant diversion of radioactivity into another nontarget organ such as liver. For instance, enhancements of tumor:kidney ratio from 0.1 to 0.7, of tumor:liver ratio from 2 to 5, of tumor:spleen ratio from 1.9 to 7.8, of tumor:lung ratio from 3.1 to 4.4, and of tumor:bone ratio from 3.8 to 9.6 are observed concurrently (24-h time point), with high tumor:nontumor ratios for most other tissues maintained, in going from $^{99m}\text{Tc-MN-14 Fab}$ to $^{99m}\text{Tc-MN-14 F(ab')}_2$ (Table 2). Stomach uptake of radioactivity, due to a small percentage of pertechnetate present in the preparation, produces a comparatively lower ratio. In a subsequent experiment using $^{99m}\text{Tc-MN-14 F(ab')}_2$, incorporation in stomach and tumor:stomach ratio of radioactivity at 24 h were <1% and 6.4, respectively, while incorporations and tumor:nontumor ratios for other organs were similar to those in Table 2 (data not shown).

In addition to these ratios which are important for radioimmunodetection, a 2-fold increase in the 24-h tumor uptake for F(ab')_2 compared to Fab was consistently observed in the comparable pair using MN-14 (Table 2, and other data not shown), leading to a 6-fold greater tumor:kidney ratio. In each of the three F(ab')_2 's examined, it is significant that the tumor uptake shows a net increase from 4 to 24 h, while that in nontarget organs decreases.

The lability of the label *in vivo*, as reflected in clearance patterns, results in a lower absolute uptake of radioactivity in tumor than could be expected of a true tracer. This tradeoff, which indeed results in the useful tumor:nontumor ratios observed, is related to the nature of the ^{99m}Tc -antibody bond. Unlike in very stable chelates, the ^{99m}Tc label in these conjugates is complexed primarily to a single thiol group and is, not surprisingly, subject to challenge *in vivo*. Transchelation of labile ^{99m}Tc label by endogenous cysteine (and glutathione), followed by the excretion of Tc-cysteine metabolite, has been proposed as a mechanism of the label loss (35). Cysteine and glutathione levels in plasma are low, but their intracellular concentrations are comparatively much higher (37). ^{99m}Tc -labeled conjugate bound to the tumor cell surface may be more inert to challenge by intracellular cysteine and glutathione than conjugate in normal organs, and this could explain the observed tumor:nontumor ratios. That there was still about 1% ID/g of radioactivity in blood at 24 h *in vivo*, as observed with $^{99m}\text{Tc-MN-14 F(ab')}_2$, showed that not all of the radioactivity was challenged readily. This is probably due to the presence of different ^{99m}Tc -thiol centers in the conjugate, some more exposed (and thereby challenged) than others. Of the two murine antibody fragments examined, $^{99m}\text{Tc-MN-14 F(ab')}_2$ showed slower clearance and higher tumor uptake than $^{99m}\text{Tc-Mu-9 F(ab')}_2$.

The method of producing $^{99m}\text{Tc-F(ab')}_2$ radioimmunoconjugates by the thiolation approach is simple and general. Reduced nontarget uptake of radioactivity (especially in kidney) also seems general, based on two

murine and one humanized fragments studied in normal mice and one nude mouse tumor model. Superiority of $^{99m}\text{Tc-MN-14 F(ab')}_2$ over the corresponding $^{99m}\text{Tc-Fab}$ fragment in the LS174T animal tumor model points to the former's potential utility in the immunoscintigraphy of colorectal cancer. The better tumor:liver ratio obtained using $^{99m}\text{Tc-MN-14 F(ab')}_2$ is especially encouraging in that it may improve detection of tumor deposits in liver. Also, its 24-h tumor-retained radioactivity (>6% ID/g) is close to what is reported for a divalent fragment containing a stable ^{99m}Tc chelate (21). The specific utility of the described methodology will obviously depend on the type of tumor or infection targeted and on the antibody used, since these are both determinants of target:nontarget ratios obtainable. Where utility is indicated, as with MN-14 F(ab')_2 , the present approach will be operationally simpler over the use of a true ^{99m}Tc tracer involving a very stable ^{99m}Tc chelate, as the latter may concurrently necessitate additional strategies to reduce accumulation of radioactivity in nontarget organs (22, 23).³

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LITERATURE CITED

- (1) Goldenberg, D. M. (1993) Monoclonal antibodies in cancer detection and therapy. *Am. J. Med.* 94, 297–312.
- (2) Serafini, A. N. (1993) Monoclonal antibody imaging: crossing the research/clinical barrier [Letter]. *Semin. Nucl. Med.* 23, 88.
- (3) Schlom, J. (1989) Innovations in monoclonal antibody tumor targeting. *J. Am. Med. Assoc.* 261, 744–746.
- (4) Griffiths, G. L.; Goldenberg, D. M.; Jones, A. L.; Hansen, H. J. (1992) Radiolabeling of monoclonal antibodies and fragments with technetium and rhenium. *Bioconjugate Chem.* 3, 91–99.
- (5) Goldenberg, D. M.; Wlodowski, T. J.; Sharkey, R. M.; Silberstein, E. B.; Serafini, A. N.; Garty, I. I.; Van Heertum, R. L.; Higginbotham-Ford, E. A.; Kotler, J. A.; Balasubramanian, N.; Swayne, L. C.; Hansen, H. J.; Pinsky, C. M. (1993) Colorectal cancer imaging with iodine-123-labeled CEA monoclonal antibody fragments. *J. Nucl. Med.* 34, 61–70.
- (6) Hansen, H. J.; Jones, A.; Sharkey, R. M.; Grebenau, R.; Blazejewski, N.; Kunz, A.; Buckley, M. J.; Newman, E. S.; Ostella, F.; Goldenberg, D. M. (1990) Pre-clinical studies of an "instant" ^{99m}Tc -labeling kit for antibody imaging. *Cancer Res. (Suppl.)* 50, 794–798.
- (7) Behr, T. M.; Becker, W. S.; Bair, H.-J.; Klein, M. W.; Stuhler, C. M.; Cidlinsky, K. P.; Wittekind, C. W.; Scheele, J. R.; Wolf, F. H. (1995) Comparison of complete versus fragmented technetium-99m-labeled anti-CEA monoclonal antibodies for immunoscintigraphy in colorectal cancer. *J. Nucl. Med.* 36, 430–441.
- (8) Fjeld, J. G.; Michaelsen, T. E.; Nustad, K. (1992) The binding parameters of radiolabeled monoclonal F(ab')_2 and Fab' fragments relative to immunoglobulin G in reactions with surface-bound antigen. *Eur. J. Nucl. Med.* 19, 402–408.
- (9) Spiegelberg, H. L. (1984) Structure and function of Fc receptors for IgG on lymphocytes, monocytes and macrophages. *Adv. Immunol.* 35, 61.
- (10) Hansen, H. J.; Goldenberg, D. M.; Newman, E. S.; Grebenau, R.; Sharkey, R. M. (1993) Characterization of second-generation monoclonal antibodies against carcinoembryonic antigen. *Cancer* 71, 3478–3485.
- (11) Greenfield, L.; Bloch, W.; Moreland, M. (1990) Thiol-containing cross-linking agent with enhanced steric hindrance. *Bioconjugate Chem.* 1, 400–410.
- (12) Penefsky, H. S. (1979) A centrifuged-column procedure for the measurement of ligand binding by beef heart F1. *Methods Enzymol.* 56, 527–530.

³ While this manuscript was in preparation, a paper describing a one-pot iminothiolane derivatization and Tc-99m labeling, and postlabeling purification involving a MAb F(ab')_2 fragment has appeared (32). Lack of information as to the formation of aggregates and Fab', if any, under the derivatizing/labeling conditions, and lack of animal biodistribution data, preclude assessment of that method's comparative utility at this stage.

- (13) Ellman, G. L. (1959) Tissue sulfhydryl groups. *Arch. Biochem. Biophys.* 82, 70–77.
- (14) Tom, B. H.; Putzky, L. P.; Jakstys, M. M. (1976) Human colonic adenocarcinoma cells. I. Establishment and description of a new line. *In Vitro (Rockville)* 12, 180–191.
- (15) Shevitz, J.; Armor, K.; King, S.; Shukla, R.; Leung, S. O.; Goldenberg, D. M.; Hansen, H. J. (1993) Successful humanization of a high-affinity anti-CEA murine monoclonal antibody, MN-14. *J. Nucl. Med.* 34, 217P (abstract).
- (16) Gold, D. V.; Nocera, M. A.; Stephens, R.; Goldenberg, D. M. (1990) Monoclonal antibodies to colon-specific antigen-p (Csa). *Cancer Res.* 50, 6405–6409.
- (17) Stein, R.; Chen, S.; Sharkey, R. M.; Goldenberg, D. M. (1990) Murine monoclonal antibodies raised against human non-small cell carcinoma of the lung: specificity and tumor targeting. *Cancer Res.* 50, 1330–1336.
- (18) Pawlak-Byczkowska, E. J.; Hansen, H. J.; Dion, A. S.; Goldenberg, D. M. (1989) Two new monoclonal antibodies, EPB-1 and EPB-2, reactive with human lymphoma. *Cancer Res.* 49, 4568–4577.
- (19) Becker, W.; Goldenberg, D. M.; Wolf, F. (1994) The use of monoclonal antibodies and antibody fragments in the imaging of infectious lesions. *Semin. Nucl. Med.* 24, 142–153.
- (20) Suzuki, S.; Hachimori, Y.; Matoba, R. (1970) The chemical modification of biopolymers III. The introduction of SH groups into Taka-amylase A. *Bull. Chem. Soc. Jpn.* 43, 3849–3852.
- (21) Fritzberg, A. R.; Abrams, P. G.; Beaumier, P. L.; Kasina, S.; Morgan, A. C.; Rao, T. N.; Reno, J. M.; Sanderson, J. A.; Srinivasan, A.; Wilbur, D. S.; Vanderheyden, J.-L. (1988) Specific and stable labeling of antibodies with technetium-99m with a diamide dithiolate chelating agent. *Proc. Natl. Acad. Sci. U.S.A.* 85, 4025–4029.
- (22) Tibben, J. G.; Massuger, L. F. A. G.; Boerman, O. C.; Claessens, R. A. M. J.; Borm, G. F.; Pak, K. Y.; Koenders, E. B.; Corstens, F. H. M. (1994) Decreased kidney uptake of technetium-99m-labeled Fab' fragments in ovarian carcinoma bearing nude mice using a cleavable chelator. *Nucl. Med. Biol.* 21, 17–24.
- (23) Abrams, M. J.; Bridger, G. J.; Padmanabhan, S.; Larsen, S. K.; Gaul, F. E.; Henson, G. W.; Schwartz, D. A.; Ultee, M. E.; Longlee, C. B.; Burton, C. A. (1994) A comparison of cleavable and noncleavable hydrazinopyridine linkers for the ^{99m}Tc labeling of Fab' monoclonal antibody fragments. *J. Nucl. Med.* 35, 29P (abstract).
- (24) Brown, B. A.; Drozynski, C. A.; Dearborn, C. B.; Hadian, R. A.; Liberatore, F. A.; Tulip, T. H.; Tolman, G. L.; Haber, S. B. (1988) Conjugation of metallothionein to a murine monoclonal antibody. *Anal. Biochem.* 177, 22–28.
- (25) Mardirossian, G.; Wu, C.; Rusckowski, M.; Hnatowich, D. J. (1992) The stability of ^{99m}Tc directly labeled to an Fab' antibody via stannous ion and mercaptoethanol reduction. *Nucl. Med. Commun.* 13, 503–512.
- (26) Rhodes, B. A.; Zamora, P. O.; Newell, K. D.; Valdez, E. F. (1986) Technetium-99m labeling of murine monoclonal antibody fragments. *J. Nucl. Med.* 27, 685–693.
- (27) Schwarz, S. W.; Connett, J. M.; Anderson, C. J.; Rocque, P. A.; Philpott, G. W.; Guo, L. W.; Welch, M. J. (1994) Evaluation of a direct method for technetium labeling intact and F(ab')_2 1A3, an anticolorectal monoclonal antibody. *Nucl. Med. Biol.* 21, 619–626.
- (28) Griffiths, G. L.; Goldenberg, D. M.; Jones, A. L.; Hansen, H. J. (1994) Preparation of a pure ^{99m}Tc - F(ab')_2 radioimmunoconjugate by direct labeling methods. *Nucl. Med. Biol.* 21, 649–655.
- (29) McCall, M. J.; Diril, H.; Meares, C. F. (1990) Simplified method for conjugating macrocyclic bifunctional chelating agents to antibodies via 2-iminothiolane. *Bioconjugate Chem.* 1, 222–226.
- (30) Larsen, S. K.; Solomon, H. F.; Caldwell, G.; Abrams, M. J. (1995) [^{99m}Tc]Tricine: A useful precursor complex for the radiolabeling of hydrazinonitricotinyl protein conjugates. *Bioconjugate Chem.* 6, 635–638.
- (31) Hnatowich, D. J.; Virzi, F.; Fogarazi, M.; Rusckowski, M.; Winnard, P., Jr. (1994) Can a cysteine challenge assay predict the in vivo behavior of ^{99m}Tc -labeled antibodies? *Nucl. Med. Biol.* 21, 1035–1044.
- (32) Tesic, M.; Sheldon, K. M.; Ballinger, J. R.; Boxen, I. (1995) Labeling small quantities of monoclonal antibodies and their F(ab')_2 fragments with technetium-99m. *Nucl. Med. Biol.* 22, 451–457.
- (33) Eckelman, W. C.; Steigman, J. (1991) Direct labeling with ^{99m}Tc . *Nucl. Med. Biol.* 18, 3–7.
- (34) Buscombe, J. R.; Lui, D.; Ensing, G.; de Jong, R.; Ell, P. J. (1990) ^{99m}Tc -human immunoglobulin (HIG)—first results of a new agent for the localization of infection and inflammation. *Eur. J. Nucl. Med.* 16, 649–655.
- (35) Hnatowich, D. J.; Mardirossian, G.; Rusckowski, M.; Fogarazi, M.; Virzi, F.; Winnard, P., Jr. (1993) Directly and indirectly technetium-99m-labeled antibodies—a comparison of in vitro and animal in vivo properties. *J. Nucl. Med.* 34, 109–119.
- (36) Fritzberg, A. R.; Beaumier, P. L. (1992) Targeted proteins for diagnostic imaging: Does chemistry make a difference? *J. Nucl. Med.* 33, 394–397.
- (37) Griffiths, G. L.; Hansen, H. J.; Goldenberg, D. M. (1993) Labeling of antibodies with technetium-99m [Letter]. *J. Nucl. Med.* 34, 1617–1618.

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Synthesis of Novel Poly(ethylene glycol) Derivatives Having Pendant Amino Groups and Aggregating Behavior of Its Mixture with Fatty Acid in Water

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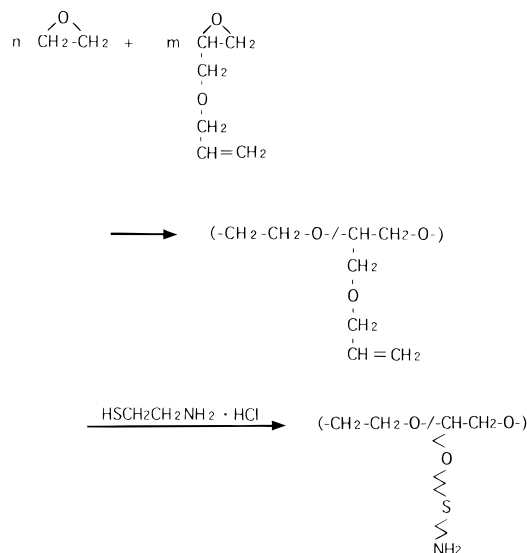
Novel poly(ethylene glycol) (PEG) derivatives having pendant amino groups were prepared by copolymerization of allyl glycidyl ether with ethylene oxide followed by chemical modification of the double bond side chains. Dropwise addition of the mixture of monomers to the anionic initiator gave an almost monodisperse ($M_w/M_n = 1.05$) random copolymer. ¹H NMR spectra showed that addition of 2-aminoethanethiol to the pendant allyl groups of the copolymer was completely carried out in methanol without catalyst, and an aminated PEG derivative with a definite structure was obtained. Acetylation of the pendant amino groups was readily performed by acetic anhydride with triethylamine. A gel permeation chromatogram of the acetylated polymer showed a very narrow molecular weight distribution ($M_w/M_n = 1.06$) of the polyamine. These cationic PEG derivatives make amphiphilic polyion complexes with fatty acids, and then aggregate in water. A fluorescence study using pyrene as a microenvironment probe revealed that the aminated PEG–lauric acid ion complex could take up the hydrophobic fluorescence probe into the lipophilic field inside, and they also had a critical aggregation concentration at [lauric acid] = 0.7 mM. It is much lower than the critical micelle concentration of the corresponding fatty acid sodium salts, indicating high stability of the polymer–fatty acid aggregate.

INTRODUCTION

Poly(ethylene glycol) (PEG)¹ is accepted as a nontoxic (1) and nonimmunogenic (2) polymer and has been applied to a variety of biomedical and biotechnical materials such as drug carriers (3) and protein modifiers (4). Preparability of the polymer with a very narrow molecular weight distribution is also attractive in many fields where the characteristics of the polymer much depend on its molecular weight. PEG has, however, only two reactive groups at both ends. That severely limits the possibility of altering its physical properties by chemical modifications. The drug-loading capacity of the polymer is also strictly limited by its poor chemical reaction equivalence.

In order to improve this low functionality of PEG, block or alternating copolymer of PEG and amino acids have been prepared using PEG having two amino groups (5) or two activated ester groups (5–8) at both ends as a monomer. These copoly(PEG–amino acid)s have several pendant functional groups with strictly controlled intervals. It seems, however, difficult to obtain the functionalized PEGs with a narrow molecular weight distribution by these methods, and the linkage along the main chain other than ether could not be excluded completely.

Scheme 1



In this study, we prepared novel PEG derivatives having pendant amino groups with only ether linkages throughout the main chain and also with a very narrow molecular weight distribution by copolymerization of allyl glycidyl ether with ethylene oxide followed by chemical modification of the double bond side chain of the copolymer. These aminated PEGs are highly hydrophilic polycations and were expected to make amphiphilic ion complexes with fatty acids. The characteristic aggrega-

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¹ Abbreviations: PEG, poly(ethylene glycol); AGE, allyl glycidyl ether; EO, ethylene oxide.

tion behavior of the ionically bonded macroamphiphiles was investigated.

EXPERIMENTAL PROCEDURES

Copolymerization of AGE and EO. PEG derivatives having double bond pendants were prepared by anionic ring-opening copolymerization of AGE and EO. Typically, the initiator, diethylene glycol (225 g), and potassium hydroxide (4.8 g) were charged into a polymerization reactor. After dehydration for 1 h at 85 °C under a nitrogen atmosphere (50 mmHg), a mixture of AGE and EO (3100 g, AGE/EO = 15.0/85.0 in moles) was added at 100–120 °C dropwise at a rate of 400 g/h. A portion of the polymer was periodically sampled out during the reaction for analysis of its molecular weight and composition. Soon after all the monomer was added, the unreacted monomer was removed at 200 mmHg, and the polymer was neutralized by HCl, dried by heating at 100 °C for 1 h under reduced pressure, and finally filtered at 80 °C. The molecular weight of the polymer was calculated from OH value analysis (9) to be 1810. AGE residue in the copolymer determined by C=C analysis (10) was 14.5 mol %.

Addition of 2-Aminoethanethiol to Copoly(AGE/EO). Copoly(AGE/EO) (AGE/EO = 12.0/88.0 in moles; M_n = 2030) (995 g) was dissolved in methanol (1300 mL), and it was added dropwise at room temperature to the solution of 2-aminoethanethiol hydrochloride (900 g) in methanol (1200 mL). After standing at 40 °C for 5 h, the reaction mixture was evaporated to remove methanol. The residual syrup was dissolved in chloroform, neutralized by sodium hydroxide, and washed three times with water. Removal of the solvent under reduced pressure gave a syrupy product (970 g). The amino group content in the product determined by titration was 1.96 mmol/(g of polymer) [theoretical, 1.95 mmol/(g of polymer)].

Acetylation of the Copoly(AGE/EO)–Aminoethanethiol Adduct. Aminated copoly(AGE/EO) (50 mg) prepared above was dissolved in 200 μ L of dry dimethylformamide, and then 200 μ L of acetic anhydride and 200 μ L of triethylamine were added to the solution. After the mixture stood for 90 min at room temperature, 3 mL of water was added. The solution was stirred for 30 min, and then the polymer product was isolated by gel filtration with a Sephadex G-50 column. Freeze-drying gave a pale yellow syrup (51 mg).

Materials. Pyrene was purified by recrystallization from ethanol. Dimethylformamide was dried over calcium hydride and then distilled under reduced pressure. Triethylamine was refluxed with sodium hydroxide and distilled. Other chemicals were used without further purification.

Measurement. The molecular weight distribution of the polymers was measured by gel permeation chromatography using a Shodex GPC System-11 apparatus equipped with columns Shodex KF-801, KF-803, and KF-804. Tetrahydrofuran was used as an eluent at a flow rate of 1.0 mL/min.

Corrected fluorescence spectra were recorded on a JASCO Spectrofluorometer FP-777 using rhodamine B as a standard as follows. An excitation spectrum was measured in advance at the emission wavelength of 390 nm. The peak wavelength around 335–340 nm in the excitation spectrum was used as an excitation wavelength for the emission spectrum measurement.

^1H NMR spectra were measured in CDCl_3 with a VARIAN VXR-500S instrument. Tetramethylsilane was used as an internal standard.

Table 1. Copolymerization of Allyl Glycidyl Ether (AGE) with Ethylene Oxide (EO)^a

sample	M_n^b	AGE in copolymer ^c (mol %)
1	510	15.2
2	940	15.1
3	1810	14.5

^a AGE/EO in feed = 15.0/85.0 in moles. ^b Determined by OH analysis (9). ^c Determined by C=C analysis (10).

RESULTS AND DISCUSSION

Anionic copolymerization of AGE and EO gave an almost monodisperse copolymer (M_w/M_n = 1.05). Polymers were sampled during the copolymerization, and their molecular weight and composition were determined. As shown in Table 1, copolymer composition was almost constant throughout the reaction and was fairly equal to the monomer composition in the feed. It would show that the almost random copolymers of AGE and EO could be obtained under these conditions.

Addition of 2-aminoethanethiol to the pendant allyl groups of copoly(AGE/EO) was readily performed in methanol without catalyst. Potentiometric titration showed that the product contained a theoretical amount of primary amino groups. A ^1H NMR spectrum of the aminated polymer shows no residual double bond peaks, and the simple spectrum indicates that the PEG derivative having pendant amino groups with a definite structure was obtained (Figure 1).

This amination reaction of the polymer seems to proceed through a free radical mechanism, considering its high conversion in methanol. A radical addition reaction might be followed by side reactions such as coupling or scission of the molecules. The aminated polymer was then acetylated by acetic anhydride, and its gel permeation chromatography was measured. A narrow molecular weight distribution (M_w/M_n = 1.06) was seen in the chromatogram (Figure 2), showing that only a few side reactions have occurred besides the addition of 2-aminoethanethiol.

In the ^1H NMR spectrum of the acetylated polymer, there are two peaks for the acetyl group at δ = 2.0 and 2.1 ppm. The higher peak at δ = 2.0 can be assigned to the methyl protons of acetamide groups on the side chain. The smaller one at δ = 2.1 should arise from the acetyl ester groups at both ends of the polymer. The ratio of these peak areas was in good accordance with that calculated from the titration results.

These PEG derivatives having pendant amino groups are thought of as novel biocompatible functional polymers, and they could be covalently bound to as many drug molecules as the pendant groups. They could form a stable polymer–drug complex as a polymeric chelating agents. Cross-linking of these polymers could be carried out by several means to give a bioinert hydrogel.

These polymers are highly hydrophilic polycations and were thought to ionically combine with fatty acids to form amphiphilic polymer complexes consisting of a hydrophilic main chain connected with hydrophobic side chains through the electrostatic bond. These polymer ion complexes would be a new type of surfactant and were expected to aggregate in water to make a micelle-like structure having an alkyl chain core with a PEG-coated surface.

Amino pendant-containing polymer (M_n = 2390, 4.65 amino groups/polymer molecule) prepared as above was then mixed with lauric acid in water at a molar ratio of NH_2/COOH = 1/1. A transparent solution was obtained, and it made stable bubbles upon agitation like a soap solution.

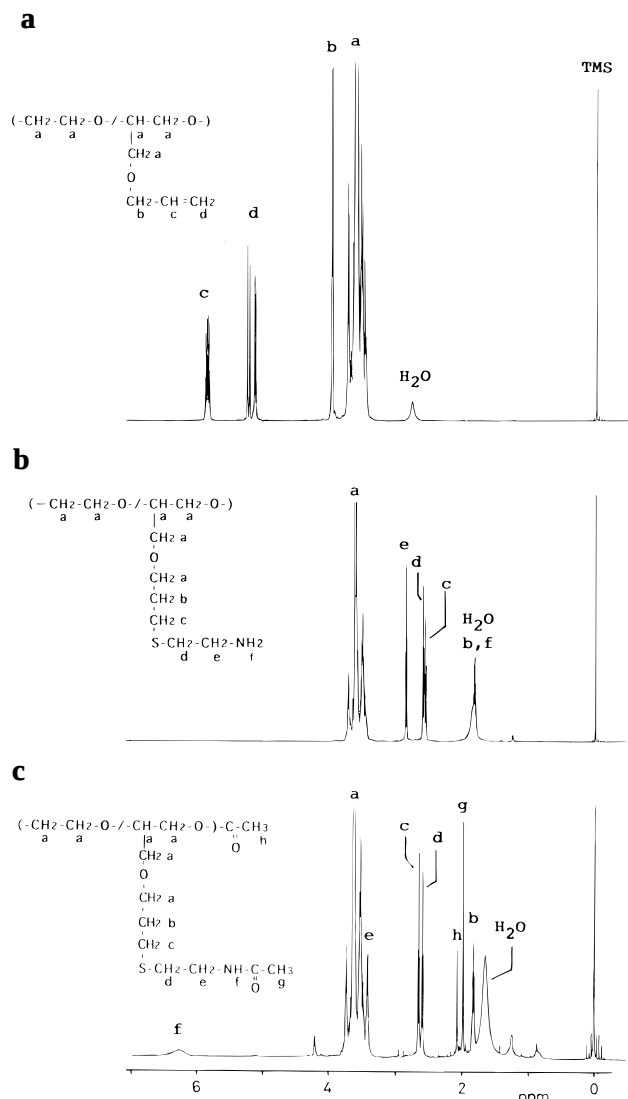


Figure 1. ^1H NMR spectra of (a) copoly(AGE/EO), (b) the addition product of copoly(AGE/EO) with aminoethanethiol, and (c) its acetylated derivative.

The solution behavior of the polymer–fatty acid complex was investigated by the fluorescence study using pyrene as a microenvironment probe. Pyrene is known as a fluorescence hydrophobicity probe, and in its emission spectrum, the intensity ratio of peak I to peak III expresses the hydrophobicity of the microenvironment of the pyrene molecule. When the polymer–fatty acid solution was added to the same volume of aqueous pyrene solution (6.0×10^{-7} M), a relatively low peak I/peak III ratio (1.0–1.2) was observed in the fluorescence spectrum. It would be caused by the polyion complexes of the aminated PEG and the fatty acid secondarily aggregating to form a microdomain structure, and the pyrene molecules entrapped in a hydrophobic field inside.

The polymer–fatty acid solution was then serially diluted before addition to pyrene solution, and the $I_{\text{I}}/I_{\text{III}}$ value was plotted vs lauric acid concentration in Figure 3. The $I_{\text{I}}/I_{\text{III}}$ value dramatically changed at around [lauric acid] = 0.7 mM. It should be the “critical secondary aggregating concentration” of the polyion complex. Such rapid change in the $I_{\text{I}}/I_{\text{III}}$ ratio was not observed with the aminated PEG without lauric acid, showing the polymer could make a hydrophobic microdomain structure only when mixed together with the fatty acid. The critical micelle concentration (cmc) for sodium laurate measured

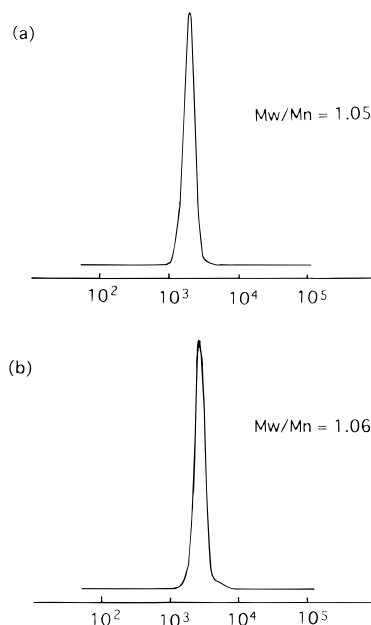


Figure 2. Gel permeation chromatograms of (a) copoly(AGE/EO) and (b) the acetylated derivative of the copoly(AGE/EO)–aminoethanethiol adduct.

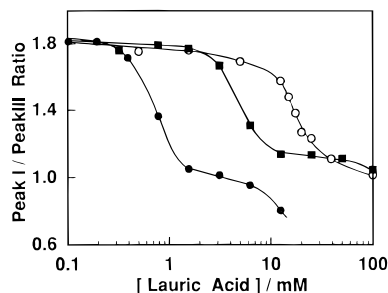


Figure 3. Peak I/peak III intensity ratio in the emission spectra for pyrene in the solution of (O) sodium laurate, (■) H_2N –PEG– NH_2 + lauric acid, and (●) amino pendant-containing PEG + lauric acid.

by the same method was around 20 mM (Figure 3). A much higher stability of such polymer complex aggregates than that of the micelle of the sodium salt was confirmed.

Similar experiments were also carried out with a PEG derivative having terminal amino groups at both ends of the polymer. This conventional type of the aminated PEG could also solubilize lauric acid being mixed at the molar ratio of $\text{NH}_2/\text{COOH} = 1/1$. A fluorescence study using pyrene was carried out with H_2N –PEG– NH_2 with a molecular weight of 1100 which has almost the same amino content [1.82 mmol/(g of polymer)] as the PEG having amino pendant groups used above [1.95 mmol/(g of polymer)]. Results are shown in Figure 3.

A similar sigmoid curve was obtained, but the critical aggregating concentration of the NH_2 -terminated PEG was 7 times higher than that of the NH_2 pendant PEG. A more significant difference between these polymers was observed in their solution stability. One day after the solution was mixed with pyrene solution, white fluffy precipitate was formed in the amino-terminated PEG–fatty acid solution, while no change was seen with the amino pendant-containing PEG over 1 month.

These differences in the critical concentration and the stability of the aggregate might be caused by the different amino group number in one polymer molecule or the structural difference between the amino-terminated and amino pendant PEG. Although an accurate reason for

the characteristic high stability and low critical aggregating concentration of the amino pendant PEG-fatty acid ion complex has not been clearly elucidated, the possibility of the aggregate as a new kind of biocompatible surfactant and also as a carrier for hydrophobic drugs was suggested. A detailed study on the aggregating behavior and their functions is now in progress.

LITERATURE CITED

- (1) Smyth, H. F., Jr., Carpenter, C. P., and Weil, C. S. (1950) The toxicology of the polyethylene glycols. *J. Am. Pharm. Assoc.* 39, 349–354.
- (2) Richter, A. W., and Akerblom, E. (1983) Antibodies against polyethylene glycol produced in animals by immunization with monomethoxy polyethylene glycol modified proteins. *Int. Arch. Allergy Appl. Immunol.* 70, 124–131.
- (3) Zalipsky, S., Gilon, C., and Zilkha, A. (1983) Attachment of drugs to polyethylene glycols. *Eur. Polym. J.* 19, 1177–1183.
- (4) Harris, J. M. (1992) Introduction to biotechnical and biomedical applications of poly(ethylene glycol). In *Poly(ethylene glycol) chemistry: Biotechnical and Biomedical Applications* (J. M. Harris, Ed.) pp 1–14, Plenum Press, New York.
- (5) Ulbrich, K., Strohalm, J., and Kopecek, J. (1986) Poly(ethylene glycol)s containing enzymatically degradable bonds. *Makromol. Chem.* 187, 1131–1144.
- (6) Nathan, A., Bolikal, D., Vyavahare, N., Zalipsky, S., and Kohn, J. (1992) Hydrogels based on water-soluble poly(ether urethane)s derived from L-lysine and poly(ethylene glycol). *Macromolecules* 25, 4476–4474.
- (7) Nathan, A., Zalipsky, S., Ertel, S. I., Agathos, S. N., Yarmush, M. L., and Kohn, J. (1993) Copolymers of lysine and polyethylene glycol: A new family of functionalized drug carriers. *Bioconjugate Chem.* 4, 54–62.
- (8) Poiani, G. J., Riley, D. J., Fox, J. D., Kemnitzer, J. E., Gean, K. F., and Kohn, J. (1994). Conjugates of *cis*-4-hydroxy-L-proline and poly(PEG-Lys), a water soluble poly(ether urethane): Synthesis and evaluation of antifibrotic effects *in vitro* and *in vivo*. *Bioconjugate Chem.* 5, 621–630.
- (9) Japan Industrial Standard, K-1557 6.4 (1970).
- (10) Japan Industrial Standard, K-1557 6.7 (1970).

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Identification of Adenine Binding Domain Peptides of the ADP Regulatory Site within Glutamate Dehydrogenase[†]

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Photoaffinity labeling with [α -³²P]-8-azidoadenosine 5'-diphosphate (8N₃ADP) and [β -³²P]-2-azidoadenosine 5'-diphosphate (2N₃ADP) was used to identify overlapping tryptic and chymotryptic generated peptides within the adenine binding domain of the regulatory ADP site of bovine liver glutamate dehydrogenase (GDH). In the absence of UV irradiation, 8N₃ADP was able to activate the reverse reaction catalyzed by GDH as well as ADP. Photoinsertion of both [α -³²P]8N₃ADP and [β -³²P]2N₃ADP was reduced best by ADP in comparison to other nucleotides. Photolabeling of GDH with [α -³²P]8N₃ADP appeared to be biphasic, with saturation occurring near 80 and 130 μ M, whereas [β -³²P]2N₃ADP showed saturation near 50 μ M. When 60 μ M [α -³²P]8N₃ADP (below the first saturation value) was used to identify peptides within the ADP binding domain, peptides corresponding to residues G¹⁵⁶–K²⁰⁰ and E¹⁷⁵–K²⁰⁰ (tryptic) and I¹⁵⁸–Y¹⁸³ (chymotryptic) were photolabeled. However, when 160 μ M [α -³²P]8N₃ADP (above the second saturation value) was used, the peptide D⁴⁰³–R⁴¹⁸ was also photolabeled. Digestion with both trypsin and chymotrypsin resulted in isolation of peptides E¹⁷⁵–Y¹⁸³ and A¹⁸⁴–I¹⁹². [β -³²P]2N₃ADP at 90 μ M also photolabeled tryptic peptides G¹⁵⁶–K²⁰⁰ and C²⁷⁰–K²⁸⁹. C²⁷⁰–K²⁸⁹ was shown earlier to be within the NAD⁺ binding site [Kim, H., and Haley, B. (1991) *Bioconjugate Chem.* 2, 142–147]. These results are consistent with the residues E¹⁷⁵–I¹⁹² being within the adenine binding domain of the ADP regulatory site.

INTRODUCTION

Mammalian glutamate dehydrogenase (GDH, EC 1.4.1.3) integrates carbon and nitrogen metabolism via the tricarboxylic acid cycle and plays a role in regulating the levels of ammonia and glutamate within the central nervous system. Bovine liver GDH catalyzes the forward and reverse reaction of oxidatively deaminating L-glutamate into α -ketoglutarate and NH₄⁺ utilizing the coenzymes NAD⁺ or NADP⁺ for oxidative power.

Bovine liver GDH is a hexamer of identical subunits (56 kDa/subunit) for which no crystal structure exists. However, the primary sequence has been published (1), and the crystal structure of the enzyme from *Clostridium symbiosum* has been determined to 1.96 Å resolution (2). The similarities between the bacterial and bovine liver enzymes as inferred from electron micrographs and sequence comparisons suggest that the two enzymes may be structurally similar (3–5).

The enzyme is regulated by numerous factors including pH, aggregation state, and a variety of molecular inducers and inhibitors (6, 7). Mammalian GDH is an NAD⁺/NADH utilizing enzyme, the activity of which is regulated by purine nucleotides. Each GDH subunit also contains both an inhibitory GTP and an activating ADP regulatory site. In addition, NADH, a substrate for the reverse reaction, has also been proposed to have an inhibitory site (8, 9).

It has been a major goal to identify the substrate and regulatory binding domains of GDH, and several classical chemical probes have been used to attempt resolution of these binding sites. The NADH inhibitory site has been proposed to be modified by an ATP analogue at Cys³¹⁹ (10, 11), by a GMP probe at Met¹⁶⁹ and Tyr²⁶² (12), and by the adenosine analogue, 5'-[p-(fluorosulfonyl)benzoyl]-adenosine (FSBA), at Lys⁴²⁰ and Tyr¹⁹⁰ (13–15). The final result using classical chemical probes to identify the NADH is a wide scatter of modified residues throughout most of the proposed three-dimensional structure of GDH. A similar fluorescent FSBA adenosine analogue has also been proposed to react covalently at the GTP inhibitory site (16), and two different AMP analogues have been proposed to modify the ADP regulatory site at His⁸² (8, 17) and Arg⁴⁵⁹ (18). With the GMP probe modifying the NADH site and an adenosine probe modifying the GTP inhibitory site, it seems that the base moiety has not been effective at directing the site of modification by classical chemical active site probes.

A contrasting approach, photoaffinity labeling, has been used to identify the base binding domains of the NAD⁺ and GTP binding sites (4, 19, 21) localizing both domains within the proposed catalytic cleft. The NAD⁺ site was identified using [³²P]2N₃NAD⁺ and was within an expected classical β - α - β ADP-binding motif; photoinsertion was only into Glu²⁷⁵ (4). The GTP inhibitory site was located using [³²P]8N₃GTP in an additional 48 residues found only in mammalian GDH (23), which is uniquely GTP regulated, and K⁴⁴⁵ appeared to be the only residue modified (21).

Photoaffinity labeling requires reversible binding before photoinsertion can occur (22). The effects of the

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¹ Abbreviations: 8N₃GTP, 8-azidoguanosine 5'-triphosphate; 8N₃ADP, 8-azidoadenosine 5'-diphosphate; 2N₃ADP, 2-azidoadenosine 5'-diphosphate; 2N₃NAD⁺, 2-azidonicotinamide adenine dinucleotide; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PTH, phenylhydantoin.

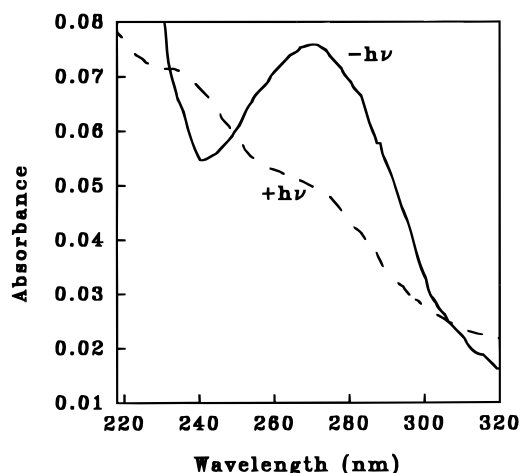


Figure 1. Spectral analysis of $[\beta\text{-}^{32}\text{P}]2\text{N}_3\text{ADP}$. $[\beta\text{-}^{32}\text{P}]2\text{N}_3\text{ADP}$ was synthesized as described (Materials and Methods). The optical density of the product was measured over a wavelength spectrum of 220–320 nm prior to (solid line) and following (dashed line) 1 min of photolysis.

reversible binding of each photoprobe can be determined using enzyme kinetic parameters. Site specificity can be validated by saturation effects and selective reduction of photoinsertion by the native nucleotide being mimicked (20). $[\text{}^{32}\text{P}]2\text{N}_3\text{NAD}^+$ is a very good substrate for GDH in the absence of activating light, and photoinsertion was appropriately reduced by NAD^+ and ADP and enhanced by GTP (4, 19). Further, $8\text{N}_3\text{GTP}$ and $8\text{N}_3\text{ADP}$ mimicked GTP and ADP, respectively, as allosteric regulators of GDH (21).

The ADP regulatory site would be expected to be more difficult to identify due to the wobble associated with ADP in its binding site (24). Also, the similarities between the structures of NAD^+ and ADP could contribute to the difficulties in identifying the specific ADP binding site. In this paper the photoprobes $[\alpha\text{-}^{32}\text{P}]8\text{N}_3\text{-ADP}$ and $[\beta\text{-}^{32}\text{P}]2\text{N}_3\text{ADP}$ are shown to mimic ADP kinetically and modify a region corresponding to $\text{E}^{175}\text{--I}^{192}$, which is distinct from the domains modified when the GTP and NAD^+ binding sites of GDH are identified (4, 19, 21). Data obtained using photoaffinity probes place all of the base binding domains of NAD^+ , GTP, and ADP at different locations within a proposed catalytic cleft defined in the structure by Teller et al. (3).

MATERIALS AND METHODS

Synthesis of Photoprobes. Nonradioactive $8\text{N}_3\text{ADP}$ was synthesized as described for $8\text{N}_3\text{AMP}$ (22, 26). $2\text{N}_3\text{-ADP}$ was synthesized as described for the preparation of $2\text{N}_3\text{ATP}$ except that inorganic phosphate was substituted for pyrophosphate (27, 28). $[\alpha\text{-}^{32}\text{P}]8\text{N}_3\text{ADP}$ (specific activity = 10–30 $\text{mCi}/\mu\text{M}$) was a byproduct formed during the synthesis of $[\alpha\text{-}^{32}\text{P}]8\text{N}_3\text{ATP}$.

$[\beta\text{-}^{32}\text{P}]2\text{N}_3\text{ADP}$ (specific activity = 5–14 $\text{mCi}/\mu\text{M}$) was synthesized by incubating $2\text{N}_3\text{AMP}$ and $[\gamma\text{-}^{32}\text{P}]2\text{N}_3\text{ATP}$ (22) with adenylate kinase in 20 mM Tris-HCl buffer in the presence of 5 mM MgCl_2 and purified as previously presented (28). The photoreactivity of the final product was measured spectrophotometrically with a Beckman DU-70 spectrophotometer (Figure 1).

Bovine liver GDH and all other chemicals were obtained from Sigma Chemical Co. and were of reagent grade unless otherwise noted.

Glutamate Dehydrogenase Assays. The activity of GDH was assayed as follows: GDH was incubated with 0.1 M K_2HPO_4 (pH 8.0), 10 mM EDTA, 5 mM α -ketoglutarate, 50 mM NH_4Cl , 0.1 mM NADPH, 0.1 mg/mL BSA,

and the appropriate effector (total volume 1 mL) in a quartz cuvette. The oxidation of NADPH to NADP^+ was determined by measuring the decrease in absorbance at 340 nm at various time points in a Beckman DU-70 spectrophotometer.

The forward reaction was assayed in 20 mM Tris-acetate buffer (pH 8.0). The incubation mixture included 10 μM EDTA, 5 mM glutamate, 0.1 mM NADP^+ , and the appropriate effector. The increase in absorbance at 340 nm was measured. The measurements were taken at 10 s intervals up to 2 min after the addition of GDH.

Photolabeling Glutamate Dehydrogenase. For GDH saturation studies, 1 μg of glutamate dehydrogenase in 5 mM Tris-acetate buffer (pH 7.1) was incubated with the appropriate concentration of the photoprobe, $[\alpha\text{-}^{32}\text{P}]8\text{N}_3\text{ADP}$ or $[\beta\text{-}^{32}\text{P}]2\text{N}_3\text{ADP}$ in Eppendorf tubes for 20 s. The sample was then irradiated with a handheld 254 nm Mineralight UVS-11 UV lamp ($I = 4600 \mu\text{W}/\text{cm}^2$) for 75 s at 4 $^\circ\text{C}$. The total reaction volume was 50 μL . The reaction was quenched by the addition of 50 μL of cold 7% trichloroacetic acid (TCA). The reaction mixture was kept at 4 $^\circ\text{C}$ for 15 min and then centrifuged in a Fisher Scientific microcentrifuge, Model 235C, for 10 min to separate the protein from unbound nucleotide. The efficiency of photoinsertion into GDH, just after acid precipitation, was determined to be about 8% under these conditions. The pellet was resuspended in a protein solubilizing mix (PSM) consisting of 200 mM Tris-HCl buffer, 10% SDS, 3.6 M urea, 2.5% (w/v) DTT, and 2% (w/v) pyronin Y (pH 8.0), in preparation for SDS-PAGE.

For effector and competition studies, 1 μg of GDH was incubated with the competitor for 15 s in buffer at 4 $^\circ\text{C}$ prior to the addition of photoprobe and then allowed to incubate with the photoprobe for 1 min. The reaction mixture was irradiated, quenched, and prepared for SDS-PAGE as described above.

Photolabeled GDH (1 μg) was subjected to SDS-PAGE in 3.5% stacking and 8% separating gels according to the method of Laemmli (29). The gels were stained, destained, and dried on a slab gel dryer. ^{32}P incorporation was determined by an AMBIS 4000 β -detector or excision of protein bands followed by liquid scintillation. ^{32}P incorporated saturated at values of 66 000–220 000 cpm depending on the specific activity of the probe. This represents a minimum of photoinsertion level of about 3% since label is lost both on electrophoresis and on staining. Bands from autoradiograms were scanned on an image acquisition densitometer (BioImage; MilliGen/Bioresearch, Ann Arbor, MI).

Identification of Photolabeled Peptides. In the determination of peptides within the binding site domain, selectivity of photoinsertion is more important than stoichiometry when enzyme is readily available. Therefore, large ratios of GDH to probe were used to decrease unbound probe concentration and nonspecific photolabeling. GDH (100–500 μg) in 5 mM Tris-acetate buffer (pH 8.0) was incubated with $[\alpha\text{-}^{32}\text{P}]8\text{N}_3\text{ADP}$ or $[\beta\text{-}^{32}\text{P}]2\text{N}_3\text{-ADP}$ (total volume 1.85 mL) for 90 s at 4 $^\circ\text{C}$ in disposable beakers from Fisher Scientific. Some experiments were performed in 5 mM K_2HPO_4 buffer (pH 8.0). Glutamate (5 mM) and NADP^+ (100 μM) were included in the incubation mixture to saturate their respective binding sites. The mixture was irradiated for 75 s and stopped by the addition of 1.85 mL of ice-cold TCA. The protein was separated from unbound nucleotide by centrifugation at 2000 rpm for 15 min in a Beckman Model TJ-6 centrifuge. The supernatant was removed, and the pellet was resuspended in a 667 μL solution containing a final concentration of 2 M urea in 75 mM NH_4HCO_3 ; the pH was adjusted to 8.5–9.0 with NH_4OH .

GDH was proteolyzed by the addition of 7.5 μg of protease (chymotrypsin or trypsin) at 25 °C for 3 h, after which time 7.5 μg of the respective protease was added again. After an additional 3 h at 25 °C, 10 μg of protease was added, and the digestion mix was kept at 25 °C for 10 h.

Immobilized Aluminum(III) Chromatography. An immobilized Al^{3+} chromatography procedure (30) modified to isolate photolabeled peptides was used as described earlier (21, 25). For preparation of the immobilized $\text{Al}(\text{III})$ resin, 0.8 mL of an epoxy-activated iminodiacetate Sepharose 6B Fast Flow resin (Sigma) was washed with H_2O and chelated to Al^{3+} by slowly passage through 20 mL of 50 mM AlCl_3 . The resin was then washed successively with H_2O and 50 mM NH_4OAc (pH 5.9) (buffer A), 0.5 M NaCl in buffer A (buffer B), buffer A, 2 M urea in buffer A (buffer C), buffer A, 5 mM glutamate in buffer A (buffer D), and buffer A. Buffer A was added to a fraction of the digestion mixture (1:1), and dithiothreitol was added to a final concentration of 1 mM. The pH was adjusted to 5.9 with acetic acid, and the sample was loaded onto the column. The column was washed with approximately 15 mL of buffers A, B, A, C, A, D, and A, respectively (0.5 mL/min). The radiolabeled peptides were eluted with 5 mM KH_2PO_4 in 50 mM NH_4OAc (pH 8.0).

Fractions from immobilized Al^{3+} chromatography were further purified by reversed-phase HPLC using either an aquapore RP 300 C₈ or C₁₈ column (Brownlee Laboratory) on an LKB HPLC system equipped with a diode array spectral detector. The mobile system consisted of a 25 μM AlCl_3 /0.1% TFA solution (X) and 0.1% TFA/70% acetonitrile (Y) solvent system. The gradient for HPLC was 0–10 min, 0% Y, and 10–70 min, 0–100% Y, at a flow rate of 0.5 mL/min. The amount of photolabeled peptide varied from experiment to experiment. Relative ^{32}P levels per fraction, as presented in each graph, were determined by Cerenkov liquid scintillation counting. When necessary, approximation of the amounts of photolabeled peptide per tube was determined as follows. First, the amount of ^{32}P in 20 μL aliquots from peak tubes was determined by liquid scintillation counting in Biosafe II cocktail (Research Products International). Second, using the specific activity of the probe used in the experiment and assuming one molecule of probe inserted per molecule of peptide, the approximate number of picomoles of peptide per fraction could be calculated.

The peak tube from HPLC fractions containing photolabeled GDH peptides, as determined by comigration with radioactivity, was sequenced using an Applied Biosystem 477A protein sequencer with an on-line PTH identification at the Macromolecular Center at the University of Kentucky. In each of the steps a fraction of the sample was saved for repeat analysis if necessary.

RESULTS

The reductive amination of α -ketoglutarate (reverse reaction) by GDH was assayed in the absence or presence of ADP, $8\text{N}_3\text{ADP}$, or $2\text{N}_3\text{ADP}$. As Table 1 shows, 50 μM ADP and $8\text{N}_3\text{ADP}$ activated GDH to the same extent. The maximal stimulatory effect for each was achieved at approximately 50 μM (data not shown). However, $2\text{N}_3\text{ADP}$, up to 200 μM , had no effect on GDH activity. $2\text{N}_3\text{ADP}$ was able, however, to prevent GDH activation by ADP (Table 1).

High micromolar concentrations of ATP or GDP had little or no effect upon the activity as did other nucleotides such as Ap_4A , adenosine, or cAMP. $\text{ATP}\gamma\text{S}$ activated GDH somewhat, while AMP only marginally activated the enzyme. $\text{ATP}-\gamma$ -benzophenone and 8N_3 -

Table 1. Effect of Nucleotides on the Activity of GDH (Reverse Reaction)^a

nucleotide	rel activity	nucleotide	rel activity
control (no ADP)	100	100 μM ATP	102
50 μM $8\text{N}_3\text{ADP}$	163	100 μM AMP	112
100 μM $2\text{N}_3\text{ADP}$	95	100 μM cAMP	100
50 μM ADP	156	100 μM $\text{ATP}\gamma\text{S}$	126
50 μM ADP + 50 μM $2\text{N}_3\text{ADP}$	110	100 μM GDP	95
50 μM ADP + 100 μM $2\text{N}_3\text{ADP}$	100	100 μM Ap_4A	89
100 μM $\text{ATP}-\gamma$ -benzophenone	158	100 μM adenosine	97
100 μM $8\text{N}_3\text{ATP}-\gamma$ -benzophenone	155		
25 μM GTP	7		
25 μM GTP + 100 μM $2\text{N}_3\text{ADP}$	9		
25 μM GTP + 200 μM $2\text{N}_3\text{ADP}$	10		

^a These results were repeated three times. Experimental error in each experiment is $\pm 10\%$.

Table 2. Effect of Nucleotides on the Activity of GDH (Forward Reaction)

nucleotide	rel activity		
	pH 8.0	pH 5.5	pH 4.5
control ^a	100 (100) ^b	100 (10) ^b	100 (5) ^b
100 μM ADP	125	83	83
100 μM $8\text{N}_3\text{ADP}$	118	104	110
100 μM $2\text{N}_3\text{ADP}$	95	85	79

^a Control values were arbitrarily assigned a value of 100% at each pH value. Each experiment was done a minimum of three times. Experimental error is $\pm 10\%$. ^b GDH activity at control values at pH 5.5 and 4.5 is shown in parentheses as a percentage of activity versus the control value at pH 8.0, which was designated 100%.

$\text{ATP}-\gamma$ -benzophenone, novel photoactive ATP analogues that modify regions at or very near the phosphate-interacting peptides within ADP/ATP binding sites (31), were able to activate GDH as well as ADP under the conditions described.

When the forward reaction was assayed, ADP and $8\text{N}_3\text{ADP}$ were able to activate GDH, although maximal activity with $8\text{N}_3\text{ADP}$ was only about 72% of maximal ADP-stimulated activity (Table 2), consistent with an earlier reported result (32). As in the case of the reverse reaction, $2\text{N}_3\text{ADP}$ alone had no effect upon the activity of GDH.

It has been reported that at lower pH values, ADP is unable to activate GDH (33). When assays (forward reaction) were performed at pH 5.5 and 4.5, the activities of GDH were, respectively, 90% and 95% lower than that at pH 8.0. At pH 4.5 and 5.5, ADP and $2\text{N}_3\text{ADP}$ inhibited activity, while $8\text{N}_3\text{ADP}$ had no effect on activity compared to activity in the absence of regulatory nucleotides (Table 2).

Photolabeling with $[\alpha\text{-}^{32}\text{P}]8\text{N}_3\text{ADP}$ and $[\beta\text{-}^{32}\text{P}]2\text{N}_3\text{ADP}$. It was very easy to photoincorporate levels of photoprobe to obtain cpm in the range of 10^6 or higher in 1 μg amounts of GDH on SDS-PAGE. To show specificity of the photoprobe-protein interaction, saturation of photoinsertion must be observed. Also, the natural nucleotide should protect the protein from photoinsertion. Photoinsertion of $[\alpha\text{-}^{32}\text{P}]8\text{N}_3\text{ADP}$ into GDH exhibited apparent biphasic saturation effects near 80 and 130 μM photoprobe (Figure 2A). Saturation of photoinsertion with $[\beta\text{-}^{32}\text{P}]2\text{N}_3\text{ADP}$ occurred at 50 μM photoprobe (Figure 2B) and did not appear to be biphasic. These experiments were repeated at least three times.

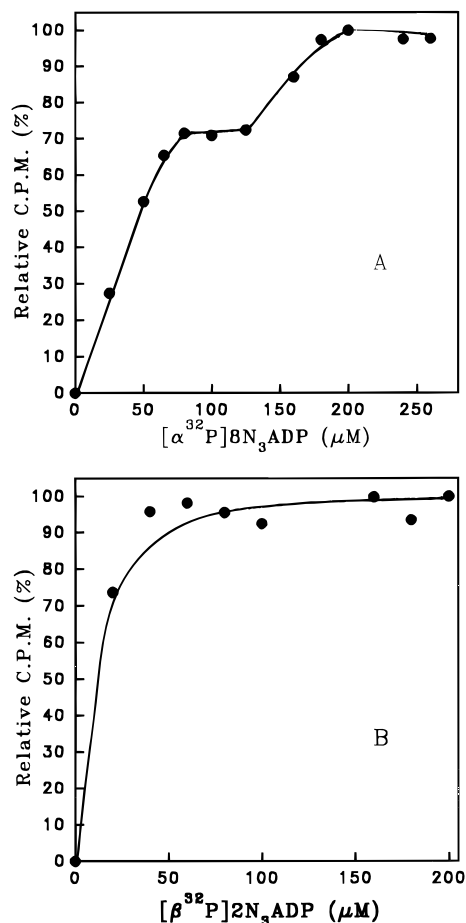


Figure 2. Saturation of photoinsertion of $[\alpha\text{-}^{32}\text{P}]8\text{N}_3\text{ADP}$ and $[\beta\text{-}^{32}\text{P}]2\text{N}_3\text{ADP}$ into GDH. One microgram of GDH was incubated in 50 μL of reaction buffer with the indicated concentration of $[\alpha\text{-}^{32}\text{P}]8\text{N}_3\text{ADP}$ (A) or $[\beta\text{-}^{32}\text{P}]8\text{N}_3\text{ADP}$ (B) and irradiated for 75 s. The reaction mixture was subjected to SDS-PAGE. ^{32}P incorporation was quantitated by an Ambis 4000 β -imager of the dried gel. 100% relative cpm in panels A and B corresponded to 130 000 and 66 284 cpm, respectively.

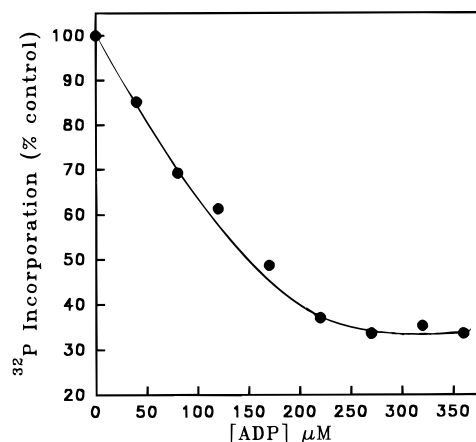


Figure 3. ADP reduction of $[\alpha\text{-}^{32}\text{P}]8\text{N}_3\text{ADP}$ photoinsertion into GDH. One microgram of GDH was incubated with 80 μM $[\alpha\text{-}^{32}\text{P}]8\text{N}_3\text{ADP}$ and the indicated concentration of ADP. The sample was irradiated and subjected to SDS-PAGE. ^{32}P incorporation was quantitated as in Figure 2. 100% relative ^{32}P incorporation corresponded to 160 000 cpm.

When 270 μM ADP (77% of total nucleotide) was present with 80 μM $[\alpha\text{-}^{32}\text{P}]8\text{N}_3\text{ADP}$, photoinsertion was reduced 65% (Figure 3). However, when GDH was photolabeled with 80 μM $[\beta\text{-}^{32}\text{P}]2\text{N}_3\text{ADP}$, only 45% protection was observed up to 300 μM ADP, indicating that 2N₃ADP was also binding at a non-ADP site (data not

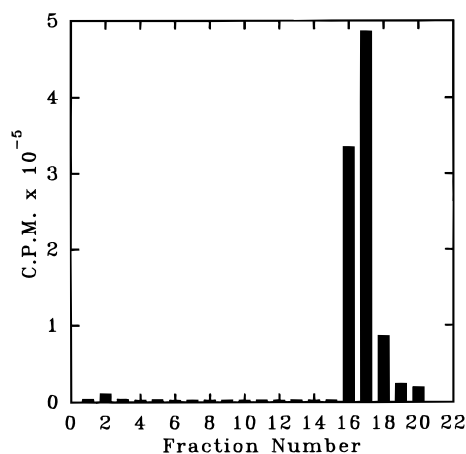


Figure 4. Immobilized Al^{3+} chromatography of GDH peptides. GDH was photolabeled with $[\alpha\text{-}^{32}\text{P}]8\text{N}_3\text{ADP}$ and subsequently proteolyzed with chymotrypsin, and the mixture was subjected to immobilized Al^{3+} chromatography. The radioactivity of each 1 mL fraction was determined by liquid scintillation. The washes were as follows: fractions 1–3, flow-through fractions; 4 and 5, 0.5 M NaCl; 6 and 7, buffer A; 8 and 9, 2 M urea; 10 and 11, buffer A; 12 and 13, 5 mM glutamate; 14 and 15, buffer A; and 16–20, 5 mM KH_2PO_4 in buffer A at pH 8.0 (see Materials and Methods).

shown). For example, NADP^+ and NAD^+ at 270 μM were able to reduce $[\beta\text{-}^{32}\text{P}]2\text{N}_3\text{ADP}$ photoinsertion by only 25%, (data not shown), whereas 250 μM GTP reduced photolabeling by only 10%. The data from multiple experiments indicated that $[\beta\text{-}^{32}\text{P}]2\text{N}_3\text{ADP}$ was binding with lower affinity to an NAD^+ site as well as the ADP site, as was confirmed by the sequencing results.

Isolation of 8N₃ADP Binding Peptides. To identify the peptides modified by 60 μM $[\alpha\text{-}^{32}\text{P}]8\text{N}_3\text{ADP}$, GDH was photolabeled in the presence of 5 mM glutamate and 100 μM NADP^+ to saturate the binding sites of the two substrates and reduce $[\alpha\text{-}^{32}\text{P}]8\text{N}_3\text{ADP}$ photoinsertion into the NADP^+ site. The photolabeled enzyme was proteolyzed, and then the photolabeled peptide was isolated by immobilized Al^{3+} chromatography. Normally, multiple photolyses are performed in these experiments to increase photoinsertion levels (21). However, due to the uncertainty of the number of ADP sites and possible wobble associated with them, GDH was incubated with photoprobe and irradiated only once. This was done to enhance site selective modification, but it also reduced the level of photoinsertion.

Acid precipitation was used to remove unbound nucleotide, and the reaction mixture was proteolyzed for 10 h with chymotrypsin. The mixture was then prepared for immobilized Al^{3+} chromatography. The immobilized Al^{3+} resin retained at least 95% of the radioactivity (Figure 4). The radioactive retentate was then eluted with 5 mM KH_2PO_4 (pH 8.0). Over 80% of the loaded radioactivity from the sample was associated with the P_i eluate (fractions 16–20) from immobilized Al^{3+} chromatography.

The flow-through fractions (1–3) from immobilized Al^{3+} chromatography were subjected to separation on a reversed-phase HPLC C₈ column. Many UV peaks with OD_{214} values between 0.1 and 1.0 were obtained, showing that the vast bulk of the peptides was not retained on the resin (Figure 5). In contrast, when the radioactive eluate (fractions 16 and 17) from immobilized Al^{3+} chromatography was subjected to HPLC (Figure 6), one minor radioactive peak and one major radioactive peak were observed. Sequence analysis of the first minor radioactive peak (I, fractions 40 and 41) yielded no identifiable residues, while the second radioactive peak (II, fractions 43–49) was identified as the chymotryptic

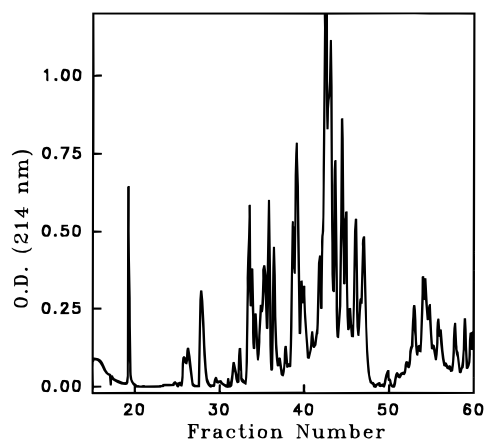


Figure 5. HPLC analysis of the flow-through fractions from immobilized Al^{3+} chromatography. The flow-through fractions from immobilized Al^{3+} chromatography (fractions 1–3 from Figure 4) of the chymotryptic digest of GDH were subjected to reversed-phase HPLC with monitoring of the optical density at 214 nm.

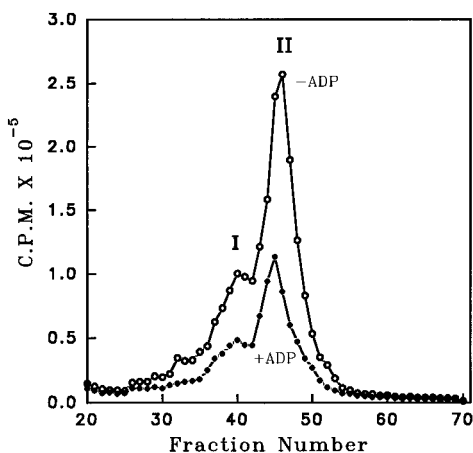


Figure 6. HPLC analysis of the radioactive P_i eluate from immobilized Al^{3+} chromatography. The chymotryptic peptides of the radioactive P_i eluate from immobilized Al^{3+} chromatography (fractions 16 and 17 from Figure 4) were separated by reversed-phase HPLC. These data represent the radioactive profile on HPLC when GDH was photolabeled in the presence (solid circles) or absence (open circles) of 250 μM ADP.

fragment $\text{I}^{158}\text{--W}^{178}$ (Table 3). When ADP was present prior to photolysis, reduction of photolabeling of this peptide was observed as shown in Figure 6.

The approximate amount of peptide tube 46 of peak II containing 2.5×10^5 cpm (Cerenkov, 27.2% efficiency, total $^{32}\text{P} = 9.19 \times 10^5$ cpm) was calculated to be 36.6 pmol using the specific activity (11.3 mCi/ μmol) of the $[\alpha\text{-}^{32}\text{P}]\text{8N}_3\text{ADP}$ as given under Materials and Methods. A total of 157 pmol was calculated to be in the six fractions of peak II containing greater than 10^5 cpm. All of the peptide in tube 46 peak II was submitted for sequencing, and the results (see Table 2) indicated about 24–34 pmol of peptide.

To confirm the site of photomodification, GDH was photolabeled with 60 μM $[\alpha\text{-}^{32}\text{P}]\text{8N}_3\text{ADP}$ and proteolyzed with trypsin to isolate a photolabeled tryptic fragment which should overlap the photolabeled chymotryptic peptide ($\text{I}^{158}\text{--W}^{178}$). The photolabeled peptides were isolated as described for the isolation of the photolabeled chymotryptic peptide. Two radioactive tryptic peptides were identified by sequence analysis. These were $\text{G}^{156}\text{--R}^{174}$ and $\text{E}^{175}\text{--K}^{200}$ (Table 3). This experiment was repeated at least three times. The usual amounts of these peptides identified from sequence were between 20 and 40 pmol.

Table 3. $[\alpha\text{-}^{32}\text{P}]\text{8N}_3\text{ADP}$ -Photolabeled Chymotryptic and Tryptic Peptide

cycle	chymotryptic peptide (peak II)		tryptic peptides			
	residue	pmol	residue	pmol	residue	pmol
1	I^{158}	24	E^{175}	43	G^{156}	30
2	G^{159}	52	M^{176}	31	F^{157}	14
3	P^{160}	31	S^{177}	28	I^{158}	18
4	G^{161}	34	W^{178}	ND	G^{159}	33
5	V^{162}	25	I^{179}	22	P^{16}	⁰ 16
6	D^{163}	17	A^{180}	20	G^{161}	20
7	V^{164}	20	D^{181}	22	V^{162}	17
8	P^{165}	25	T^{182}	18	D^{163}	25
9	A^{166}	13	Y^{183}	9	V^{16}	⁴ 14
10 ^a	P^{167}	14	A^{184}	11	P^{165}	10
11			S^{185}	10	A^{166}	11
12			T^{186}	9	P^{167}	8
13			I^{187}	10	N^{168}	6
14			G^{188}	12	M^{169}	4
15 ^b			H^{189}	2	S^{170}	ND ^c
16					T^{171}	7
17					G^{172}	11
18					E^{173}	ND
19					R^{174}	ND

^a Only the first 10 cycles were sequenced. ^b Only the first 15 cycles were sequenced. ^c Residue not detected.

Since saturation of GDH with $[\alpha\text{-}^{32}\text{P}]\text{8N}_3\text{ADP}$ appeared to be biphasic (Figure 2A), an attempt was made to isolate GDH peptides photolabeled with a higher concentration of photoprobe. When GDH was photolabeled with 160 μM $[\alpha\text{-}^{32}\text{P}]\text{8N}_3\text{ADP}$ and proteolyzed with trypsin, another peptide was sometimes identified in addition to $\text{G}^{156}\text{--R}^{174}$ and $\text{E}^{175}\text{--K}^{200}$. This peptide was identified as $\text{D}^{403}\text{--R}^{418}$ (data not shown), a region unique to only the vertebrate enzymes (23).

Since proteolysis of $[\alpha\text{-}^{32}\text{P}]\text{8N}_3\text{ADP}$ -photolabeled GDH with two different proteases afforded identification of the same photolabeled region, a double proteolysis (chymotrypsin and trypsin) of $[\alpha\text{-}^{32}\text{P}]\text{8N}_3\text{ADP}$ -photolabeled GDH was performed in an attempt to narrow the photomodified region. One milligram of GDH was incubated with 60 μM $[\alpha\text{-}^{32}\text{P}]\text{8N}_3\text{ADP}$ in the presence of 5 mM glutamate and 100 μM NAD^+ . The protein was separated from unbound nucleotide and proteolyzed for 6 h with chymotrypsin followed by 10 h with trypsin, and the photolabeled peptides were isolated by immobilized Al^{3+} chromatography and HPLC as before. The UV-absorbing and radioactive profiles from the HPLC are shown in Figure 7. No peptide was identified from sequence analysis of peak III. However, peaks IV and V were identified as $\text{A}^{184}\text{--K}^{200}$ and $\text{E}^{175}\text{--Y}^{183}$, respectively (Table 4). To demonstrate specificity, 500 μM ADP was added to an identical incubation mixture prior to photolysis and the radioactivity of each peak was reduced about 80% compared to that of controls with no competing ADP present (data not shown).

Isolation of $2\text{N}_3\text{ADP}$ Binding Domains. The photoprobe $[\beta\text{-}^{32}\text{P}]\text{2N}_3\text{ADP}$ was used to further define the adenine binding domain of the ADP site. This analogue is thought to predominate in an anti conformation as opposed to the more even syn/anti equilibrium conformation of $8\text{N}_3\text{ADP}$ (34). It should therefore label peptides in close proximity to those modified by $[\alpha\text{-}^{32}\text{P}]\text{8N}_3\text{ADP}$ but not necessarily the same one(s).

Five hundred micrograms GDH was photolabeled with 90 μM $[\beta\text{-}^{32}\text{P}]\text{2N}_3\text{ADP}$ in phosphate buffer alone (pH 7.5) and subjected to tryptic proteolysis. The sample was prepared for immobilized Al^{3+} chromatography. The radioactive profile was similar to that of Figure 4, with about 90% of the radioactivity being retained on the resin. The radioactive retentate was eluted with 5 mM

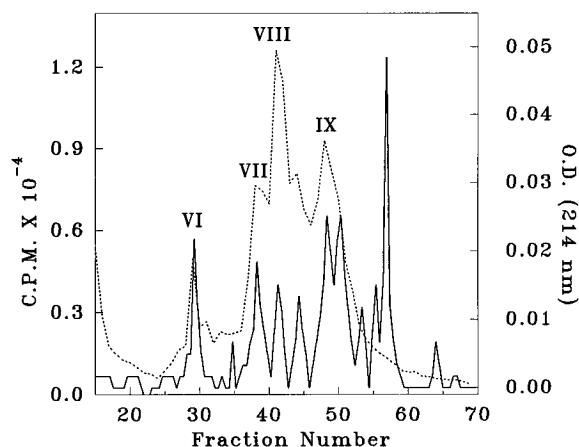


Figure 7. Radioactive profile of $[\alpha\text{-}^{32}\text{P}]8\text{N}_3\text{ADP}$ -photolabeled GDH peptides on HPLC. GDH was photolabeled with $80\ \mu\text{M}$ $[\alpha\text{-}^{32}\text{P}]8\text{N}_3\text{ADP}$. The sample was proteolyzed with both chymotrypsin and trypsin followed by separation by immobilized Al^{3+} chromatography. The peptides of the radioactive P_i eluate were separated by reversed-phase HPLC. The optical density at 214 nm (solid line) and radioactive (dashed line) profiles are shown.

Table 4. Sequence Analyses of Peaks IV and V from Figure 7

cycle	peak IV		peak V	
	identified residue	pmol	identified residue	pmol
1	A ¹⁸⁴	10	E ¹⁷⁵	11
2	S ¹⁸⁵	5	M ¹⁷⁶	3
3	T ¹⁸⁶	3	S ¹⁷⁷	5
4	I ¹⁸⁷	4	W ¹⁷⁸	ND ^a
5	G ¹⁸⁸	6	I ¹⁷⁹	6
6	H ¹⁸⁹	1	A ¹⁸⁰	4
7	Y ¹⁹⁰	2	D ¹⁸¹	4
8	D ¹⁹¹	5	T ¹⁸²	2
9	I ¹⁹²	5	Y ¹⁸³	2

^a Not detected.

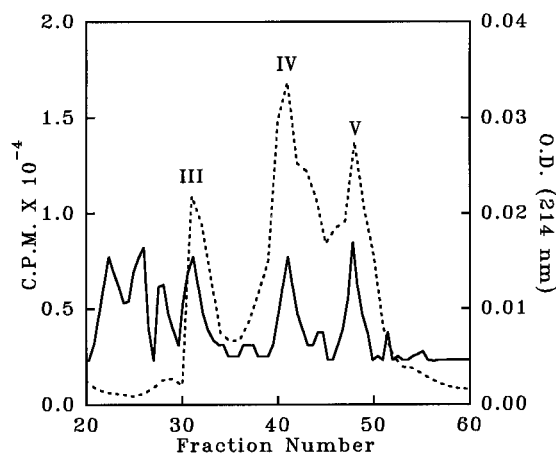


Figure 8. HPLC analysis of $[\beta\text{-}^{32}\text{P}]2\text{N}_3\text{ADP}$ -photolabeled GDH peptides. GDH was photolabeled with $[\beta\text{-}^{32}\text{P}]2\text{N}_3\text{ADP}$ and proteolyzed with trypsin, and photolabeled peptides were isolated by immobilized Al^{3+} chromatography. The peptides of the radioactive P_i eluate from immobilized Al^{3+} chromatography were purified by reversed-phase HPLC. The optical density at 214 nm (solid line) and radioactive (dashed line) profiles are shown.

KH_2PO_4 and subsequently subjected to C_8 reversed-phase HPLC. The radioactive and UV absorbance profiles from HPLC are shown in Figure 8. Radioactive peaks appeared in the flow-through and at 29, 38, 42, and 48 min (peaks VI, VII, VIII, and IX, respectively). Over 90% reduction of radiolabel in these peaks occurred when

Table 5. Sequence Analysis of Radioactive Peak VIII from Figure 8

cycle	identified residue	pmol	cycle	identified residue	pmol
1	G ¹⁵⁶	88	11	A ¹⁶⁶	26
2	F ¹⁵⁷	69	12	P ¹⁶⁷	13
3	I ¹⁵⁸	77	13	N ¹⁶⁸	3
4	G ¹⁵⁹	60	14	M ¹⁶⁹	4
5	P ¹⁶⁰	59	15	S ¹⁷⁰	6
6	G ¹⁶¹	62	16	T ¹⁷¹	6
7	V ¹⁶²	47	17	G ¹⁷²	11
8	D ¹⁶³	27	18	E ¹⁷³	3
9	V ¹⁶⁴	32	19	R ¹⁷⁴	6
10	P ¹⁶⁵	30			

competing ($327\ \mu\text{M}$) ADP was present during photolysis (data not shown).

Three tryptic peptides were identified in sequence analyses. No residues were identified in peak VI (similar to peak III, Figure 7). Peak VII contained two peptides. The peptides were identified as portions of the tryptic peptides E¹⁷⁵–K²⁰⁰ and C²⁷⁰–K²⁸⁹. C²⁷⁰–K²⁸⁹ has been previously identified as the $[\text{P}^{32}]\text{N}_3\text{NAD}^+$ binding peptide (20; see Discussion). Peak VIII contained both the highest level of radioactivity and the tryptic peptide G¹⁵⁶–R¹⁷⁴ (Table 5). Finally, peak IX contained the same partial tryptic peptides, E¹⁷⁵–K²⁰⁰ and C²⁷⁰–K²⁸⁹, as peak VII. In peak IX both recovered peptides were longer versions of the peptides in peak VII (data not shown).

DISCUSSION

The activity of bovine GDH, a complex multimeric enzyme that regulates amino acid metabolism, can be modulated by a variety of factors including the purine nucleotides ADP, GTP, and NADH. This laboratory has previously identified peptides of the base binding domains of the NAD^+ and GTP sites using the photoprobes $[\text{P}^{32}]\text{N}_3\text{NAD}^+$ and $[\text{P}^{32}]\text{N}_3\text{GTP}$, respectively, which were biological mimics of NAD^+ and GTP (4, 19, 21). The ADP site was more difficult to efficiently photolabel and identify due to the lower affinity of the ADP site, similarities between NAD(P)^+ and ADP, and wobble of ADP within the ADP binding region (24). A further complication was the possibility of multiple ADP binding sites as suggested by Batra and Colman (8).

In the present study, the photoprobes $[\alpha\text{-}^{32}\text{P}]8\text{N}_3\text{ADP}$ and $[\beta\text{-}^{32}\text{P}]2\text{N}_3\text{ADP}$ were used to determine the domain of GDH involved in the GDH–ADP interactions. The specificity of $8\text{N}_3\text{ADP}$ and the utility of this probe as a good candidate for determining the ADP site were demonstrated by the following: First, in the absence of activating light, $8\text{N}_3\text{ADP}$ was able to activate GDH similarly to ADP. The ability to mimic a native compound before photolysis has an advantage over determination of the enzyme function after modification. With the activation constant known, it is easy to show that photoinsertion occurs in correlation with the known affinity. Also, it is well-known that many enzymes are inhibited by stoichiometric amounts of modification with chemical probes without active site involvement and such could also occur with activation. Second, saturation of photoinsertion by $[\alpha\text{-}^{32}\text{P}]8\text{N}_3\text{ADP}$ into GDH was observed. Third, photoinsertion of $80\ \mu\text{M}$ $[\alpha\text{-}^{32}\text{P}]8\text{N}_3\text{ADP}$ was 65% reduced with $270\ \mu\text{M}$ ADP as determined after SDS–PAGE. This measures ^{32}P photoinsertion only into the GDH subunits and eliminates the possibility of photolabeling contaminants. At these concentrations, ADP represents 77% of total nucleotide; therefore, a 65% reduction is 86% of the theoretical maximum if $8\text{N}_3\text{ADP}$ and ADP had the same affinity with GDH. Also, reduction by other nucleotides was not nearly as effective as

that observed with ADP. Fourth, only a select domain of GDH was photomodified by both [α - ^{32} P]8N₃ADP and [β - ^{32} P]2N₃ADP, demonstrating a lack of nonspecific photoinsertion and site selectivity corresponding to ADP site occupancy. Also, addition of ADP to the photolabeling mixture greatly reduced the quantity of photolabeled peptides isolated. Finally, a circular dichroism study by others showed that 8N₃ADP and ADP induced similar effects on the CD spectra of GDH, and 8N₃ADP was also shown to effectively activate GDH in the forward reaction, although not as well as ADP (32). This is consistent with the results in Table 2.

The nucleotide, 2N₃ADP, has no effect on the activity of GDH at pH 8.0. However, 2N₃ADP was able to prevent the activation of GDH by ADP. It is possible that at pH 8.0, 2N₃ADP bound to the ADP binding domain but was unable to elicit a response due to a different binding orientation or inability to induce the proper protein conformational change necessary for increased activity. Similar related differences between binding and activity of 8- versus 2-azidoadenosine analogues have been observed with the F1-ATPase (34). Further, to eliminate the possibility that 2N₃ADP was interacting at the GTP site (due to substitution at the 2-position of the purine ring), the reduction of [β - ^{32} P]2N₃ADP photoinsertion by GTP was examined. Photolabeling with 80 μ M [β - ^{32} P]2N₃ADP was reduced only 10% by 250 μ M GTP. Also at pH 8.0, 2N₃ADP did not inhibit the activity of GDH as does GTP, and at acidic pH both ADP and 2N₃ADP inhibited the enzyme (Table 2). These observations indicate that 2N₃ADP binds at the ADP site and was not interacting at the GTP site.

Besides ADP and 8N₃ADP, only ATP-benzophenone and 8N₃ATP-benzophenone, novel photoactive ATP analogues that modify regions at or very near the phosphate-interacting peptides within ADP/ATP binding sites (31), were able to fully activate GDH under the conditions described in this paper. ATP γ S activated GDH about half as well as ADP. ATP, GDP, Ap₄A, cAMP, or adenosine had no effect on the activity of the enzyme. AMP was only able to marginally activate the enzyme. It is unclear why ATP- γ -benzophenone or 8N₃ATP- γ -benzophenone should activate the enzyme. However, they have the same net charge as ADP at pH 8.0 as does ATP γ S.

The crystal structure of *C. symbiosum* GDH (2) has been aligned with the primary sequence of the bovine liver GDH (3). The structures of the two were suggested to be similar due to considerable homology and the conservation of 13 glycine residues, which probably conserves the structure among species, and to consist of two domains. The first domain has been proposed to contain the catalytically important residues, while the second domain contains a β - α - β motif that is responsible for coenzyme binding (5).

The primary sequence of bovine liver GDH also suggests the presence of a β - α - β motif corresponding to the motif within the bacterial subunit. This laboratory previously labeled within the putative β - α - β motif (E²⁷⁴-G²⁷⁵) in bovine liver GDH with [^{32} P]2N₃NAD⁺ a photoactive substrate, with E²⁷⁸ being the residue photomodified (4, 19). This lends credence to the determination of this motif as the NAD⁺ binding domain. This site is near the active site portion of GDH as depicted by Singh *et al.* (35).

Previously, 8N₃GTP was shown to be an effective biomimic of GTP in inhibiting GDH activity. Using [α - ^{32} P]8N₃GTP, a guanine-binding peptide within the GTP regulatory site of (bovine liver) GDH was identified as the peptide containing residues I⁴³⁹-Y⁴⁵⁴ with probable

point photomodification at K⁴⁴⁵ (21). Since only the vertebrate enzymes contain the sequence corresponding to G³⁹⁴-G⁴⁴¹ of bovine liver GDH, and only GDHs from these sources are regulated by GTP, it is most likely that this region plays a role in GTP binding. The ADP and GTP regulatory sites are thought to be distinct but proximal and possibly overlapping (36). Both nucleotides also elicit different conformational changes within the enzyme (37).

The region spanning tryptic peptide G¹⁵⁶-K²⁰⁰ was photolabeled with both [α - ^{32} P]8N₃ADP and [β - ^{32} P]2N₃ADP. Tryptic plus chymotryptic digestion reduced the photolabeled domain to E¹⁷⁵-I¹⁹². The peptides within this region were specifically protected from photomodification by competing amounts of ADP. Comparisons of this sequence with the bacterial enzyme suggest that these peptides lie within the first globular domain. The E¹⁷⁵-I¹⁹² domain was isolated as two photolabeled peptides clipped between residues Y¹⁸³ and A¹⁸⁴, indicating that photoinsertion was into more than one residue. This is possibly due to the wobble or the binding orientation.

The affinity of ADP, 2N₃ADP, and 8N₃ADP to GDH was not as tight as that of NAD⁺, GTP, and their corresponding photoaffinity probes. This was likely due to fewer points of protein-nucleotide contact, which would account for "loose" binding which may lead to photoinsertion into more than one residue of a specific peptide. While certain residues of the photolabeled peptides showed decreases compared to other residues, the exact residues photomodified within the adenine ring domain within the ADP binding site remain uncertain.

When excess (160 μ M) [α - ^{32} P]8N₃ADP was used to photolabel the enzyme, another peptide was identified (D⁴⁰³-R⁴¹⁸). Our laboratory has also labeled this peptide with [γ - ^{32} P]ATP- γ -benzophenone (31), suggesting this peptide may be important for recognition of the phosphate domain of ADP. This peptide is absent in the corresponding bacterial enzyme and is adjacent to the site (near K⁴⁴⁵) identified as within the base binding domain of the GTP site. It starts just after helix α _{15b}, which lies near beta strand β ₆ and helix α ₈ (3), the domain photolabeled by both [β - ^{32} P]2N₃ADP and [α - ^{32} P]8N₃ADP, and is a region that has been suggested to be important in nucleotide regulation and possibly subunit interaction (23). Therefore, from the proposed structure of the bacterial enzyme, D⁴⁰³-R⁴¹⁸ could be very near G¹⁵⁶-K²⁰⁰ in three-dimensional orientation (3).

Since peptides G¹⁵⁶-K²⁰⁰ and D⁴⁰³-R⁴¹⁸ could be identified in the same experiment, it is unclear whether their concomitant identification could be due to two ADPs bound at the same time at separate, distinct sites or the photoinsertion into two nearby peptides due to the low-affinity, high-wobble binding at the same ADP site. Wobble would allow different base orientation that could result in photoinsertion into different residues. For example, using 2- and 8-azido-ATP (both effective substrates) to probe the singular ATP site of creatine kinase clearly identified two different peptides (25). Other possibilities exist which include complexities that occur due to possible intersubunit contacts.

Another region, C²⁷⁰-K²⁸⁹, was labeled with [β - ^{32} P]2N₃ADP only. This peptide has already been determined to be within the adenine binding domain of the NAD⁺ binding site (4) as predicted by others (2, 5). It has been suggested that under certain conditions ADP acts as a competitive inhibitor of the coenzyme (38). Since 2N₃ADP is about two-thirds portion of a 2N₃NAD⁺ molecule and since 2N₃NAD⁺ binds to the NAD⁺ binding site with higher affinity than NAD⁺ (4), one would expect some labeling of the NAD(P)⁺ site with this ADP probe. In

support of this was the observation that the presence of NAD⁺ or NADP⁺ reduced the amount of this peptide that was isolated.

In contrast to our results, two earlier studies using two different adenosine monophosphate chemical probes modified residues H⁸² and Arg⁴⁵⁹, and it was suggested that these residues lie within the regulatory ADP site (17, 18). These two residues are outside the catalytic cleft and markedly distal from the peptide region identified herein using two ADP photoaffinity probes. It is possible that 8N₃ADP, 2N₃ADP, and the chemical probes all bind within the same ADP site. Classical chemical probes selectively modify only specific reactive residues and may label residues near or outside the binding domain due to their long-lived chemical reactivity. They usually have much lower affinity and many times require solvents such as dimethyl sulfoxide for solubility. Their lack of specificity may be the reason for the wide three-dimensional distribution of the residues identified using classical chemical probes as being in the NADH inhibitory site of GDH (10–15). In contrast, azidopurine photoprobes generate short-lived, very reactive nitrenes which will modify any residue placed near the generated nitrene by binding. The observed lack of photolabeling outside the catalytic cleft and the fact that the photoprobes of NAD⁺, GTP, and ADP each identify appropriate, but different, binding domain peptides in GDH support the contention that binding within specific domains must occur before photomodification can be effected. The ability of the photoaffinity probes, in the absence of activating light, to mimic the native nucleotides as substrate and regulatory constituents further supports their specificity. Also, saturation of photoinjection at concentrations corresponding to that expected from the reversible binding affinities strongly supports the site being labeled is within the binding domain.

The results presented in this work confirm the validity of photolabeling with azidonucleotide analogues to locate specific binding domains on complex enzymes. Different peptides were identified within the base binding domain of the coenzyme (NAD⁺) and two regulatory (GTP, ADP) sites of one homologous enzyme, GDH, through this methodology. The peptides identified within the purine ring binding domain of each nucleotide binding site were distinct from the others, yet all fell within the proposed catalytic cleft. The GTP site and one of the two isolated 8N₃ADP binding peptides was also located in a region whose primary sequence (residues 400–500) is unique to vertebrate GDHs, the only GDHs that are regulated by these nucleotides. The correlation of this structural data with the three-dimensional structure of mammalian GDH should aid in the determination of the nucleotide regulatory mechanism utilized by this enzyme.

LITERATURE CITED

- Moon, K., Piszkiwicz, D., and Smith, E. (1972) Glutamate dehydrogenase: amino-acid sequence of the bovine enzyme and comparison with that from chicken liver. *Proc. Natl. Acad. Sci. U.S.A.* 69, 1380–1383.
- Britton, K., Baker, P., Rice, D., and Stillman, T. (1992) Structural relationship between the hexameric and tetrameric family of glutamate dehydrogenases. *Eur. J. Biochem.* 209, 851–859.
- Teller, J., Smith, R., McPherson, M., Engel, P., and Guest, J. (1992) The glutamate dehydrogenase gene of *Clostridium symbiosum*: cloning by polymerase chain reaction, sequence analysis and over-expression in *Escherichia coli*. *Eur. J. Biochem.* 206, 151–159.
- Kim, H., and Haley, B. (1991) Identification of peptides in the adenine ring binding domain of glutamate and lactate dehydrogenase. *Bioconjugate Chem.* 2, 142–147.
- Wierenga, R., Terpstra, P., and Hol, W. G. J. (1986) Prediction of the occurrence of the ADP-binding $\beta\alpha\beta$ -fold in proteins, using an amino acid sequence fingerprint. *J. Mol. Biol.* 187, 101–107.
- Erecinska, M., and Nelson, D. (1990) Activation of glutamate dehydrogenase by leucine and its nonmetabolizable analogue in rat brain synaptosomes. *J. Neurochem.* 54, 1335–1343.
- Fahien, L., Teller, J., MacDonald, M., and Fahien, C. (1990) Regulation of glutamate dehydrogenase by Mg²⁺ and magnification of leucine activation by Mg²⁺. *Mol. Pharmacol.* 37, 943–949.
- Batra, S., and Colman, R. (1986) Affinity labeling of an allosteric ADP site of glutamate dehydrogenase by 2-(4-bromo-2,3-dioxobutylthio)adenosine 5'-monophosphate. *J. Biol. Chem.* 261, 15565–15571.
- Colman, R. F. (1991) in *A Study in Enzymes, Vol. II, Mechanism of Enzyme Action* (S. A. Kuby, Ed.) Chapter 7, pp 173–192, CRC Press, Boca Raton, FL.
- Ozturk, D. H., Safer, D., and Colman, R. F. (1990) Affinity labeling of bovine liver glutamate dehydrogenase by 8-(bromo-2,3-dioxobutylthio)adenosine 5'-diphosphate and 5'-triphosphate. *Biochemistry* 29, 7112–7118.
- Ozturk, D. H., and Colman, R. F. (1991) Identification of cysteine-319 as the target amino acid of 8-[(4-bromo-2,3-dioxobutylthio)adenosine 5'-triphosphate in bovine glutamate dehydrogenase. *Biochemistry* 30, 7126–7134.
- Ozturk, D. H., Park, I., and Colman, R. F. (1992) Guanosine 5'-O-[S-(3-bromo-2-oxopropyl)thiophosphate]: a new reactive purine nucleotide analog labeling Met-169 and Tyr-262 in bovine liver glutamate dehydrogenase. *Biochemistry* 31, 10544–10555.
- Pal, P. K., Wechter, W. J., and Colman, R. F. (1975) Affinity labeling of the DPNH inhibitory site of bovine liver glutamate dehydrogenase by 5'-p-fluorosulfonylbenzoyl adenosine. *J. Biol. Chem.* 250, 8140–8147.
- Saradambal, K. V., Bednar, R. A., and Colman, R. F. (1981) Lysine and tyrosine in the NADH inhibitory site of bovine liver glutamate dehydrogenase. *J. Biol. Chem.* 256, 11866–11872.
- Schmidt, J. A., and Colman, R. F. (1984) Identification of the lysine and tyrosine peptides labeled by 5'-p-fluorosulfonylbenzoyl adenosine in the NADH inhibitory site of glutamate dehydrogenase. *J. Biol. Chem.* 259, 14515–14519.
- Jacobson, M. A., and Colman, R. F. (1982) Affinity labeling of a guanosine 5'-triphosphate site of glutamate dehydrogenase by a fluorescent nucleotide analogue, 5'-[p-(fluorosulfonyl)benzoyl]-1,N⁶-ethenoadenosine. *Biochemistry* 21, 2177–2186.
- Batra, S., Lark, R., and Colman, R. (1989) Identification of histidyl peptide labeled by 2-(4-bromo-2,3-dioxobutylthio)adenosine 5'-triphosphate in an ADP regulatory site of glutamate dehydrogenase. *Arch. Biochem. Biophys.* 270, 277–285.
- Wrzeszczynski, K. O., and Colman, R. F. (1994) Activation of bovine liver glutamate dehydrogenase by covalent reaction of adenosine 5'-O-[S-(4-bromo-2,3-dioxobutyl)thiophosphate] with arginine-459 at an ADP regulatory site. *Biochemistry* 33, 11544–11553.
- Kim, H., and Haley, B. (1990) Synthesis and properties of 2-azido-NAD⁺: a study of interactions with glutamate dehydrogenase. *J. Biol. Chem.* 265, 3636–3641.
- King, S. M., Kim, H., and Haley, B. E. (1991) Strategies and reagents for photoaffinity labeling of mechanochemical proteins. *Methods Enzymol.* 196, 449–466.
- Shoemaker, M., and Haley, B. (1993) Identification of a guanine binding domain peptide of the GTP binding site of glutamate dehydrogenase. *Biochemistry* 32, 1883–1890.
- Potter, R. L., and Haley, B. E. (1982) Photoaffinity labeling of nucleotide binding sites with 8-azidopurine analogs. *Methods Enzymol.* 91, 613–633.
- Smith, E., Austen, B., Blumenthal, K., and Nyc, J. (1975) Glutamate dehydrogenase, in *The Enzymes*, Vol. 11 (P. Boyer, Ed.) pp 293–367, Academic Press, New York.
- Colen, A., Cross, D., and Fisher, H. (1974) Two-step binding of adenosine diphosphate to L-glutamate dehydrogenase. Effect on the binding of reduced nicotinamide adenine diphosphate and on enzymatic catalysis. *Biochemistry* 13, 2341–2347.

- Olcott, M., Bradley, M., and Haley, B. (1994) Identification of two peptides from the ATP-binding domain of creatine kinase. *Biochemistry* 33, 11935–11941.
- Czarnecki, J., Geahlen, R., and Haley, B. (1979) Synthesis and use of azido photoaffinity analogs of adenine and guanine nucleotides. *Methods Enzymol.* 56, 642–653.
- Michelson, A. (1964) Synthesis of nucleotide anhydrides by anion exchange. *Biochim. Biophys. Acta* 91, 1–13.
- Salvucci, M., Chavan, A., and Haley, B. (1992) Identification of peptides for the adenine binding domains of ATP and AMP in adenylate kinase: isolation of photoaffinity labeled peptides by metal chelate chromatography. *Biochemistry* 31, 4479–4487.
- Laemmli, U. (1970) Cleavage of structural proteins during the assembly of the bacteriophage T4. *Nature* 227, 680–685.
- Anderson, L. (1991) Recognition of phosphate groups by immobilized aluminum(III) ions. *J. Chromatogr.* 539, 327–334.
- Rajagopalan, K., Chavan, A., Haley, B., and Watt, D. (1993) Bidentate cross-linking reagents: non-hydrolyzable nucleotide photoaffinity probes with two photoactive groups. *J. Biol. Chem.* 268, 14230–14238.
- Koberstein, R., Cobianchi, L., and Sund, H. (1976) Interaction of the photoaffinity label 8-azido-ADP with glutamate dehydrogenase. *FEBS Lett.* 64, 176–180.
- Bailey J., Bell, E., and Bell, J. (1982) Regulation of bovine glutamate dehydrogenase. The effects of pH and ADP. *J. Biol. Chem.* 257, 5579–5583.
- Hartog, A., Edel, C., Lubbers, F., and Berden, J. (1992) Characteristics of the non-exchangeable nucleotide binding sites of mitochondrial F1 revealed by dissociation and reconstitution with 2-azido-ATP. *Biochim. Biophys. Acta* 1100, 267–277.
- Singh, N., Maniscalco, S., and Fisher, H. (1993) The real-time resolution of proton-related transient-state steps in an enzymatic reaction: the early steps in the oxidative deamination reaction of bovine liver glutamate dehydrogenase. *J. Biol. Chem.* 268, 21–28.
- Cross, D., and Fisher, H. (1970) The mechanism of glutamate dehydrogenase reaction. III. The binding of ligands at multiple subsites and resulting kinetic effects. *J. Biol. Chem.* 245, 2612–2621.
- Cioni, P., and Strambini, G. (1989) Dynamic structure of glutamate dehydrogenase as monitored by tryptophan phosphorescence: signal transmission following binding of allosteric effectors. *J. Mol. Biol.* 207, 237–247.
- George, A., and Bell, E. (1980) Effects of adenosine 5'-diphosphate on bovine glutamate dehydrogenase: diethyl pyrocarbonate modification. *Biochemistry* 19, 6057–6061.

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Magnetically Labeled Secretin Retains Receptor Affinity to Pancreas Acinar Cells[†]

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Previously, we have developed a colloidal dextran-stabilized monocrystalline iron oxide nanocompound (MION-46) as a magnetic label for magnetic resonance imaging (MRI). In an effort to use this magnetic label to visualize pancreatic receptor function by MRI *in vivo*, we investigated the potential of secretin as a vector molecule. Secretin receptors, abundant on exocrine pancreas cells, recognize secretin through its amidated carboxyl terminal. In order to conjugate secretin to MION, we utilized the specific interaction between biotin and streptavidin, since direct conjugation of human secretin to MION has previously resulted in low yields and low affinity of the conjugate (unpublished results). Initially, we biotinylated the N-terminal primary amino group of secretin (60% yield). In a separate step, streptavidin (SA) was immobilized onto the surface dextran molecules of MION (79% yield) by reductive amination. Each secretin molecule was conjugated to one biotin molecule and each MION particle to an average of two SA molecules. The biotinylated secretin was then conjugated to MION through the biotin–streptavidin interaction (90% yield). The secretin–biotin–streptavidin–MION construct thus contained approximately two secretin molecules per MION. An *in vitro* competitive binding assay of pancreatic acinar cells demonstrated that the magnetically labeled secretin retained affinity to the secretin receptors. *In vivo* distribution studies in rats showed a significantly higher pancreatic accumulation of the secretin–biotin–streptavidin–MION construct as compared to the control group that had received unmodified MION. Our data indicate that bioactive peptides can be attached to dextran-coated iron oxide particles through the biotin–streptavidin interaction while retaining receptor affinity. Such target-specific agents have potential use in MR imaging to probe for a variety of receptor systems.

INTRODUCTION

Receptor-specific magnetic resonance (MR) contrast agents targeted to the pancreas could improve the accuracy of cancer detection and tumor characterization and facilitate therapeutic drug delivery to the pancreas. Early detection of pancreatic cancer is of prime clinical importance (1). Unfortunately, the sensitivity of tumor detection by conventional imaging methods is relatively low primarily due to the lack of cell-specific contrast agents (2).

We have previously developed a dextran-coated (B-512F, molecular mass of 11 kDa) monocrystalline iron oxide nanocompound (MION) (3), a stable magnetopharmaceutical which consists of a single crystal core of iron oxide (4.6 ± 1.1 nm) coated with an average of 25 surface dextran molecules (hydrodynamic radius of 20 ± 4 nm). The induced magnetization of MION is 68 emu/g of Fe at 1.5 T, and proton relaxivities are 16.5 (R_1) and 34.8 $\text{mM}^{-1}\cdot\text{s}^{-1}$ (R_2), respectively (3). MION-46 can be detected *in vivo* by MR imaging at tissue concentrations as low as 50 nmol of Fe/g of tissue (4), and it has been used as a magnetic label for a variety of MR imaging applications such as imaging of axonal transport (5), ASG receptor function (4), and myocardial infarction imaging (6). When given intravenously in its unmodified form, MION has a blood half-life of 4.5 h in rats and is largely phagocytosed by phagocytic cells in the liver and spleen.

In an effort to direct MION to the pancreatic cells, early feasibility studies utilized cholecystokinin (CCK) as a vector molecule (7). MION–CCK has been shown to be recognized by receptors on exogenous pancreas cells and to decrease the T_2 relaxation time of the pancreas (33.8 ± 1.4 ms) but not that of adenocarcinoma (78 ± 6.3 ms). In these early preparations, CCK was chemisorbed onto MION. Although this provides for simple synthesis, the chemisorbed CCK was shown to dissociate from MION. Furthermore, the CCK receptor density on pancreas is not as high as that of other receptor systems, such as secretin receptors (8).

In the current study, we investigated the potential of the 27-amino acid peptide secretin as a pancreaticotropic vector molecule. Receptor recognition of this peptide occurs primarily through the amidated carboxyl terminal of secretin which interacts with the receptor present on pancreatic parenchymal cells (9). Previous attempts to covalently conjugate secretin to MION yielded low attachment efficiency (<5%; T. Shen, unpublished results) as well as low affinity of the conjugate which may be explained by steric hindrance caused by surface-bound dextran molecules on MION. We therefore explored possibilities of indirect conjugation using the biotin–streptavidin system.

Biotin, naturally occurring vitamin H, has the unique property of binding streptavidin (60 kDa protein) with high affinity ($K_a = 10^{15} \text{ M}^{-1}$) and specificity (10, 11). Initially, we biotinylated the N-terminal primary amino group of secretin. In a separate step, streptavidin (SA) was immobilized onto the MION surface and then biotinylated secretin was conjugated to MION through the biotin–streptavidin interaction. The affinity of the conjugation product was tested in a competitive cell binding

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assay using pancreas acinar cells. *In vivo* distribution patterns of various conjugates were finally tested in rats.

MATERIALS AND METHODS

Materials. MION-46 was synthesized and characterized as described previously (3). Synthetic human secretin (MW = 3039.4), Hanks balanced salt solution (HBSS), collagenase type II, and soybean trypsin inhibitor (STI) were obtained from Sigma, St. Louis, MO. IODO-GEN and sulfosuccinimidyl-6-(biotinamido) hexanoate (Biotin-LC-NHS, molecular weight of 244) were obtained from Pierce, Rockford, IL. Streptavidin (MW 60 000) was obtained from Molecular Probes, Eugene, OR. Ham's F-12 medium and Waymouth's medium 752/1 were obtained from Gibco, Grand Island, NY. Na¹²⁵I was obtained from Amersham, Arlington Heights, IL. Sephadex G25 was obtained from Pharmacia, Piscataway, NJ. Male Sprague-Dawley rats (150–200 g) were obtained from Charles River Laboratories, Wilmington, MA. Binding buffer, freshly prepared before each competitive cell binding experiment, contained 10 mM glucose, 100 mM NaCl, 5 mM KCl, 2.5 mM NaH₂PO₄, 1 mM MgCl₂, 0.5 mM CaCl₂, 25 mM HEPES, 5 mM sodium glutamate, 5 mM sodium pyruvate, 2 mM glutamine, 1% (w/v) bovine serum albumin, and a 1% (w/v) amino acid mixture without L-glutamine and 0.01% (w/v) STI.

Methods. The individual steps of the synthesis methods described below were developed by initial optimization of reaction conditions. Subsequently, several batches of the conjugate were synthesized to produce the quantity required for the *in vivo* experiments. The consistency of the product was monitored at each synthesis step.

Secretin Iodination. Synthetic human secretin [100 μ L, 0.1 mg/mL in 50 mM sodium borate buffer (pH 8.0)] was radiolabeled with ¹²⁵I (1 mCi) by solid phase catalysis (ODO-GEN, 0.1 mg) at room temperature for 15 min, positioning the ¹²⁵I on the N-terminal histidine residue of secretin (12). After iodination, the [¹²⁵I]secretin was separated from unreacted ¹²⁵I and unlabeled secretin using a Sephadex G15/G50 column [7:3 w/w, equilibrated and eluted with 50 mM sodium borate buffer (pH 8.5), containing 80 mM NaCl and 0.1% bovine serum albumin (BSA)], followed by reverse phase high-performance liquid chromatography (HPLC). The column (Dynamax C-18, Rainin, MA) was eluted with an acetonitrile gradient in 1% trifluoroacetic acid (TFA), and simultaneous chromatograms were acquired using a UV-vis detector (215 nm) and a Bicon detector for radioactivity (Model PGM, Bicon Corp., Solon, Ohio).

Biotinylation of Secretin. [¹²⁵I]Secretin solution [900 μ L, 2 μ Ci, 0.1 mg/mL in 50 mM sodium bicarbonate (pH 8.5)] was added directly to dry biotin-LC-NHS (0.2 mg) and incubated on ice for 2 h, followed by incubation at 4 °C overnight under stirring. Biotin-LC-NHS was chosen for biotinylation because of its extended spacer arm (23.4 Å), thus minimizing steric hindrance. The reaction mixture was then vacuum concentrated to 100 μ L (HPLC loading volume) for characterization and purification by reverse phase HPLC.

The biotinylated secretin was then tested in binding assays using streptavidin-coated poly(vinyl chloride) plates (13). Free ¹²⁵I and [¹²⁵I]secretin were used as controls. For this assay, 100 μ L/well of 10 μ g/mL streptavidin in 50 mM carbonate buffer (pH 9.3) was incubated with the plates for 3 h (37 °C), followed by additional incubation in 100 μ L/well of 0.1% BSA in phosphate-buffered saline (PBS) (pH 7.6) for 30 min. At the end of the incubation, each well was washed with 100 μ L of PBS (pH 7.6) five times. The biotinylated secretin fraction and the controls were then added to the

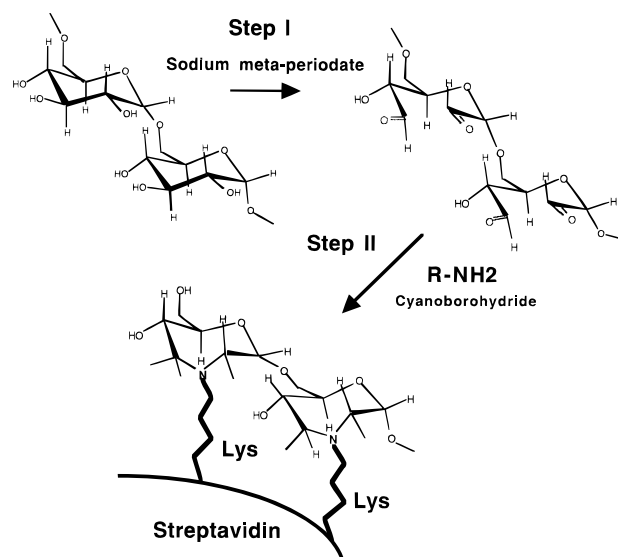


Figure 1. Streptavidin immobilization. SA was immobilized onto the MION surface by reductive amination. Surface bond dextran molecules were first oxidized with sodium metaperiodate (NaIO₄), generating two aliphatic aldehydes per glucose molecule of dextran (step I). Addition of streptavidin to the activated MION established a reversible Schiff base between the aldehydes and the primary amino groups available from surface lysine groups of streptavidin. The Schiff base is then reduced to form a stable covalent bond between MION and streptavidin (step II).

streptavidin-coated wells and the wells incubated at room temperature for 30 min. The wells were washed extensively (5 times), and the radioactivity associated with the wells was measured with a scintillation counter.

MION-Streptavidin. Streptavidin was immobilized onto the MION surface by reductive amination (Figure 1). The surface dextran molecules on MION were first partially oxidized with sodium metaperiodate (NaIO₄), generating two aliphatic aldehydes per glucose molecule of dextran. NaIO₄ solution (170 μ L, 20 mg/mL in water) was added to MION [830 μ L, 30 mg of Fe/mL in 0.3 M NaCl (pH 8.0)], and the mixture was stirred at room temperature in the dark for 40 min. The reaction was then stopped by separation of free NaIO₄ from activated MION through a 12 mL Sephadex G-25 spin column equilibrated with 10 mM NaHCO₃ and 0.15 M NaCl buffer (pH 8.7). Activated MION (110 μ L, 30 mg/mL) was then incubated with streptavidin [500 μ L, 7.0 mg/mL in 0.1 M NaHCO₃ buffer (pH 8.7)] at room temperature for 15 min. The reaction was allowed to proceed at 4 °C overnight. Conjugation of streptavidin to the activated MION proceeds via a reversible Schiff base between the aldehydes and the primary lysine ϵ -amino groups of streptavidin. The Schiff bases were then reduced by sodium cyanoborohydride (NaCNBH₃, 1 μ L of a 10 mg/mL solution in H₂O) to form a stable covalent bond between MION and streptavidin.

The MION-streptavidin conjugate was analyzed and purified by size-exclusion HPLC. The iron concentration of MION was determined by spectrophotometry (Hitachi Model U-3000, Tokyo, Japan) using a HCl/H₂O₂ assay (3), and the concentration of streptavidin was determined spectrophotometrically using a BCA assay with bovine serum albumin as a standard. The overall size distribution of the MION-SA complex in aqueous media was determined by photon correlation spectroscopy using 90° angle scattering (Model N4MD Submicron Particle Analyzer, Coulter Electronics, Hialeah, FL). Relaxation times of MION-SA in aqueous solution at 40 °C and 0.47 T were measured with a Bruker Minispec (NMS100 NMR

analyser, Bruker Instruments, Rheinstetten, Germany). Before each measurement, the spectrometer was tuned and calibrated. T_1 (longitudinal relaxation time) was measured from 20 data points generated by an inversion-recovery pulse sequence. T_2 (transverse relaxation time) was measured by a Carr-Purcell-Meiboom-Gill pulse sequence with a τ of 1 ms. The inverse of the relaxation time was plotted as a function of iron concentration to yield the relaxivities (R_1 and R_2) from the slopes.

MION-Streptavidin-Biotin-Secretin Conjugate. HPLC-purified biotinylated secretin containing trace amounts of radioiodinated biotinylated secretin (80 μ g) was incubated with HPLC-purified MION-SA (100 μ L, 15 mg of Fe/mL) at room temperature for 1 h and dialyzed with Spectra Por membrane tubing (MWCO of 100 000, Spectrum Medical Industries, Inc., Houston, TX) against 1x PBS (pH 7.6) at 4 °C overnight. Photon correlation spectroscopy, relaxation time measurements, and radioactivity were also obtained on the final dialyzed product.

Pancreatic Cell Isolation. The affinity of the secretin-biotin-streptavidin-MION conjugate to secretin receptors was evaluated in a competitive cell binding assay using isolated pancreatic acini. Dispersed pancreatic cells were prepared by a modified method previously described (14). Pancreatic tissue pooled from two male Sprague-Dawley rats was used for each cell isolation. The freshly harvested and minced rat pancreatic tissue (1–2 mm pieces) was subjected to two cycles of enzymatic digestion by collagenase (type II), and pancreatic cells were isolated by filtration and centrifugation. The final cell pellet was resuspended in 10 mL of Waymouth's medium. The pancreatic acinar cell suspension was evaluated by light microscopy, and viable cells were counted using a standard hemocytometer chamber. Typically, 3×10^7 cells were harvested using this protocol. Cell binding experiments were always performed within 1 h of cell isolation.

Competitive Cell Binding. Freshly isolated pancreatic acini were resuspended in a binding buffer at 37 °C. The cell suspensions (500 μ L, containing 8×10^5 viable cells) were incubated with the ^{125} I-labeled secretin-MION conjugate (4×10^5 cpm), [^{125} I]secretin, and an unlabeled MION preparation. Serial dilutions of nonradioactive secretin were then added, followed by incubation at 37 °C for 30 min. All cell suspensions were then washed twice with the binding buffer at 4 °C, and radioactivity associated with the cells was determined with a crystal scintillation counter (LKB-Wallac, Turku). Tests at each concentration point were carried out in triplicate, and the average value was used for the binding curve. The cell suspensions without addition of cold secretin were used as controls. Data were expressed as bond percent of the control.

In Vivo Biodistribution. HPLC-purified [^{125} I]secretin (20 μ g/animal), the secretin-biotin-SA-MION complex (1 mg of Fe/animal), and unaltered MION (control, 1 mg of Fe/animal) were tested *in vivo* in male Sprague-Dawley rats ($n = 18$ rats). Animal experiments were approved by the Institutional Review Committee and were in accordance with the USDA Animal Welfare Act. Following intravenous administration, animals were sacrificed by overdose of pentobarbital and the harvested organs (pancreas, liver, spleen, heart, lungs, small intestine, and stomach) were weighed immediately. Radioactivity of the samples was measured (60 s counting time/sample) with a crystal scintillation counter (LKB-Wallac, Turku). The activity of each organ was expressed as the percentage of injected dose per gram of tissue.

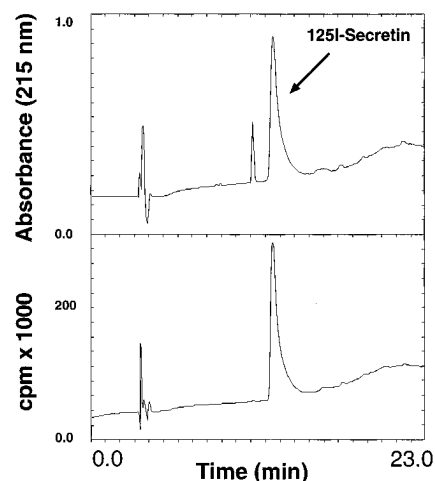


Figure 2. Chromatogram of [^{125} I]secretin using reverse phase HPLC. The top chromatogram was acquired with UV absorption at 215 nm, and the bottom shows radioactivity. The peak of radioactivity and the peak from absorption correspond in elution time, typical for [^{125} I]secretin.

RESULTS AND DISCUSSION

Modification of Secretin. The initial step in the synthesis of the construct was the iodination of secretin to yield an easily traceable ligand. Because secretin does not contain tyrosine residues (which can be easily iodinated), iodination was carried out through the N-terminal histidine residue as reported previously (15, 16). Using a solid phase catalysis, we achieved up to 80% (range of 60–80%) iodination of secretin. This compares favorably to the 20–60% yield reported for other methods, such as chloramine T or enzymatic iodination with lactoperoxidase (12). Figure 2 shows the HPLC chromatogram of the reaction product containing [^{125} I]secretin, ^{125} I, and secretin; because HPLC allows separation of the three peaks, this method was used to purify [^{125} I]secretin.

The second step in the synthesis was to biotinylate [^{125} I]secretin. Because secretin contains only one primary amino group at its N-terminal, point modification with biotin-NHS is possible since the latter only reacts with primary amino groups. This point modification theoretically preserves the receptor recognition site of secretin located at the C-terminal. To optimize the yield, we used a 30-fold molar excess of biotin in the initial reaction. HPLC analysis (Figure 3) indicated that 60% modification (range of 50–80%) of secretin could be achieved. Biotinylated secretin eluted at 13.4 min on the reverse phase HPLC column and could thus be separated from free biotin (elution time of 2.9–3.5 min).

In subsequent experiments, we determined the binding affinity of [^{125} I]secretin-biotin to streptavidin-coated plates using a similar assay as previously reported (13). As expected, binding of secretin-biotin to SA plates was 2 orders of magnitude higher than that of free secretin ([^{125} I]secretin-biotin, $21.1 \pm 0.4\%$ bound; [^{125} I]secretin, $0.380 \pm 0.004\%$ bound; ^{125} I, $0.060 \pm 0.001\%$ bound). This assay confirmed that secretin was indeed biotinylated and that conjugated biotin still retained affinity to SA.

Streptavidin Modification of MION. In parallel to the above synthesis, we immobilized streptavidin onto dextran, the surface coating of MION, using oxidation of dextran and subsequent reductive amination. Using HPLC and analytical assays for iron and protein, we determined a 79% yield of streptavidin immobilization onto MION. Figure 4 shows the HPLC chromatogram of the MION-streptavidin reaction mixture. Uncoupled MION eluted at 4.8 min, whereas MION-streptavidin

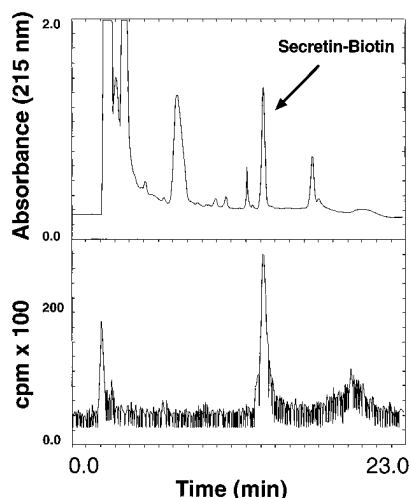


Figure 3. HPLC chromatograms of biotinylated [^{125}I]secretin. The chromatograms shown were obtained simultaneously using a radioactive detector as well as an UV detector at 215 nm. Free unreacted biotin eluted first because of its higher polarity, followed by several polar hydrolysis byproducts of biotin. The biotin–secretin peak appears at 13.4 min, corresponding closely to the radioactivity peak, whereas unmodified secretin appears at 12.5 min.

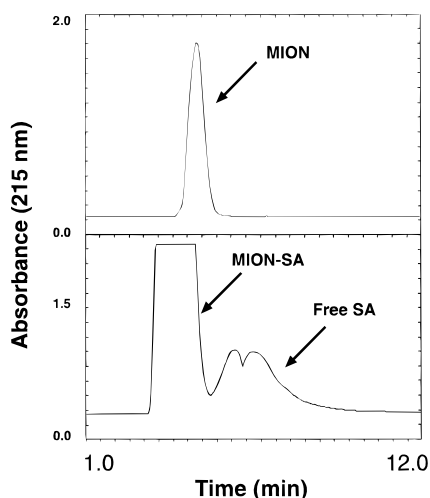


Figure 4. Chromatograms of MION and MION–streptavidin using HPLC. Two chromatograms (215 nm) of unaltered MION (top) and the MION–SA reaction mixture (bottom) are shown. Note that the larger MION–SA elutes earlier than the unaltered MION (4.1 versus 4.8 min). Free SA elutes as a double peak (tetramer and dimer) at 6.1 and 6.8 min, respectively.

eluted at 4.1 min, consistent with its higher molecular weight. Unreacted streptavidin eluted in two peaks at 6.1 and 6.8 min (corresponding to the tetrameric and dimeric forms).

Previous analysis of MION showed that each 4.6 nm MION core consists of 2064 iron atoms (on the basis of an inverse spinel crystal structure of the core) (17). Assuming this composition, a molar ratio of streptavidin to MION of approximately 2:1 can be calculated (Table 1). It appears possible to position larger amounts of SA onto MION; however, this may not be desirable for *in vivo* targeting purposes. Although little data is currently

available regarding ideal structure and composition of pancreaticotrophic labels, overloading the MION surface with SA is expected to result in steric hindrance for biotin–SA binding and thus decrease possible recognition of the contrast agent by secretin receptors.

Magnetically Labeled Secretin. *Conjugation.* In the final step of the synthesis, we combined the purified [^{125}I]secretin–biotin and streptavidin–MION. The product of reaction was then dialyzed to eliminate unbound biotinylated secretin. On the basis of radioactivity measurements of the reaction product and the dialysate, the efficiency of conjugation was calculated to be approximately 90%. This value is lower than the theoretical efficiency (100%) which may be explained by a loss of binding sites due to SA immobilization onto the MION surface. Data analysis indicates that an average of two secretin molecules are associated with each MION particle.

On the basis of the above experimental data, a possible structure of the construct is shown in Figure 5. Because the primary amino group available for biotinylation of secretin can only be contributed from its N-terminal, the biotinylated secretin is specifically oriented with the active binding site (its amidated C-terminal). The illustration highlights the lysine groups available on the surface (yellow) allowing conjugation to activated dextran molecules on MION. The illustration also shows that part of the biotin-binding sites on streptavidin could conceivably be sterically hindered.

Physical Properties. Analysis of MION–SA–biotin–secretin by photon correlation spectroscopy (Table 1) showed little change in particle size distribution (20 ± 4 versus 25 ± 4 nm), indicating that there was no significant particle aggregation during modification. Relaxivity measurements of MION, MION–SA, and the MION–secretin construct are also very similar, indicating that peptide modification of MION does not change its magnetic properties significantly as would be expected by particle aggregation or dissociation (Table 1).

Competitive Cell Binding. To test the ability of magnetically labeled secretin to bind to the secretin receptor, we performed competitive binding assays on the isolated pancreatic cells (Figure 6). Our results indicate that the conjugate binds to secretin receptors with high affinity (K_D in the nanomolar range). Both [^{125}I]secretin and the secretin–biotin–SA–MION conjugate showed a percentage binding (89 ± 5 and $84 \pm 13\%$) similar to that of isolated pancreatic acini at low cold secretin concentrations (0.01 pM). This binding was inhibited with increased concentrations of cold secretin. The concentration of cold secretin required to achieve 50% inhibition (IC_{50}) for the secretin–biotin–SA–MION conjugate (1.1 nM) was 1 order of magnitude lower than that of [^{125}I]secretin (10 nM), possibly as a result of the steric effect of the surface dextran of the secretin–biotin–streptavidin–MION conjugate.

Biodistribution. Figure 7 summarizes the *in vivo* biodistribution of the three compounds 4 h after iv administration. As expected, pancreatic accumulation was highest for [^{125}I]secretin (2.5% ID/g) and lowest for unmodified MION ($<0.25\%$ ID/g). Magnetically labeled secretin showed a significantly higher pancreatic accumulation (0.88% ID/g) than unmodified MION ($p <$

Table 1. Analysis of MION and MION Complexes

	size by PCS (nm) (90° angle)	ratio (SA:MION)	ratio (secretin:MION)	R_1 ($\text{M}^{-1} \text{s}^{-1}$)	R_2 ($\text{M}^{-1} \text{s}^{-1}$)
MION	20.0 ± 4	—	—	34.8	16.5
MION–SA	23.8 ± 4	1.98	—	32.0	15.9
MION–SA–biotin–secretin	25.0 ± 4	1.98	1.85	34.0	15.3

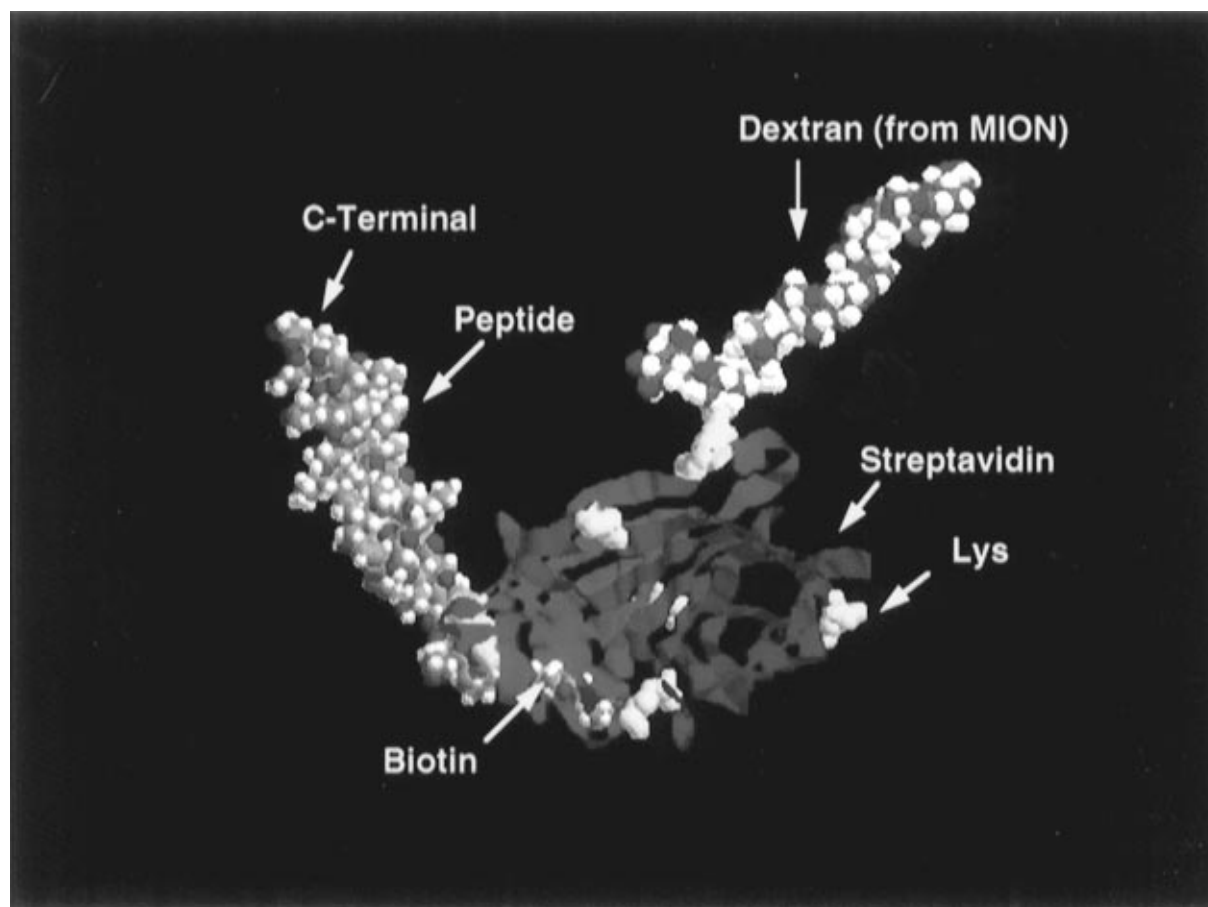


Figure 5. Hypothetical structure of the streptavidin-biotin-secretin complex. The biotinylated secretin is shown in space-fill representation on the left, and the streptavidin is shown in ribbon representation (two subunits) with its lysine residues highlighted on the surface. These groups allow the conjugation with activated dextran molecules on MION. A branch of a MION surface dextran molecule is shown in space-fill representation on the right.

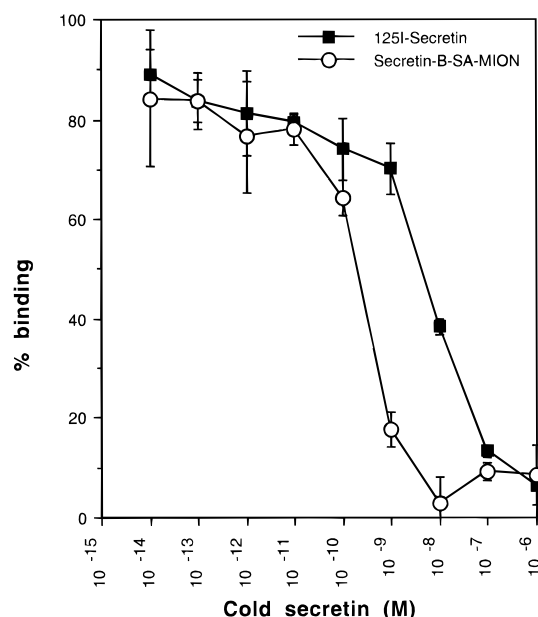


Figure 6. Competitive binding assay. Competitive inhibition of attachment of [¹²⁵I]secretin and secretin-biotin-SA-MION to the rat pancreas acini. At the lowest concentration of inhibitor (cold secretin), [¹²⁵I] attachment was higher than that of magnetically labeled secretin. With increasing concentration of inhibitor, attachment decreased as a function of added inhibitor.

0.001), presumably because of secretin immobilization. Although it could be argued that unmodified MION has a longer blood half-life than the other two compounds and thus requires a longer time to accumulate in pancreas,

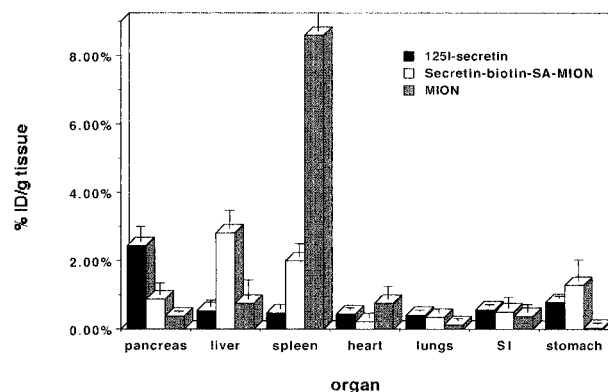


Figure 7. Biodistribution. The percentage of injected dose per gram of tissue is plotted for various organs in rats. MION-secretin shows significantly more accumulation in the pancreas than the unaltered MION, however, not as much as secretin alone.

this possibility was excluded by biodistribution experiments at later time points. Assuming that 0.88% of the injected dose accumulates per gram of pancreas, this would result in local concentrations of 160 nmol of Fe/g of tissue, 3 times higher than that detectable by conventional MR imaging at clinical field strengths. This local iron concentration is well within limits of feasibility as dextran-coated iron oxides are currently administered at doses of up to 1.7 mg of Fe/kg of body weight in clinical trials.

Of particular interest is the distribution of secretin and secretin-MION to other tissues that are known to express secretin receptors. These tissues include stom-

ach and heart (18). Accumulation of secretin or its degradation products was also detected in small bowel which is probably best explained by excretion of pancreatic fluid through the ampulla of Vater into the duodenum.

Unmodified MION accumulates primarily in the spleen and liver, where it is phagocytosed and stored within the lysosomal compartment (19). Our biodistribution data suggest that some of the secretin-modified MION also undergoes this distribution which may be ultimately exploited for diagnostic purposes. Prior research has shown that MION is biodegradable and that iron from the particle's core is ultimately incorporated into the body's iron pool (19). Toxicity of MION and other dextran-coated iron oxides in clinical trials is low. Although the toxicity of our secretin-modified agent has not been directly determined, we expect a similarly low toxicity like that of the parent compound.

SUMMARY

Our results indicate that secretin can be specifically biotinylated at its N-terminal and conjugated to a bio-compatible dextran-coated superparamagnetic label through interaction of biotin and streptavidin. Competitive cell binding assays demonstrated that the magnetically labeled secretin still retains its affinity to secretin receptors. *In vivo* biodistribution data confirm *in vitro* observations showing accumulation of the construct within the pancreas and other secretin receptor positive sites such as the stomach. The described method of biotinylation of peptide and linkage to MION-SA opens exciting possibilities to magnetic labeling of a variety of other bioactive peptides or proteins.

LITERATURE CITED

- (1) Klein, G. M., and Stein, G. N. (1985) Bockus Gastroenterology. In *Conventional Radiology of the Pancreas* (J. E. Berk, Ed.) pp 3893–3906, W. B. Saunders, New York.
- (2) Freeny, P. C. (1988) Radiology of the pancreas: Two decades of progress in imaging and intervention. *AJR, Am. J. Roentgenol.* 150, 975.
- (3) Shen, T., Weissleder, R., Papisov, M., Bogdanov, A., and Brady, T. J. (1993) Monocrystalline iron oxide nano-compounds (MION): physicochemical properties. *Magn. Reson. Med.* 29, 599–604.
- (4) Schaffer, B. K., Linker, C., Papisov, M., Tsai, E., Nossiff, N., Shibata, T., Bogdanov, A., Brady, T. J., and Weissleder, R. (1993) MION-ASF: Biokinetics of an MR receptor agent. *Magn. Reson. Imaging*, 11, 411–417.
- (5) van Everdingen, K., Enochs, W. S., Bhide, P. G., Nossiff, N., Papisov, M., Bogdanov, A., Brady, T. J., and Weissleder, R. (1994) Determinants of *in vivo* MR imaging of slow axonal transport. *Radiology* 193, 485–491.
- (6) Weissleder, R., Lee, A. S., Khaw, B. A., Wittenberg, J., and Brady, T. J. (1991) MR antibody imaging: Antimyosin labeled with monocrystalline iron oxide allows detection of myocardial infarct. Association of University Radiologists, 39th Annual Meeting, March 17–23, Orlando, FL.
- (7) Reimer, P., Weissleder, R., Shen, T., Knoefel, W. T., and Brady, T. J. (1994) Pancreatic receptors: Initial feasibility studies with targeted contrast agent for MR imaging. *Radiology* 193, 527–531.
- (8) Robberecht, P., Conlon, T. P., and Gardner, J. D. (1976) Interaction of porcine vasoactive intestinal peptide with dispersed pancreatic acinar cells from the guinea pig: Structural requirements for effects of VIP and secretin on cellular adenosine 3':5'-monophosphate. *J. Biol. Chem.* 251, 4635–4639.
- (9) Gardner, J. D., Conlon, T. P., Beyerman, H. C., and van Zon, A. (1977) Interaction of synthetic 10-tyrosol analogs of secretin with hormone receptors on pancreatic acinar cells. *Gastroenterology* 73, 52–56.
- (10) Chaiet, L., and Wolf, F. J. (1964) The properties of streptavidin, a biotin-binding protein produced by streptomycetes. *Arch. Biochem. Biophys.* 106, 1–5.
- (11) Green, N. M. (1975) *Advances in Protein Chemistry, Avidin*, pp 85–133, Academic Press, New York.
- (12) Chang, T. M., and Chey, W. Y. (1980) Radioimmunoassay of Secretin. A critical Review and current status. *Dig. Dis. Sci.* 25, 529–552.
- (13) Klibanov, A. L., Bogdanov, A. A., Torchilin, V. P., and Huang, L. (1989) Biotin-bearing pH-sensitive liposomes: high-affinity binding to avidin layer. *J. Liposome Res.* 1, 233–244.
- (14) Brannon, P. M., Orrison, B. M., and Kretchmer, N. (1985) Primary cultures of rat pancreatic acinar cells in serum-free medium. *In Vitro Cell. Dev. Biol.* 21, 6–14.
- (15) Holohan, K. N., Murphy, R. F., Flanagan, R. W. J., Buchanan, K. D., and Elmore, D. T. (1973) Enzymic iodination of the histidyl residue of secretin: A radioimmunoassay of the hormone. *Biochim. Biophys. Acta.* 322, 178–180.
- (16) Li, C. H. (1944) Kinetics of reactions between iodine and histidine. *J. Am. Chem. Soc.* 66, 225–227.
- (17) Verwey, E. J. W., and Heilmann, E. L. (1947) Physical Properties and Cation Arrangement of Oxides with Spinel Structures. *J. Chem. Phys.* 15, 174–180.
- (18) Ishihara, T., Nakamura, S., Kaziyo, Y., Takahashi, T., Takahashi, K., and Nagata, S. (1991) Molecular cloning and expression of a DNA encoding the secretin receptor. *EMBO J.* 10, 1635–1641.
- (19) Weissleder, R., Stark, D. D., Engelstad, B., Compton, C. C., White, D., Jacobs, P., and Lewis, J. (1989) Superparamagnetic iron oxide: pharmacokinetics and toxicity. *AJR, Am. J. Roentgenol.* 152, 167–173.

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A General Method for the Spatially Defined Immobilization of Biomolecules on Glass Surfaces Using “Caged” Biotin

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A method has been developed to spatially define the immobilization of proteins on surfaces using the classic avidin–biotin link, for which a wide variety of biochemical reagents are commercially available. A derivative of biotin bearing a photoremovable nitrobenzyl group (MeNPOC-biotin) has been prepared in a form suitable for simple linkage to biomolecules and surfaces. It has been used to functionalize bovine serum albumin (BSA) to form MeNPOC-biotin–BSA, which can then be coated onto glass. On photolithographic patterning of the surface, biotins are freed in the irradiated areas, permitting avidin to be localized at the irradiated sites. Subsequent addition of a biotinylated molecule permits its site-specific localization. Patterning of a biotinylated antibody and dye-labeled avidins or streptavidin using this reagent has been demonstrated by fluorescence microscopy.

INTRODUCTION

A crucial technology for the production of a new generation of “biochips” (1–6) is the integration of biological and chemical materials on the microscopic scale. Such devices could have uses ranging from biomolecular electronics (7–10) to studies in cell adhesion to micromachines. Because of the power of parallel processing in the patterning of semiconductors, similar methods have been considered for the patterning of biological components. A method that permits the immobilization of multiple proteins in predefined patterns is essential to construct biochips of any significant complexity. The work reported here permits immobilization of multiple proteins at photolithographically determined sites without risk of photochemical damage. We accomplished this by temporally separating the patterning and binding steps using “caged” biotin linked to the glass surface through a conventional serum albumin coating process. This biotin derivative is protected from its normal binding to avidin by a photoremovable nitrobenzyl group. When exposed to light through a mask, this group is lost, the patterned, irradiated sites gain affinity for avidin, and they hereby can immobilize biotinylated substances.

BACKGROUND

Several reports of photolithographic protein immobilization have utilized surface-bound photoactivated reagents like nitroarylazides (11, 12). A technique for attaching biomolecules to surfaces using light but without patterning has been reported; it involves attachment of nitrophenylazide photoaffinity reagents to agarose beads and irradiation of the modified solid in the presence of an antibody to immobilize it for immunoassays. The obvious drawbacks of this technique are the low yields known for the nitroarylazide photo-cross-linking process and possible photochemical damage to the protein. Likewise, nitroarylazides have been connected to urokinase, hyaluronic acid, collagen, fibronectin, or serum albumin by polyethylene glycol spacers and the conju-

gates irradiated in the presence of polyurethane tubing and other plastics to generate biomolecule-functionalized surfaces. This method suffers from the fact that molecules may be photoactivated when distant from the surface and are therefore wasted. The former strategy, involving generation of reactive sites on the surface, makes more efficient use of the reactants.

Performing similar nitroarylazide photochemistry in a spatially localized way on the gate of a field effect transistor has been used to form a BIOCHEMFET (13). The direct photochemical cross-linking (14) of benzophenone-derivatized glass surfaces to proteins in solution has been described (15), and a similar use of light-activatable nitroarylazide compounds attached to a surface through an avidin–biotin link has been reported (16). Patterning such substrates with light permits the creation of $\sim 2\ \mu\text{m}$ features, but these methods require the protein to be present during the irradiation, potentially damaging it. Two methods temporally separate the irradiation and immobilization steps. Keana has used the efficient perfluorophenylazide photochemistry (17, 18) to attach NHS active esters to lithographically patterned regions on a polystyrene film. The active esters are then used to immobilize a protein in the patterned areas (19, 20). Ligler has reported a photolithographic method for the production of patterned surfaces that resist protein adsorption in irradiated areas and permit adsorption in masked areas, but this method is limited to the patterning of one protein per surface (21, 22). Whitesides has used self-assembled monolayers on gold to prepare a number of biofunctionalized surfaces, in some cases involving patterning by stamping (23).

The use of light to prepare sites on a surface that can react with biomolecules by well-established, conventional chemistry was a guiding principle in this work. We aimed to use the extensively exploited interactions between avidin and biotin (24) in assembling biomolecular aggregates. Other recent reports describe the preparation of a photoactivatable biotin derivative (caged biotin) based on nitrobenzyl photochemistry and its use for covalent derivatization of aminopropylsilanized glass surfaces (25, 26). This work was aimed at the derivatization of bovine serum albumin (BSA), which is commonly coated onto glass surfaces in biological derivati-

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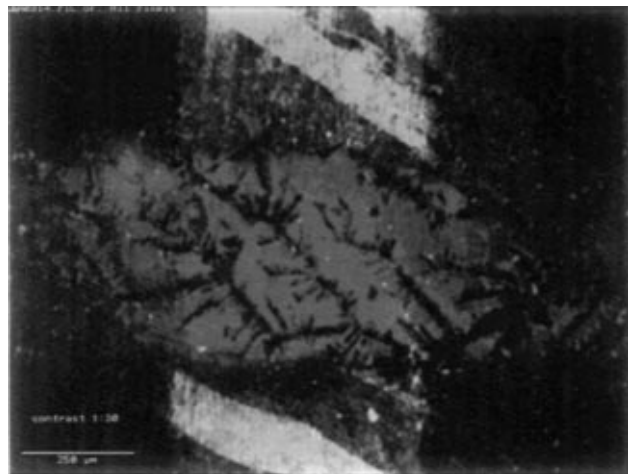


Figure 3. Two-color fluorescence micrograph after exposure of a microscope slide coated with MeNPOC-biotin-BSA through a 500 μm stripe mask, treatment with avidin-Texas Red, followed by another exposure through a 500 μm stripe mask rotated 90° to the former pattern, and treatment with avidin-fluorescein. The signal:noise ratio is 30:1 for the fluorescein image (300 μm $\times$ 200 μm light area:150 μm $\times$ 100 μm dark area) and 30:1 for the Texas Red image (200 μm $\times$ 133 μm light area: 200 μm $\times$ 133 μm dark area).

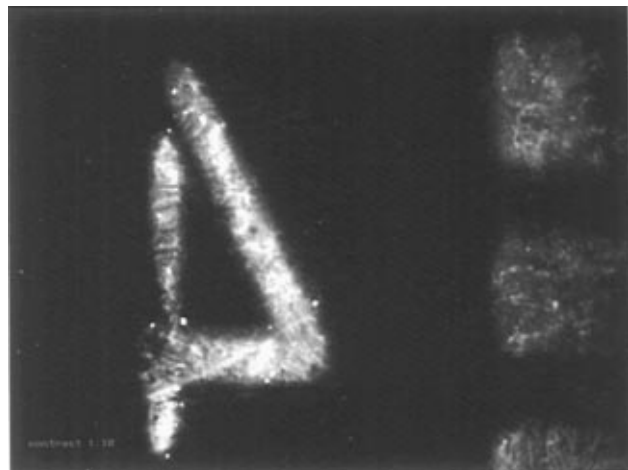


Figure 4. Fluorescence micrograph of a MeNPOC-biotin-BSA-coated microscope slide after exposure (350 nm, Oriel arc lamp, 20 min) through a resolution target and staining with 100 $\mu\text{g}/\text{mL}$ FITC-streptavidin.

(s:n > 30:1) between sites and demonstrates that essentially all of the biotins are released in the first irradiation step, since no fluorescein-avidin is immobilized in the Texas Red region. When avidin-fluorescein is used as the first stain and avidin-Texas Red is used as the second stain, green fluorescence is seen in the area of intersection (data not shown). This control demonstrates that the red fluorescence in the area of intersection in the micrograph shown in Figure 3 is not due to energy transfer from fluorescein to Texas Red. Treatment of the first slide with avidin-Texas Red overnight ensured saturation of free biotin groups on the surface. The control shows some cross-talk between green and red because the slide was treated only for 1 h with avidin-fluorescein before the second patterning step.

More complex features of even smaller dimensions were written by exposure through a resolution target as a mask (Figure 4).

Significance. This paper reports a novel and general technique for localization of biomolecules at microscopic locations on glass surfaces utilizing biotinylated biomol-

ecules, which are available by either chemical derivatization or recombinant DNA techniques (31). The general interest of the method lies in its potential to construct complex patterns of proteins on the micrometer scale without risk of photochemical damage and its convergence with reagents widely used for the linking of biomolecules, namely bovine serum albumin, avidin, and biotin. The avidin-biotin link and the absorption of BSA to glass used in this method are quite robust, though denaturants are still capable of disrupting these interactions. Possible applications of patterned proteinaceous surfaces prepared using this method include mimicking of cell surfaces and the development of multianalyte fluoroimmunosensors.

MATERIALS AND METHODS

General. Ether and THF were distilled from a deep blue solution resulting from benzophenone and sodium. Dichloromethane and chloroform were refluxed and distilled from calcium hydride. DMF was stirred with calcium sulfate, filtered, and distilled from calcium hydride. BSA, streptavidin, streptavidin-fluorescein, bovine IgG-FITC, and anti-bovine IgG-biotin were purchased from Sigma. Bodipy avidin-Texas Red, Bodipy avidin-fluorescein, and biotin-FITC were purchased from Molecular Probes, Inc. All washings were accomplished by dipping the slide in the specified solution.

3',4-(Methylenedioxy)-6'-nitroacetophenone. To a solution of 3',4'-(methylenedioxy)acetophenone (Aldrich, 5 g, 30.4 mmol) being stirred in 50 mL of TFA was added sodium nitrite (6.3 g, 91.3 mmol) in one portion. A red-brown gas was generated. The mixture was stirred overnight. Water was added, and the solution was extracted with CH_2Cl_2 (100 mL $\times$ 3). The organic layer was washed with saturated NaHCO_3 and water and dried (MgSO_4). Solvent was removed under reduced pressure. Purification by flash column chromatography (hexane:EtOAc = 2:1) afforded 3.75 g of yellow solid (60%). ^1H NMR (CDCl_3 , 300 MHz): δ 7.53 (s, 1 H), 6.75 (s, 1 H), 6.19 (s, 1 H), 2.49 (s, 3 H). $^{13}\text{C}\{^1\text{H}\}$ NMR (CDCl_3 , 75 MHz): δ 199.2, 152.6, 148.7, 139.9, 134.9, 106.0, 104.7, 103.6, 30.1. This compound is known (32, 33).

Methylnitropiperonyl Alcohol (1). The above ketone (3.1 g, 14.8 mmol) was dissolved in a mixture of dry THF and absolute EtOH (50 mL each). To this solution was added 560 mg (14.8 mmol) of NaBH_4 , and it was stirred at room temperature overnight. The reaction was quenched with 1 N HCl, and solvent was removed under reduced pressure. The residue was partitioned between CH_2Cl_2 and water. The organic layer was dried (MgSO_4) and solvent removed under reduced pressure. Purification by flash chromatography (hexane:EtOAc = 3:1) afforded 3.53 g of yellow solid (96%). ^1H NMR (CDCl_3 , 300 MHz): δ 7.43 (s, 1 H), 7.25 (s, 1 H), 6.11 (dd, J = 3.3 Hz, J = 1.2 Hz, 2 H), 5.43 (q, J = 6.3 Hz, 1 H), 2.50 (br, 1 H), 1.51 (d, J = 6.3 Hz, 3 H). $^{13}\text{C}\{^1\text{H}\}$ NMR (CDCl_3 , 75 MHz): δ 152.3, 146.8, 141.3, 139.0, 106.3, 105.1, 102.9, 65.6, 24.1. This compound is known (28, 34).

MeNPOC-biotin-OMe (2). Four reactions of the same scale were set up simultaneously. To methylnitropiperonyl alcohol (1) (715 mg, 3.39 mmol) in 5 mL of dry THF was added KO^tBu (43 mg, 0.39 mmol). After 10 min, carbonyldiimidazole (829 mg, 5.08 mmol) was added. The mixture was stirred at room temperature for 3 h. THF was removed under reduced pressure and the residue triturated with CCl_4 . The precipitate was filtered through Celite, and the filtrate was evaporated. The residue was redissolved in 15 mL of dry CHCl_3 (amylene-stabilized). Biotin-OMe (500 mg, 1.94 mmol) and NaH

(91 mg, 60% dispersion in mineral oil) were added. The mixture was heated to reflux under argon for 72 h. The reaction was quenched with a few drops of acetic acid. The four reaction mixtures were combined, and the solvent was removed under reduced pressure. Purification by flash chromatography on silica (3% acetone, 3% MeOH in CHCl₃) afforded 1.4 g (37%) of a yellow foam. Sp: 79–80 °C. IR (film): 3284, 1779, 1730 cm⁻¹. ¹H NMR (CDCl₃, 300 MHz): δ 7.51 (d, J = 3.9 Hz, 1 H), 7.38 (d, J = 9.0 Hz, 1 H), 4.51 (q, J = 6.3 Hz, 1 H), 6.11 (s, 2 H), 5.93 (d, J = 12.3 Hz, 1 H), 4.84 (m, 1 H), 4.21 (m, 1 H), 3.66 (s, 3 H), 3.21 (m, 1 H), 3.10 (m, 1 H), 2.97 (m, 1 H), 2.33 (t, J = 7.5 Hz, 2 H), 1.67 (m, 7 H), 1.47 (m, 2 H). ¹³C{¹H} NMR (CDCl₃, 75 MHz): δ 173.8, 155.4, 155.3, 152.5, 147.2, 141.1, 135.2, 106.8, 105.0, 103.0, 71.1, 62.6, 57.6, 55.1, 51.5, 38.6, 33.3, 28.1, 27.8, 24.5, 22.4. MS-FAB: m/z 494 (M⁺ - H). Anal. Calcd for C₂₁H₂₅N₃O₉S: C, 50.91; H, 5.05; N, 8.48. Found: C, 50.99; H, 5.12; N, 8.52.

MeNPOC-biotin-OH (3). MeNPOC-biotin-OMe (1.21 g, 2.45 mmol) was dissolved in 15 mL of THF and 15 mL of 1 N HCl. The mixture was heated at reflux for 24 h, and the solvent was removed under reduced pressure. Purification by flash chromatography (10% MeOH in CHCl₃) afforded 897 mg (76%) of a yellow foam. Sp: 91–92 °C. IR (film): 3280, 1775, 1723 cm⁻¹. ¹H NMR (CDCl₃/CD₃OD, 300 MHz): δ 7.50 (d, J = 3.3 Hz, 1 H), 7.31 (d, J = 5.1 Hz, 1 H), 6.49 (q, J = 3 Hz, 1 H), 6.14 (s, 2 H), 4.81 (m, 1 H), 4.22 (m, 1 H), 3.20 (m, 1 H), 3.03 (m, 2 H), 2.33 (t, J = 7.2 Hz, 2 H), 1.68 (m, 7 H), 1.49 (m, 2 H). ¹³C{¹H} NMR (CDCl₃, 75 MHz): δ 177.7, 157.2, 157.1, 152.6, 147.2, 141.2, 134.9, 106.6, 105.1, 103.0, 71.3, 62.8, 57.8, 54.9, 38.6, 33.5, 28.3, 28.1, 24.9, 22.3. MS-FAB: m/z 480 (M⁺ - H). Anal. Calcd for C₂₀H₂₃N₃O₉S: C, 49.89; H, 4.78; N, 8.73. Found: C, 49.66; H, 4.87; N, 8.54.

MeNPOC-biotin-AC-OH (4). To a stirring solution of MeNPOC-biotin-OH (400 mg, 0.83 mmol) and *N*-hydroxysuccinimide (105 mg, 0.92 mmol) in 5 mL of anhydrous dimethoxyethane and 3 mL of anhydrous DMF was added 1,3-dicyclohexylcarbodiimide (171 mg, 0.83 mmol). The mixture was stirred under argon overnight and filtered through Celite. The filtrate was transferred to a round bottom flask, and 6-aminocaproic acid (109 mg, 0.83 mmol) was added. The mixture was stirred under Ar overnight and filtered through Celite. The filtrate was evaporated and placed in a high vacuum to remove DMF. Purification by flash chromatography (10% MeOH in CHCl₃ to 20% MeOH in CHCl₃) afforded 349 mg (70%) of a brown syrup. IR (film): 3419, 1768, 1648 cm⁻¹. ¹H NMR (CD₃OD, 300 MHz): δ 7.97 (t, J = 5.4 Hz, 1 H), 7.48 (s, 1 H), 7.24 (s, 1 H), 6.37 (q, J = 6.3 Hz, 1 H), 6.14 (s, 2 H), 4.88 (m, 1 H), 4.26 (dd, J = 7.8 Hz, J = 3.6 Hz, 1 H), 3.29 (t, J = 1.5 Hz, 2 H), 3.16 (m, 2 H), 3.06 (m, 1 H), 2.25 (t, J = 7.5 Hz, 2 H), 2.16 (t, J = 7.5 Hz, 2 H), 1.67 (m, 11 H), 1.49 (m, 2 H), 1.36 (m, 2 H). ¹³C{¹H} NMR (CDCl₃, 75 MHz): δ 176.8, 173.8, 156.2, 152.5, 150.5, 147.2, 140.9, 134.7, 106.4, 104.8, 103.0, 70.9, 62.5, 57.6, 54.9, 50.1, 38.9, 35.5, 33.6, 29.4, 28.6, 27.8, 27.6, 25.9, 24.1, 22.0. MS-FAB: m/z 593 (M⁺ - H). Anal. Calcd for C₂₆H₃₄N₄O₁₀S·2H₂O: C, 49.52; H, 5.40; N, 8.89. Found: C, 49.53; H, 5.66; N, 8.83.

MeNPOC-biotin-AC-NHS (5). MeNPOC-biotin-AC-OH (113 mg, 0.19 mmol) and *N*-hydroxysuccinimide (24 mg, 0.21 mmol) were dissolved in 5 mL of dry DMF. Dicyclohexylcarbodiimide (39 mg, 0.19 mmol) were added with stirring under Ar. The mixture was stirred at room temperature overnight and filtered through Celite, washing with 2 mL of dry DMF. The filtrate was brought to

a total volume of 8 mL with dry DMF. This solution was kept in a dessicator at 4 °C for direct derivatization of BSA.

MeNPOC-biotin-AC-BSA (6). MeNPOC-biotin-AC-NHS (200 mL of a 23.75 mM solution in DMF) was mixed with 4 mg of BSA in 2 mL of 0.1 M NaHCO₃/0.2 M NaCl solution; the reaction mixture was brought to a total volume of 3 mL with PBS, and it was allowed to stand at 4 °C overnight. The product was purified by ultrafiltration (Centricon-30 microconcentrator at 4 °C, 6000 rpm). Alternatively, a gel filtration column (Sephadex G-50, fine) could be used to purify the product. The concentrated product was diluted in PBS to about 2 mg of BSA/mL and kept at 4 °C for further use. The ratio of MeNPOC-biotin-AC to BSA can be varied by using different amounts of the active ester (Table 1). The loading was determined by comparison of the unique MeNPOC absorption at 346 nm (ϵ = 4595 M⁻¹ cm⁻¹) with the protein band at 280 nm.

Immobilization of MeNPOC-biotin-AC-BSA on a Solid Support. A microscopic slide (precleaned with absolute EtOH) was treated with 1 mL of 30 μ M MeNPOC-biotin-AC-BSA in phosphate-buffered saline (PBS) solution (pH 7.4) for 2 h, washed with PBS for 5 min, and dried.

Spatially Addressable Immobilization of Fluorescein-Streptavidin on a Solid Support. A MeNPOC-biotin-AC-BSA-derivatized slide was exposed to UV light at 350 nm (Oriel arc lamp, 4 mW/cm²) through a mask (stripe mask or resolution target) for 20 min in PBS. The slide was washed in PBS for 5 min and treated with 1 mL of 100 μ g/mL FITC-streptavidin in PBS for 1 h. The slide was then washed in PBS for 10 min, washed with deionized water for 1 min, and dried. Table 1 shows the different contrast ratios of the fluorescence images which were collected when MeNPOC-biotin-AC-BSA conjugates with different biotin:BSA ratios were used.

Spatially Addressable Immobilization of Fluorescein-Biotin on a Solid Support. A MeNPOC-biotin-AC-BSA-coated slide was exposed through a 350 μ m stripe mask, treated with streptavidin (200 μ g/mL PBS) for 1 h, washed in PBS for 10 min, and dried. The slide was then treated with biotinylated fluorescein (100 μ g/mL PBS) for 2 h and washed in PBS for 10 min and deionized water for 1 min.

Spatially Addressable Immobilization of Fluorescein-Antibody on a Solid Support. A MeNPOC-biotin-AC-BSA-coated slide was exposed through a 350 μ m stripe mask, treated with streptavidin (100 μ g/mL PBS) for 3 h, washed in PBS for 10 min, and dried. The slide was then treated with biotinylated anti-bovine IgG (50 μ g/mL in PBS) for 2 h and washed in PBS for 10 min. The slide was stained with bovine IgG-FITC (180 μ g/mL PBS) for 1 h and washed in PBS for 10 min and deionized water for 1 min.

Two-Color Experiments: Immobilization of Fluorescein-Streptavidin and Fluorescein-Texas Red on a Solid Support. A MeNPOC-biotin-AC-BSA-derivatized slide was exposed to UV light at 350 nm through a mask for 20 min in contact with PBS. The slide was washed in PBS for 5 min and treated with 1 mL of 100 μ g/mL avidin-Texas Red in PBS overnight. The slide was then washed in PBS for 10 min and exposed again at 350 nm through a mask with a stripe 90° to the first mask. The slide was washed in PBS for 5 min and treated with 1 mL of 100 μ g/mL avidin-fluorescein in PBS for 1 h. A similar experiment with green stain first and red stain second gave a similar outcome.

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LITERATURE CITED

- (1) Wangermann, G. (1989) Topical aspects of bioelectronics. *Stud. Biophys.* 132, 946.
- (2) Hamann, C. (1987) Bioelectronics – perspectives and reality. *Naturwiss. Reihe* 51, 95–102.
- (3) Gil'manshin, R. I., and Lazarev, D. I. (1987) Biotechnology as a source of materials for electronics. *Biotechnologiya* 3, 421–32.
- (4) Drexler, K. E. (1986) *Engines of Creation*, Anchor/Doubleday, New York.
- (5) *Molecular Electronic Devices* (1987) Vol. I, II, Marcel-Dekker, New York.
- (6) Wrighton, M. S. (1985) Prospects for a new kind of synthesis: assembly of molecular components to achieve functions. *Comments Inorg. Chem.* 4, 269–94.
- (7) Stayton, P. S., Olinger, J. M., Wollman, S. T., Bohn, P. W., and Sligar, S. G. (1994) Engineering proteins for electrooptical biomaterials. *Adv. Chem. Ser.* 240, 475–90.
- (8) Hong, H. G., Jiang, M., Sligar, S. G., and Bohn, P. W. (1994) Cysteine-specific surface tethering of genetically engineered cytochromes for fabrication of metalloprotein nanostructures. *Langmuir* 10, 153–8.
- (9) Bohn, P. W., Sligar, S. G., Hong, H. G., Jiang, M., Thurman, E. M., and Cong, Y. (1993) Oriented monolayers of genetically engineered cytochrome b5. Implications for molecular devices. *Proc.-Electrochem. Soc.* 93–7, 845–8.
- (10) Stayton, P. S., Olinger, J. M., Jiang, M., Bohn, P. W., and Sligar, S. G. (1992) Genetic engineering of surface attachment sites yields oriented protein monolayers. *J. Am. Chem. Soc.* 114, 9298–9.
- (11) Swanson, M. J., Dunkirk, S. G., Pietig, J. A., and Guire, P. E. (1992) Use photochemistry to modify membranes. *CHEMTACH* 22, 624–6.
- (12) Dunkirk, S. G., Gregg, S. L., Duran, L. W., Monfils, J. D., Haapala, J. E., Marcy, J. A., Clapper, D. L., Amos, R. A., Guire, P. E. (1991) Photochemical coatings for the prevention of bacterial colonization. *J. Biomater. Appl.* 6, 131–56.
- (13) Lowe, C. R., and Earley, F. G. P. (1985) Diagnostic device incorporating a biochemical ligand. U.S. Patent 4,562,157.
- (14) Sigrist, H., Gao, H., and Wegmüller, B. (1992) Light-dependent, covalent immobilization of biomolecules 'inert' surfaces. *Bio/Technology* 10, 1026–8.
- (15) Rozsnyai, L. F., Benson, D. R., Fodor, S. P. A., and Schultz, P. G. (1992) Photolithographic immobilization of biopolymers on solid supports. *Angew. Chem., Int. Ed. Engl.* 31, 759.
- (16) Pritchard, D. J., Morgan, H., and Cooper, J. M. (1995) Micron-scale patterning of biological molecules. *Angew. Chem., Int. Ed. Engl.* 34, 91–93.
- (17) Young, M. J. T., and Platz, M. S. (1989) Polyfluorinated aryl azides as photoaffinity labeling reagents; the room-temperature carbon-hydrogen insertion reactions of singlet pentafluorophenyl nitrene with alkanes. *Tetrahedron Lett.* 30, 2199–202.
- (18) Leyva, E., Munoz, D., and Platz, M. S. (1989) Photochemistry of fluorinated aryl azides in toluene solution and in frozen polycrystals. *J. Org. Chem.* 54, 5938–45.
- (19) Yan, M., Cai, S. X., Wybourne, M. N., and Keana, J. F. W. (1993) Photochemical functionalization of polymer surfaces and the production of biomolecule-carrying micrometer-scale structures by deep-UV lithography using 4-substituted perfluorophenyl azides. *J. Am. Chem. Soc.* 115, 814.
- (20) Yan, M., Cai, S. X., Wybourne, M. N., and Keana, J. F. W. (1994) N-Hydroxysuccinimide ester functionalized perfluorophenyl azides as novel photoactive heterobifunctional cross-linking reagents. The covalent immobilization of biomolecules to polymer surfaces. *Bioconjugate Chem.* 5, 151.
- (21) Bhatia, S. K., Hickman, J. J., and Ligler, F. S. (1992) New approach to producing patterned biomolecular assemblies. *J. Am. Chem. Soc.* 114, 4432–3.
- (22) Bhatia, S. K., Teixeira, J., Anderson, M., Shriver-Lake, L. C., Calvert, J. M., Georger, J. H., Hickman, J. J., Dulcey, C., Schoen, P., and Ligler, F. S. (1993) Fabrication of surfaces resistant to protein adsorption and application to two-dimensional protein patterning. *Anal. Biochem.* 208, 197–205.
- (23) Kumar, A., Abbott, N. L., Biebuyck, H. A., Kim, E., and Whitesides, G. M. (1995) Patterned Self-Assembled Monolayers and Meso-Scale Phenomena. *Acc. Chem. Res.* 28, 219–26.
- (24) Wilchek, M., and Bayer, E. (1990) Application of Avidin-Biotin Technology. *Methods in Enzymology*, Vol. 184, pp 3–746, Academic Press, San Diego.
- (25) Barrett, R. W., Pirrung, M. C., Stryer, L., Holmes, C. P., and Sundberg, S. A. (1993) Spatially-addressable Immobilization of Anti-ligands on Surfaces. U.S. Patent 5,252,743.
- (26) Sundberg, S. A., Barrett, R. W., Stryer, L., Pirrung, M. C., Liu, A. T., Kiangsoontra, B., and Holmes, C. P. (1995) Spatially-addressable immobilization of macromolecules on solid supports. *J. Am. Chem. Soc.* 117, 12050–7.
- (27) Lin, J. N., Andrade, J. D., and Chang, I. N. (1989) The influence of adsorption of native and modified antibodies on their activity. *J. Immunol. Methods* 125, 67.
- (28) Pease, A. C., Solas, D., Sullivan, E. J., Cronin, M. T., Holmes, C. P., and Fodor, S. P. A. (1994) Light-generated oligonucleotide arrays for rapid DNA sequence analysis. *Proc. Natl. Acad. Sci. U.S.A.* 91, 5022–6.
- (29) Wilchek, M., and Bayer, E. (1990) Protein Biotinylation. *Methods in Enzymology*, Vol. 184, pp 138–160, Academic Press, San Diego.
- (30) Plowman, T. E., Pierrincenzi, R. T., Reichert, W. M., Peters, C. R., Wang, H. K., Christensen, D. A., and Herron, J. N. (1996) Femtomolar sensitivity using a channel-etched thin film integrated optical waveguide fluorosensor. *Bio-sens. Bioelectron.* (in press).
- (31) Yamano, N., Kawata, Y., Kojima, H., Yoda, K., and Yamasaki, M. (1992) In vivo biotinylation of fusion proteins expressed in *Escherichia coli* with a sequence of *Propionibacterium freudenreichii* transcarboxylase 1.3S biotin subunit. *Biosci., Biotechnol., Biochem.* 56, 1017–26.
- (32) Fetter, J., Lempert, K., and Möller, J. (1975) N-(6,7-Methylenedioxy-3-quinazolinio)amidates – I Synthesis spectra and some dark reactions. *Tetrahedron* 31, 2559–69.
- (33) Mosettig, R., and Czadek, K. (1931) Über die Einwirkung von diazo-methan auf piperonal III. *Monatsh. Chem.* 57, 291–303.
- (34) Holmes, C. P., and Kiangsoontra, B. (1994) Development of a new photoremovable protecting group for the amino and carboxyl groups of amino acids. *Pept. Chem., Struct. Biol., Proc. Am. Pept. Symp.*, 13th, 110–2.

BC960013V

DNA Cleavage by an $\alpha,3$ -Dehydrotoluene Precursor Conjugated to a Minor Groove Binding Element†

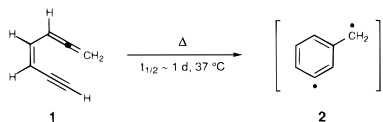
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Arnold and Mabel Beckman Laboratories of Chemical Synthesis, California Institute of Technology, Pasadena, California 91125. Received November 21, 1995[⊗]

The (*Z*)-allene-ene-yne **10**, a precursor to a reactive $\alpha,3$ -dehydrotoluene biradical, was covalently linked to a minor groove binding element, and the resultant conjugate (**9**) was shown to bind and cleave DNA with sequence selectivity.

INTRODUCTION

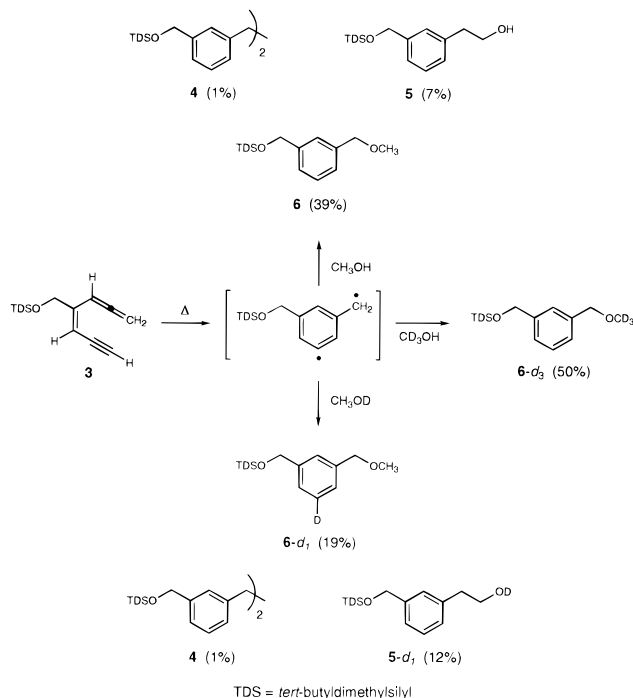
The (*Z*)-allene-ene-yne functional group has been shown to undergo a facile unimolecular thermal isomerization reaction to form an $\alpha,3$ -dehydroalkylbenzene biradical intermediate (1–3). The cycloaromatization of (*Z*)-1,2,4-heptatrien-6-yne (**1**) to $\alpha,3$ -dehydrotoluene (**2**) may be considered prototypical, occurring with a half-life of about 1 day at 37 °C. The $\alpha,3$ -dehydroalkylbenzene



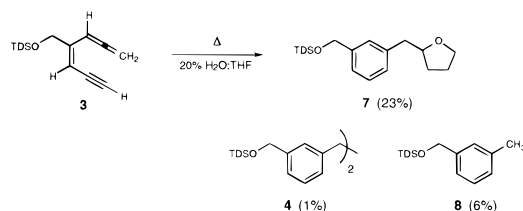
biradicals produced in these cyclization reactions exhibit reactivity that is consistent with traditional definitions of both radical and polar (zwitterionic) species. For example, thermal cyclization of the (*Z*)-allene-ene-yne **3** in methanol (Scheme 1) leads to the formation of products consistent with a free radical description of the biradical intermediate, the bibenzyl derivative **4** (1% yield) and the alcohol **5** (7% yield), in addition to the methyl ether **6** (39% yield), a product anticipated from a polar description of the biradical intermediate wherein the benzylic carbon is δ^+ and the *m*-aryl carbon is δ^- . The distribution of polar versus free radical products varies with subtle changes in the reaction medium in an entirely predictable fashion. Substitution of deuterium for protium on the methanol carbon (CD_3OH) leads to the exclusive formation of the product derived from a polar reaction mechanism, the (deuterated) methyl ether **6-*d*₃**, while substitution of the hydroxylic proton of methanol with deuterium skews the product distribution toward the products of a free radical reaction mechanism (Scheme 1).

The ability of $\alpha,3$ -dehydroalkylbenzene biradicals to abstract a hydrogen atom from the methyl group of methanol raises the possibility that these species might function as DNA-damaging agents, in analogy to biradical intermediates arising from the enediyne antibiotics. However, the variation of the branching ratio between polar and radical reaction pathways with relatively small changes in the bond dissociation energy of the hydrogen atom donor and with the acidity of the medium (3) makes it difficult to predict just how an $\alpha,3$ -dehydrotoluene intermediate might react with double-stranded DNA in an aqueous environment. It is easy to imagine that the

Scheme 1



primary reaction pathway might involve the addition of water by a polar mechanism. On the other hand, if the biradical were formed in the minor groove of DNA, then it may well react in a manner analogous to that of the enediyne antibiotics and abstract a hydrogen atom from the ribose backbone of DNA (4, 5). In a preliminary effort to explore this issue, the (*Z*)-allene-ene-yne **3** was heated at 60 °C in 20% aqueous tetrahydrofuran solution. The purpose of the experiment was to see if the biradical intermediate would react preferentially with water in a polar mechanism or by hydrogen atom abstraction from the labile α -CH bonds of tetrahydrofuran. In the event, only products arising from a free radical reaction mechanism (adducts **4**, **7**, and **8**) were observed. Encouraged

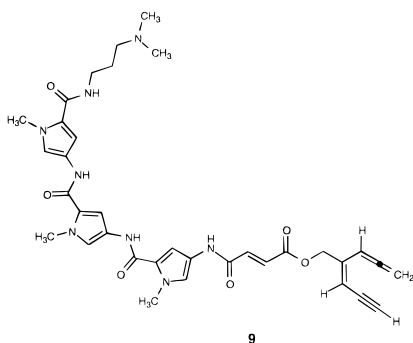


† Contribution no. 9103.

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by this result, we set out to address in a more direct fashion the question of whether an $\alpha,3$ -dehydrotoluene

intermediate might lead to free radical damage of double-stranded DNA. Because $\alpha,3$ -dehydrotoluene itself was anticipated to have a low affinity for double-stranded DNA (a speculation verified below), the well-established *N*-methylpyrrolecarboxamide DNA binding motif of the natural products distamycin and netropsin was incorporated covalently into the substrates of our work. This binding motif was first used by Dervan and co-workers in their development of the affinity-cleaving concept and in the design of DNA-cleaving molecules (6, 7) and has subsequently been used for the synthesis of several bioconjugates incorporating biradical precursors (8–12). The synthesis of conjugate **9**, consisting of an $\alpha,3$ -dehydrotoluene precursor covalently linked to an *N*-methylpyrrolecarboxamide minor groove binding element, and its DNA-binding and -cleaving properties are described herein.



MATERIALS AND METHODS

General Procedures. All reactions were performed in flame-dried round bottom or modified Schlenk (Kjeldahl shape) flasks fitted with rubber septa under a positive pressure of argon, unless otherwise noted. Air- and moisture-sensitive liquids and solutions were transferred via syringe or stainless steel cannula. Where necessary (so noted), reaction mixtures were degassed by alternate evacuation/argon flush cycles (five or more iterations). Organic solutions were concentrated by rotary evaporation at ≈ 25 Torr (water aspirator). Flash chromatography was performed as described by Still et al. (13), employing 230–400 mesh silica gel. NMR tubes for sealed tube experiments were fabricated by attachment of a standard 18 cm [5 mm outside diameter (o.d.)] NMR tube to a standard taper female 14/20 joint using a 7 cm extension of 5 mm o.d. medium-wall borosilicate glass. Sealed NMR samples were prepared by flame-sealing a frozen (liquid nitrogen), evacuated (≤ 10 mTorr) sample that had been degassed by five successive freeze–pump–thaw cycles.

Materials. Commercially available reagents were used as received with the following exceptions. Ethyl ether and tetrahydrofuran were distilled from sodium benzophenone ketyl. Benzene, toluene, dichloromethane, triethylamine, *N,N*-diisopropylethylamine, and hexanes were distilled from calcium hydride at 760 Torr. Methanol was distilled from magnesium at atmospheric pressure. Methanesulfonyl chloride was distilled from phosphorus pentoxide at atmospheric pressure. Anhydrous *N,N*-dimethylformamide was purchased from Aldrich Chemical Co. and was stored over 4 Å molecular sieves. Cuprous iodide was purified by continuous extraction (48 h) with tetrahydrofuran in a Soxhlet apparatus. 1,4-Cyclohexadiene was purified by passage through a short column of neutral alumina immediately prior to use.

Instrumentation. Infrared spectra were obtained using a Perkin-Elmer 1600 FT-IR spectrophotometer

internally referenced to a polystyrene standard. ^1H NMR and ^{13}C NMR spectra were obtained on General Electric QE-300 (300 MHz), JEOL JX-400 (400 MHz), or Bruker AM-500 (500 MHz) NMR spectrometers; chemical shifts are expressed in parts per million (δ scale) relative to an internal standard of chloroform (^1H , 7.26; ^{13}C , 77.0), benzene- d_6 (^1H , 7.20; ^{13}C , 128.0), or dimethyl sulfoxide- d_6 (^1H , 2.49; ^{13}C , 39.5). Multiplicities were reported as follows: s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), and br s (broad singlet). Coupling constants are reported in hertz (Hz). Mass spectrometry was performed at the University of California, Riverside, Mass Spectrometry Facility or at the University of Nebraska, Lincoln, Midwest Center for Mass Spectrometry (with partial support by the National Science Foundation, Biology Division, Grant DIR9017262). High-pressure liquid chromatography (HPLC) was carried out on a Waters Delta Prep 3000 instrument using Vydac analytical (10 μm , 0.46×25 cm) and preparative (10 μm , 2.2×25 cm) reverse-phase C18 (201HS) columns. UV spectra were obtained on a Shimadzu UV160U spectrophotometer.

(Z)-Bromoene 12. *tert*-Butyldimethylsilyl chloride (0.895 g, 5.95 mmol, 1.1 equiv) was added to a solution of alcohol **11** (1.26 g, 5.41 mmol, 1 equiv, ref 3) and triethylamine (1.14 mL, 8.12 mmol, 1.5 equiv) in dichloromethane (100 mL) at 0 °C. After 5 min, the reaction mixture was warmed to 23 °C, 4-dimethylaminopyridine (0.179 g, 1.46 mmol, 0.27 equiv) was added, and the resulting solution was stirred for 17 h. The reaction mixture was then poured into water (100 mL), and the product was extracted with a 1:1 mixture of ethyl acetate and hexanes (3×150 mL). The combined organic layers were dried over sodium sulfate and were concentrated. The residue was purified by flash chromatography (5% ethyl acetate in hexanes) to yield the (Z)-bromoene **12** (1.76 g, 94%) as a yellow oil: $R_f = 0.89$ (20% ethyl acetate/hexanes); IR (neat) 2956 (s), 2858 (m), 2145 (m, C $\equiv$ C), 1471 (m), 1375 (m), 1251 (s), 1132 (s), 1092 (s), 1032 (m), 842 (s), 779 (m); ^1H NMR (300 MHz, CDCl_3) 6.38 (t, 1H, $J = 2.0$ Hz, C=CH), 4.31 (d, 2H, $J = 2.0$ Hz, SiOCH $_2$), 0.92 (s, 9H, SiC(CH $_3$) $_3$), 0.24 (s, 9H, Si(CH $_3$) $_3$), 0.10 (s, 6H, Si(CH $_3$) $_2$); ^{13}C NMR (500 MHz, CDCl_3) 137.2 (RCH=CBrR), 108.8 (RCH=CBrR), 101.2 (C $\equiv$ C), 101.1 (C $\equiv$ C), 67.5 (H $_2$ COTBS), 25.7 ((CH $_3$) $_3$ CSi), 22.6 ((CH $_3$) $_3$ CSi), -0.2 (CSi(CH $_3$) $_3$), -5.5 ((CH $_3$) $_2$ Si); HRMS (chemical ionization; ammonia gas) observed $[\text{MH}]^+$ 347.0849 (-3.8 ppm), calculated 347.0862.

(Z)-Enediene 13. A solution of the (Z)-bromoene **12** (1.76 g, 5.07 mmol, 1 equiv), propargyl alcohol (0.58 mL, 10.1 mmol, 2.0 equiv), and *n*-propylamine (1.67 mL, 20.3 mmol, 4.0 equiv) in tetrahydrofuran (50 mL) was deoxygenated at 23 °C and then was cooled in ice. Cuprous iodide (0.145 g, 0.760 mmol, 0.15 equiv) and bis-(triphenylphosphine)palladium(II) chloride (0.178 g, 0.254 mmol, 0.05 equiv) were added sequentially to the reaction mixture, deoxygenating thoroughly after each addition. Upon completion of the addition, the reaction mixture was warmed from 0 to 23 °C and then was stirred for 15 h. The reaction mixture was then poured into a 1:1 mixture of saturated aqueous ammonium chloride solution and saturated aqueous potassium carbonate solution (60 mL). The product was extracted with a 1:1 mixture of ethyl acetate and hexanes (3×100 mL). The combined organic layers were dried over sodium sulfate and were concentrated. The residue was purified by flash chromatography (10% ethyl acetate/hexanes) to give the (Z)-enediye **13** (1.31 g, 80%) as an oil: $R_f = 0.38$ (20% ethyl acetate/hexanes); IR (neat) 3358 (br, OH), 2956 (s), 2858 (m), 2134 (m, C $\equiv$ C), 1469 (w), 1252 (s), 1120 (s), 1034

(w), 851 (s), 778 (m); ^1H NMR (500 MHz, CDCl_3) 6.06 (t, 1H, $J = 2.1$ Hz, $\text{C}=\text{CH}$), 4.46 (s, 2H, $\text{C}=\text{CCH}_2\text{OH}$), 4.20 (d, 2H, $J = 2.1$ Hz, SiOCH_2R), 0.91 (s, 9H, $\text{Si}(\text{CH}_3)_3$), 0.22 (s, 9H, $\text{Si}(\text{CH}_3)_3$), 0.07 (s, 6H, $\text{Si}(\text{CH}_3)_2$); ^{13}C NMR (500 MHz, CDCl_3) 135.0 ($\text{RCH}=\text{C}$), 113.3 ($\text{RCH}=\text{C}$), 102.4 ($\text{C}\equiv\text{CTMS}$), 101.7 ($\text{C}\equiv\text{CTMS}$), 95.5 ($\text{C}=\text{CCH}_2\text{OH}$), 82.1 ($\text{C}=\text{CCH}_2\text{OH}$), 64.5 (RCH_2OTBS), 51.6 (RCH_2OH), 25.8 ($(\text{CH}_3)_3\text{CSi}$), 18.3 ($(\text{CH}_3)_3\text{CSi}$), -0.1 ($(\text{CH}_3)_3\text{Si}$), -5.5 ($(\text{CH}_3)_2\text{Si}$); HRMS (chemical ionization; ammonia gas) observed $[\text{MH}]^+$ 323.1859 (-1.1 ppm), calculated 323.1863.

(Z)-Eneidyne 14. Aqueous sodium hydroxide solution (50% w/w, total volume of 0.25 mL) was added in five portions over a 2 h period to an ice-cooled solution of the (Z)-eneidyne **13** (0.650 g, 2.02 mmol, 1 equiv) in methanol (25 mL). The reaction mixture was then poured into water (100 mL), and the product was extracted with a 1:1 mixture of ethyl acetate and hexanes (3×100 mL). The combined organic layers were dried over sodium sulfate and were concentrated. The residue was purified by flash chromatography using 20% ethyl acetate/hexanes as the eluent to provide the (Z)-eneidyne **14** as an oil (0.45 g, 89%): $R_f = 0.29$ (20% ethyl acetate/hexanes); IR (neat) 3289 (br, OH, and s, $\text{C}=\text{CH}$), 2932 (s), 2858 (s), 2217 (w), 2103 (w), 1467 (m), 1257 (s), 1118 (s), 1032 (m), 840 (s), 780 (s); ^1H NMR (500 MHz, CDCl_3) 6.04 (dd, 1H, $J = 4.5$ Hz, 2.1 Hz, $\text{C}=\text{CH}$), 4.47 (d, 2H, $J = 6.0$ Hz, RCH_2OH), 4.22 (d, 2H, $J = 1.5$ Hz, SiOCH_2), 3.29 (m, 1H, $\text{C}=\text{CH}$), 1.62 (t, 1H, $J = 6.1$ Hz, RCH_2OH), 0.92 (s, 9H, $\text{Si}(\text{CH}_3)_3$), 0.08 (s, 6H, $\text{Si}(\text{CH}_3)_2$); ^{13}C NMR (500 MHz, CDCl_3) 135.6 ($\text{RCH}=\text{C}$), 112.0 ($\text{RCH}=\text{C}$), 95.5 ($\text{C}=\text{CCH}_2\text{OH}$), 83.6 ($\text{C}=\text{CH}$), 81.7 ($\text{C}=\text{CCH}_2\text{OH}$), 81.0 ($\text{C}=\text{CH}$), 64.6 (RCH_2OTBS), 51.6 (RCH_2OH), 25.8 ($(\text{CH}_3)_3\text{CSi}$), 18.3 ($(\text{CH}_3)_3\text{CSi}$), -5.5 ($(\text{CH}_3)_2\text{Si}$); HRMS (FAB; 3-nitrobenzyl alcohol matrix) observed $[\text{M} + \text{Na}]^+$ 273.1284 (-1.1 ppm), calculated 273.1287.

Allene 15. Methanesulfonyl chloride (0.17 mL, 2.3 mmol, 3.0 equiv) was added dropwise over 10 min to an ice-cooled solution of the (Z)-eneidyne **14** (0.19 g, 0.75 mmol, 1 equiv) and triethylamine (0.55 mL, 3.8 mmol, 5.0 equiv) in dichloromethane (8 mL). The resulting suspension was stirred for 15 min at 0°C . A solution of anhydrous hydrazine in methanol (1:1 v/v, 1.5 mL, 23.9 mmol of hydrazine, 31.8 equiv) was then added directly by syringe, and the reaction mixture was stirred at 0°C for 18 h. The reaction solution was poured into half-saturated brine (100 mL), and the product alkylhydrazine was extracted with 5% methanol in dichloromethane (2×100 mL). The combined organics were washed with brine (100 mL), dried over sodium sulfate, and concentrated in vacuo.

The crude alkylhydrazine (theory, 0.75 mmol, 1 equiv) was taken up in ethyl ether (70 mL), and the resulting solution was deoxygenated at 23°C and then was cooled to -78°C . A deoxygenated solution of 4-methyl-1,2,4-triazoline-3,5-dione (MTAD, 0.170 g, 1.5 mmol, 2.0 equiv) in ethyl ether (10 mL) was added quickly to the reaction mixture via cannula. The resulting pink solution was stirred at -78°C for 15 min and then was poured into half-saturated brine (150 mL). The product allene was extracted with pentane (3×200 mL). The combined organic layers were dried over sodium sulfate and were concentrated. The residue was purified by flash chromatography (5% ethyl acetate/hexanes), affording the allene **15** (70.0 mg, 40%) as a yellow oil: $R_f = 0.93$ (50% ethyl acetate/hexanes); IR (neat) 3310 (m), 2955 (s), 2929 (s), 2857 (s), 2100 (w), 1934 (m), 1666 (w), 1604 (w), 1464 (m), 1390 (w), 1362 (w), 1255 (s), 1192 (m), 1118 (s), 1076 (m), 1006 (w), 939 (w), 841 (s), 778 (s), 671 (w), 638 (w); ^1H NMR (400 MHz, C_6D_6) 6.86 (t, 1H, $J = 6.8$ Hz, $\text{HC}=\text{C}=\text{CH}_2$), 6.08 (s, 1H, $\text{C}=\text{CH}$), 4.69 (d, 2H, $J = 6.8$

Hz, $\text{HC}=\text{C}=\text{CH}_2$), 4.35 (s, 2H, SiOCH_2), 2.98 (s, 1H, $\text{C}=\text{CH}$), 0.94 (s, 9H, $\text{Si}(\text{CH}_3)_3$), 0.01 (s, 6H, $\text{Si}(\text{CH}_3)_2$); ^{13}C NMR (400 MHz, C_6D_6) 210.5 ($\text{C}=\text{C}=\text{C}$), 146.5 ($\text{RCH}=\text{C}$), 104.2 ($\text{RCH}=\text{C}$), 90.8 ($\text{C}=\text{C}=\text{CH}_2$), 84.6 ($\text{C}=\text{CH}$), 81.0 ($\text{C}=\text{CH}$), 78.3 ($\text{C}=\text{C}=\text{CH}_2$), 62.9 (RCH_2OTBS), 26.0 ($(\text{CH}_3)_3\text{CSi}$), 18.4 ($(\text{CH}_3)_3\text{CSi}$), -5.5 ($(\text{CH}_3)_2\text{Si}$); HRMS (chemical ionization; ammonia gas) observed $[\text{MH}]^+$ 235.1516 (-0.9 ppm), calculated 235.1518.

Alcohol 10. A total volume of 1.75 mL of triethylamine trihydrofluoride was added in four portions over a period of 2 h to a solution of the allene **15** (70.0 mg, 0.299 mmol, 1 equiv) in dichloromethane (10 mL) at 23°C with periodic monitoring of the progress of the reaction by thin-layer chromatography. After being stirred for an additional 1 h, the reaction mixture was poured into water (100 mL), and the product was extracted with dichloromethane (3×100 mL). The combined organics were dried over sodium sulfate and were concentrated. The residue was purified by flash chromatography (30% ethyl acetate/hexanes), providing the alcohol **10** as a (volatile) yellow oil (27.3 mg, 76%): $R_f = 0.44$ (30% ethyl acetate/hexanes); IR (neat) 3294 (br, OH, and s, $\text{C}=\text{CH}$), 2924 (s), 2092 (w), 1932 (s, $\text{C}=\text{C}=\text{C}$), 1718 (m), 1600 (w), 1385 (m), 1292 (m), 1056 (s), 852 (s); ^1H NMR (500 MHz, C_6D_6) 6.76 (t, 1H, $J = 6.7$ Hz, $\text{HC}=\text{C}=\text{CH}_2$), 5.69 (s, 1H, $\text{C}=\text{CH}$), 4.64 (d, 2H, $J = 6.7$ Hz, $\text{HC}=\text{C}=\text{CH}_2$), 3.93 (d, 2H, $J = 5.6$ Hz, HOCH_2), 2.96 (s, 1H, $\text{C}=\text{CH}$), 0.70 (t, 1H, $J = 5.8$ Hz, HOCH_2); ^{13}C NMR (500 MHz, $\text{DMSO}-d_6$) 209.8 ($\text{C}=\text{C}=\text{C}$), 147.4 ($\text{RCH}=\text{C}$), 87.0 ($\text{RCH}=\text{C}$), 87.0 ($\text{C}=\text{C}=\text{CH}_2$), 80.6 ($\text{RC}=\text{CH}$), 79.1 ($\text{RC}=\text{CH}$), 75.6 ($\text{C}=\text{C}=\text{CH}_2$), 60.5 (CH_2OH); HRMS (electron impact) observed $[\text{M}]^+$ 120.05725 (-2.2 ppm), calculated 120.05751.

Bis(pentafluorophenyl) Ester 20. Fumaric acid (0.200 g, 1.72 mmol, 1 equiv), 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (0.992 g, 5.16 mmol, 3.0 equiv), and 4-(dimethylamino)pyridine (7.5 mg, 0.061 mmol, 0.04 equiv) were added sequentially to a solution of pentafluorophenol (0.955 g, 5.19 mmol, 3.0 equiv) in dichloromethane (50 mL). After being stirred at 23°C for 12 h, the reaction mixture was poured into water (100 mL), and the product was extracted with dichloromethane (3×100 mL). The combined organic layers were dried over sodium sulfate and were concentrated to a volume of 100 mL. The concentrated product solution was extracted with saturated aqueous sodium bicarbonate solution (3×90 mL), then was dried over sodium sulfate, and was concentrated. Upon addition of cold dichloromethane (-20°C , 10 mL), the product precipitated as a pale yellow solid. The solid was collected, and the filtrate was concentrated. The residue upon concentration was precipitated with cold dichloromethane, as before, and the precipitate was collected and combined with the first crop of product. The solid product was dried under vacuum, yielding 0.489 g (63%) of bis(pentafluorophenyl) ester **20**: mp $161\text{--}163.5^\circ\text{C}$ dec; $R_f = 0.94$ (0.4% acetic acid/ethyl acetate); IR (neat) 3097 (w), 1769 (m), 1516 (s), 1324 (w), 1282 (s), 1120 (s), 1026 (w), 1002 (s), 907 (w); ^1H NMR (400 MHz, CDCl_3) 7.34 (s, 2H, $\text{RCH}=\text{CHR}$); ^{13}C NMR (400 MHz, CDCl_3) 160.2 (CO_2R), 134.0 ($\text{RCO}_2\text{CH}=\text{CHCO}_2\text{R}$), 143–124 (complex, pentafluorophenyl carbons); HRMS (FAB; 3-nitrobenzyl alcohol matrix) observed $[\text{M}]^-$ 447.9823 (6.6 ppm), calculated 447.9793.

Pentafluorophenyl Ester 21. Bis(pentafluorophenyl) ester **20** (0.286 g, 0.638 mmol, 3.0 equiv) and 4-(dimethylamino)pyridine (~ 2 mg, 0.02 mmol, 0.01 equiv) were added sequentially to a pale yellow solution of alcohol **10** (25 mg, 0.21 mmol, 1 equiv) and triethylamine (1.48 μL , 1.07 mmol, 5.0 equiv) in dichloromethane (25

mL) at 23 °C. After being stirred for 3.5 h at 23 °C, the reaction mixture was poured into water (90 mL), and the product was extracted with a 1:1 mixture of ethyl acetate and hexanes (3 $\times$ 100 mL). The combined organic layers were dried over sodium sulfate and were concentrated. The residue was purified by flash chromatography using 20% toluene/hexanes as the eluent to provide 48 mg (60%) of pentafluorophenyl ester **21** as an oil: R_f = 0.67 (20% ethyl acetate/hexanes); IR (neat) 3294 (m), 3077 (w), 2995 (w), 2933 (w), 2667 (w), 2462 (w), 2092 (w), 1933 (m), 1773 (s), 1732 (s), 1645 (m), 1604 (w), 1522 (s), 1471 (w), 1376 (w), 1285 (s), 1201 (s), 1146 (m), 1121 (s), 1005 (s), 856 (m), 760 (m); ^1H NMR (400 MHz, CDCl_3) 7.11 (ABq, 2H, J = 15.9 Hz, $\Delta\nu$ = 18.9 Hz, $\text{RCO}_2\text{CH}=\text{CHCO}_2\text{R}'$), 6.54 (t, 1H, J = 7.2 Hz, $\text{HC}=\text{C}=\text{CH}_2$), 5.59 (s, 1H, $\text{RCH}=\text{C}$), 5.13 (d, 2H, J = 6.8 Hz, $\text{C}=\text{C}=\text{CH}_2$), 4.86 (s, 2H, CH_2OR), 3.36 (d, 1H, J = 2.4 Hz, $\text{C}=\text{CH}$); ^{13}C NMR (400 MHz, CDCl_3) 211.0 ($\text{C}=\text{C}=\text{C}$), 163.4 ($\text{RCO}_2\text{CH}_2\text{R}$), 160.6 ($\text{RCO}_2\text{C}_6\text{F}_5$), 141.1 ($\text{RCH}=\text{C}$), 137.0 and 130.4 ($\text{RCO}_2\text{CH}=\text{CHCO}_2\text{R}'$), 107.6 ($\text{RCH}=\text{C}$), 90.9 ($\text{C}=\text{C}=\text{CH}_2$), 85.5 ($\text{RC}=\text{CH}$), 79.5 ($\text{RC}=\text{CH}$), 79.2 ($\text{C}=\text{C}=\text{CH}_2$), 65.0 (CH_2OR), 143–124 (complex, pentafluorophenyl carbons); HRMS (FAB; 3-nitrobenzyl alcohol matrix) observed $[\text{M}]^-$ 384.0445 (6.2 ppm), calculated 384.0421.

Conjugate 9. Palladium (10%) on activated carbon (11.6 mg) was added to a 100 mL Schlenk-type flask (the use of an oversized flask appears to be important for success in this reaction) containing a deoxygenated solution of 4-nitropyrrole-2-carboxamide **16** (33.0 mg, 66.3 mmol, 1 equiv) (**14**) in methanol (8.0 mL), and the resultant suspension was deoxygenated. The reaction flask was then purged with hydrogen gas, and the reaction solution was stirred under 1 atm of hydrogen for 2.5 h at 23 °C. The reaction mixture was filtered through Celite, and the filtrate was concentrated. The residue was concentrated twice from dichloromethane (10 mL) and then was lyophilized from benzene (10 mL) to afford the 4-aminopyrrole-2-carboxamide **17** (R_f = 0.30, 3% ammonium hydroxide in methanol) as an air-sensitive yellow powder that was immediately coupled with the pentafluorophenyl ester **21**.

Pentafluorophenyl ester **21** (25.5 mg, 66.3 mmol, 1.0 equiv) was added to a deoxygenated solution of the crude 4-aminopyrrole-2-carboxamide **17**, prepared above, in *N,N*-dimethylformamide (6.0 mL). The reaction mixture was observed to darken to a golden color. After being stirred for 1 h at 23 °C, the reaction mixture was poured into water (65 mL), and the product was extracted with dichloromethane (3 $\times$ 60 mL). The combined organic layers were washed with water (2 $\times$ 100 mL), dried over sodium sulfate, and then concentrated. The residue was purified by flash chromatography (1% ammonium hydroxide in 1:1 acetonitrile/methanol) wherein acetic acid (2–3 drops) was added to each fraction as it eluted. Fractions containing product **9** were pooled and concentrated, and the residue was lyophilized from dimethyl sulfoxide (2 mL) to afford the conjugate **9** as its acetate salt (yellow film, 17 mg, 36%): R_f = 0.38 (3% ammonium hydroxide/methanol); IR (neat) 3401 (br), 3284 (br), 2943 (m), 1931 (w), 1720 (m), 1641 (s), 1578 (s), 1555 (s), 1465 (m), 1435 (s), 1405 (s), 1349 (w), 1290 (m), 1263 (s), 1208 (m), 1165 (m), 1149 (m), 1106 (w), 1049 (w), 1020 (m), 1005 (m), 885 (w), 814 (w), 774 (m), 668 (w), 609 (w); ^1H NMR (400 MHz, $\text{DMSO}-d_6$) 10.66 (s, 1H, $\text{CH}=\text{CHCONH}$), 9.99 (s, 1H, NH), 9.90 (s, 1H, NH), 8.07 (br s, 1H, CONHCH_2), 7.34 (s, 1H, aromatic), 7.24 (s, 1H, aromatic), 7.19 (d, 1H, J = 14.6 Hz, $\text{CH}=\text{CH}$), 7.17 (s, 1H, aromatic), 7.03 (s, 1H, aromatic), 6.97 (s, 1H, aromatic), 6.81 (s, 1H, aromatic), 6.68 (d, 1H, J = 15.8 Hz, $\text{CH}=\text{CH}$), 6.41 (t, 1H, J = 7.0 Hz, $\text{HC}=\text{C}=\text{CH}_2$), 5.74 (s, 1H, $\text{C}=\text{CHC}=\text{C}$),

5.30 (d, 2H, J = 7.0 Hz, $\text{HC}=\text{C}=\text{CH}_2$), 4.77 (s, 2H, CH_2OR), 4.48 (s, 1H, $\text{C}=\text{CH}$), 3.86 (s, 3H, NCH_3), 3.83 (s, 3H, NCH_3), 3.78 (s, 3H, NCH_3), 3.17 (m, 2H, CONHCH_2), 2.23 (t, 2H, J = 6.6 Hz, $\text{CH}_2\text{N}(\text{CH}_3)_2$), 2.12 (s, 6H, $\text{N}(\text{CH}_3)_2$), 1.82 (s, 3H, acetate), 1.60 (m, 2H, $\text{CH}_2\text{CH}_2\text{N}(\text{CH}_3)_2$); ^{13}C NMR (400 MHz, $\text{DMSO}-d_6$) 210.4 ($\text{C}=\text{C}=\text{C}$), 172.5 ($-\text{OCOCH}_3$), 164.5 ($\text{RCO}_2\text{CH}_2\text{R}$), 161.2 (CONH), 159.5 (CONH), 158.4 (CONH), 158.2 (CONH), 141.2 ($\text{HC}=\text{CCH}=\text{C}$), 137.8 ($\text{RCO}_2\text{CHCHCO}_2\text{R}$), 127.7 ($\text{RCO}_2\text{CHCHCO}_2\text{R}$), 123.3 (aromatic), 123.0 (aromatic), 122.8 (aromatic), 122.1 (aromatic), 122.0 (aromatic), 121.3 (aromatic), 118.8 (aromatic), 118.4 (aromatic), 117.7 (aromatic), 107.5 (aromatic), 104.6 (aromatic and $\text{HC}=\text{CCH}=\text{C}$), 104.0 (aromatic), 90.0 ($\text{C}=\text{C}=\text{CH}_2$), 88.6 ($\text{RC}=\text{CH}$), 79.8 ($\text{RC}=\text{CH}$), 79.6 ($\text{C}=\text{C}=\text{CH}_2$), 63.9 (CH_2OR), 57.1 (CONHCH_2), [45.1, 37.1, 36.2, 36.0, 35.9 (3 NCH_3 and $\text{CH}_2\text{N}(\text{CH}_3)_2$ and $\text{N}(\text{CH}_3)_2$), 27.1 ($\text{CH}_2\text{CH}_2\text{N}(\text{CH}_3)_2$), 21.9 ($-\text{OCOCH}_3$); HRMS (FAB; 3-nitrobenzyl alcohol matrix) observed $[\text{MH}]^+$ 669.3142 (–1.0 ppm), calculated 669.3149.

Thermal Cyclization of Conjugate 9: Product Analysis and Kinetics. Thermal cyclization studies of conjugate **9** (3.0–3.8 mM) were conducted in dimethyl sulfoxide- d_6 solution containing 1,4-cyclohexadiene (30–300 mM) in sealed NMR tubes. Each reaction solution was thoroughly deoxygenated by several freeze–pump–thaw cycles prior to flame-sealing under vacuum. Samples prepared for kinetics runs contained *N,N*-dimethylformamide (25 mM) as an internal standard and were analyzed periodically by ^1H NMR, integrating against the formyl proton resonance. Thermolyses were conducted at temperatures from 30–37 °C in a regulated sand bath for a total reaction period of 85–90 h. Upon the completion of the reaction, the product solutions were analyzed by ^1H NMR and by reverse-phase high-performance liquid chromatography (rp-HPLC). rp-HPLC was conducted with a Waters Delta Prep 3000 instrument using Vydac analytical (10 μm , 0.46 $\times$ 25 cm, flow rate of 0.4 mL/min) or preparative (10 μm , 2.2 $\times$ 25 cm, flow rate of 7.0 mL/min) reverse-phase C18 (201HS) columns, employing acetonitrile and aqueous ammonium acetate buffer solution (10 mM, pH 5.5) as eluent in a linear gradient of 30:70 to 60:40, respectively, over a 30 min period followed by isocratic elution (60:40). Peaks were detected with a Waters 994 programmable photodiode array detector. Cyclization product **22** was typically formed in 25% yield. Isomers **23** and **24** were also formed in 25% (combined) yield. Product **22** (retention time of 25 min) and the mixture of isomers **23** and **24** (inseparable, retention time of 37 min) were separated by HPLC and were fully characterized, as follows.

Cyclization product 22: IR (neat) 3384 (br), 2919 (m), 2849 (w), 1725 (w), 1660 (m), 1637 (s), 1584 (s), 1549 (s), 1531 (m), 1467 (m), 1437 (s), 1402 (s), 1349 (w), 1296 (w), 1261 (m), 1208 (w), 1155 (w), 1102 (w); ^1H NMR (400 MHz, $\text{DMSO}-d_6$) 10.65 (s, 1H, $\text{CH}=\text{CHCONH}$), 9.99 (s, 1H, NH), 9.89 (s, 1H, NH), 8.07 (br s, 1H, NHCH_2), 7.33 (s, 1H, aromatic), 7.30–7.15 (5H, aromatic plus $\text{CH}=\text{CH}$), 7.23 (s, 1H, aromatic), 7.17 (s, 1H, aromatic), 7.02 (s, 1H, aromatic), 6.96 (s, 1H, aromatic), 6.81 (s, 1H, aromatic), 6.69 (d, 1H, J = 15.0 Hz, $\text{CH}=\text{CH}$), 5.18 (s, 2H, CH_2OR), 3.85 (s, 3H, NCH_3), 3.83 (s, 3H, NCH_3), 3.78 (s, 3H, NCH_3), 3.18 (m, 2H, CONHCH_2), 2.31 (s, 3H, ArCH_3), 2.23 (t, 2H, J = 7.0 Hz, $\text{CH}_2\text{N}(\text{CH}_3)_2$), 2.12 (s, 6H, $\text{N}(\text{CH}_3)_2$), 1.82 (s, acetate), 1.59 (m, 2H, $\text{CH}_2\text{CH}_2\text{N}$); HRMS (FAB; 3-nitrobenzyl alcohol matrix) observed $[\text{MH}]^+$ 671.3297 (–1.2 ppm), calculated 671.3306.

Isomers 23 and 24: IR (neat) 3383 (br), 2954 (w), 2908 (s), 2837 (s), 1655 (w), 1631 (m), 1578 (s), 1561 (s), 1543 (m), 1525 (m), 1467 (w), 1402 (m), 1349 (w), 1249 (w), 1061 (br), 861 (m); ^1H NMR (400 MHz, $\text{DMSO}-d_6$) 10.67 (s, 1H, $\text{CH}=\text{CHCONH}$), 9.99 (s, 1H, NH), 9.89 (s, 1H, NH), 8.07 (br s, 1H, NHCH_2), 7.33 (s, 1H, aromatic),

7.32–7.17 (5H, aromatic plus CH=CH), 7.24 (s, 1H, aromatic), 7.17 (s, 1H, aromatic), 7.02 (s, 1H, aromatic), 6.96 (s, 1H, aromatic), 6.81 (s, 1H, aromatic), 6.70 (d, 1H, $J = 15.0$ Hz, CH=CH), 5.87–5.55 (m, cyclohexadienyl protons), 5.20 (s, 2H, CH₂OR), 3.85 (s, 3H, NCH₃), 3.83 (s, 3H, NCH₃), 3.78 (s, 3H, NCH₃), 3.18 (m, 2H, NHCH₂R), 2.65 (d, ArCH₂CH), 2.22 (t, 2H, $J = 7.0$ Hz, CH₂CH₂N(CH₃)₂), 2.12 (s, 6H, N(CH₃)₂), 1.76–1.74 (–OAc (buffer)), 1.59 (m, 2H, CH₂CH₂N(CH₃)₂); HRMS (FAB; 3-nitrobenzyl alcohol matrix) observed $[MH]^+$ 749.3777 (0.2 ppm), calculated 749.3775.

Reagents and Materials for DNA Studies. All reaction solutions were prepared with ultrapure water, obtained from a Millipore Milli-Q Plus water purification system. Sonicated, deproteinized calf thymus DNA was purchased from Pharmacia and was dissolved in water to a concentration of 1 mM base pairs (bp) in DNA. Plasmid pBR322 was purchased from Boehringer Mannheim. Enzymes were obtained from Boehringer Mannheim and New England Biolabs and were used with the supplied buffers, with the exception of Klenow enzyme, which was used with *Hae*III buffer. Deoxyadenosine 5'-[α -³²P]triphosphate and adenosine 5'-[γ -³²P]triphosphate were purchased from Amersham or NEN. Other reagents were used as received. Standard techniques were employed for DNA manipulations (15).

DNA Fragment Preparation. Plasmid pBR322 was linearized with *Eco*RI restriction endonuclease and then was labeled on the 3' terminus with [α -³²P]dATP using the Klenow fragment of DNA polymerase I. Following removal of the 5'-phosphate with calf alkaline phosphatase (CAP), a separate batch of *Eco*RI-digested plasmid was labeled at the 5' end with [γ -³²P]ATP using T4 polynucleotide kinase. After labeling, the DNA was digested with *Rsa*I to yield two end-labeled restriction fragments. The 517 bp fragment was excised from a 5% nondenaturing polyacrylamide gel (17.0 $\times$ 15.0 cm $\times$ 3 mm thickness) and further isolated according to standard procedures (15). Chemical-sequencing reactions were performed according to published methods (16, 17). A molecular weight standard for the nondenaturing gels was prepared as follows. Equal amounts of the 3'-radiolabeled 517 bp fragment and 0.25 μ g of pBR322 were combined and digested individually with restriction endonucleases *Ssp*I, *Bsp*HI, and *Xmn*I. The reaction mixtures were ethanol precipitated, counted, diluted to the same concentration [counts per minute (cpm) per microliter], and then combined in equal volumes with the same cpm of intact 517 bp fragment.

Footprinting and Cleavage Reactions. Reactions were executed in a total volume of 20 μ L with final concentrations of each component as noted. The conjugate **9** was added to solutions of radiolabeled restriction fragment (~18 000 cpm), calf thymus DNA (100 μ M bp), Tris-acetate buffer (25 mM, pH 7.0), and NaCl (10 mM), and the resulting solutions were incubated for 15 min at 37 °C. Footprinting reactions were initiated by addition of MPE·Fe(II) solution (4 μ M, prepared by mixing 20 μ L of a 100 μ M MPE solution with 20 μ L of a freshly made, 100 μ M ferrous ammonium sulfate solution and then diluting to 100 μ L with water; see ref 18) and dithiothreitol (4 mM) and proceeded for 9 min at 37 °C. Cleavage reaction mixtures were incubated at 37 °C for various defined lengths of time, up to 70 h. All reactions were terminated by freezing (dry ice), and the frozen solution was lyophilized on a Savant rotary speed-vac. The reaction mixtures were resuspended in 80% denaturing formamide loading buffer (4 μ L), assayed for radioactivity (Beckman LS 6000SC scintillation counter), and electrophoresed on 8% polyacrylamide denaturing gels (5% cross-link, 7 M urea; 31 $\times$ 38.5 cm $\times$ 0.4 mm thickness) at 1700 V for 1.5 h. The gels were dried and

analyzed using standard phosphor technology. Dried gels were also exposed to preflashed X-ray film at –80 °C with an intensifying screen.

Quantitative MPE·Fe(II) Footprint Titrations. Reaction mixtures (20 μ L total volume) consisted of the following final concentrations: serially diluted conjugate **9** (500 μ M to 1 nM) or distamycin analog **25** (100 μ M to 0.1 nM), 3'-labeled 517 bp restriction fragment (20 000 cpm), calf thymus DNA (100 μ M bp), Tris-acetate buffer (25 mM, pH 7.0), and NaCl (10 mM). After incubation at 37 °C for 15 min, freshly prepared MPE·Fe(II) solution (2 μ M) and dithiothreitol (2 mM) were added to initiate the footprinting reactions. After the reactions proceeded for 9 min at 37 °C, the mixtures were frozen (dry ice) and dried by speed-vac. Reaction mixtures were electrophoresed and analyzed as described above.

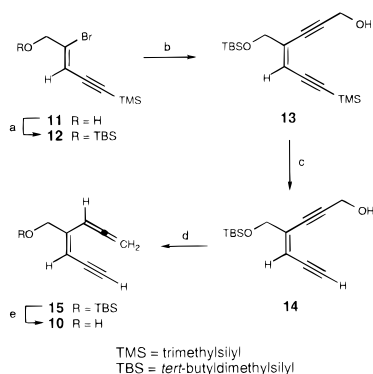
Calculation of Relative Binding Affinities. Binding isotherms were created following published methods (19). Briefly, standard phosphor-imaging technology was used to analyze both binding sites (5'-TTTTT-3' and 5'-AATAA-3') and a reference site (5'-CGTCA-3') by performing volume integrations. The apparent fraction of DNA bound (θ_{app}) was calculated and then fit to a Langmuir binding titration isotherm over the range of substrate concentrations. Binding affinities were determined from averaged θ_{app} values from three different acceptable (according to the χ^2 criterion, ref 20) gels. Values from the two binding sites were then compared for conjugate **9** and the distamycin analog **25**.

Cleavage Trials under Nondenaturing Conditions. Reactions were executed in a total volume of 20 μ L with the final concentrations of each component as follows, coupled product **9** (20 or 10 μ M), 3'-labeled 517 bp restriction fragment (8000 cpm), calf thymus DNA (100 μ M bp), Tris-acetate buffer (25 mM, pH 7.0), and NaCl (10 mM), and incubated at 37 °C for 60–70 h. Reactions were terminated by ethanol precipitation, and mixtures were dried on a rotary speed-vac. The dried pellet was resuspended in water (10 μ L) and Ficoll (6 $\times$) loading buffer (2 μ L), and the resulting suspension was assayed for radioactivity. Samples were electrophoresed on an 8% nondenaturing polyacrylamide gel (125 V, 17.0 $\times$ 15.0 cm $\times$ 1.5 mm thickness) positioned behind a cooling fan until the bromophenol blue dye had migrated within 1–2 cm of the bottom of the gel. The gel was then exposed to preflashed X-ray film at –80 °C with an intensifying screen.

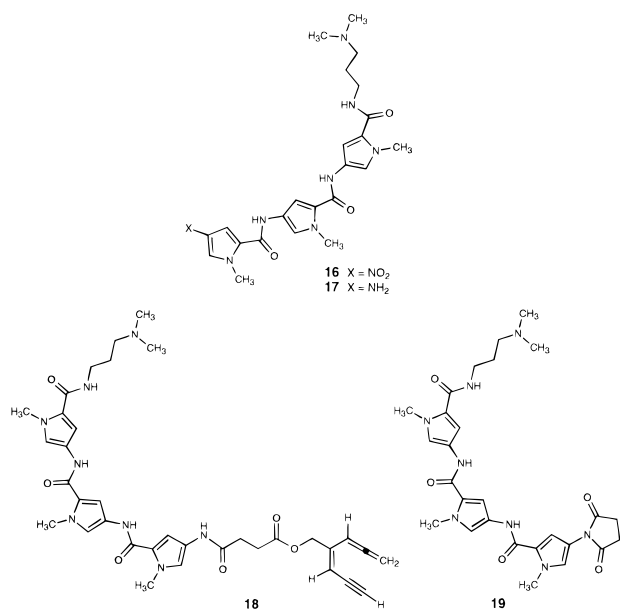
Quantitation by Storage Phosphor Technology Autoradiography. Photostimulatable storage phosphor-imaging plates (Kodak S0230) were pressed flat against dried gels and exposed in the dark at 23 °C for 12–18 h. Data from storage plates were obtained from a Molecular Dynamics 400S PhosphorImager. Data analysis was performed by calculation of volume integrations of all bands using the ImageQuant v.3.1 software running on an AST Premium 386/33 computer.

RESULTS

Synthesis of **9.** The (Z)-allene-ene-yne alcohol **10** was prepared using methodology previously developed for the synthesis of **3** (Scheme 2,⁴ ref 3). Protection of the known alcohol **11** (**3**) with *tert*-butyldimethylsilyl chloride, triethylamine, and 4-(dimethylamino)pyridine (DMAP) afforded the silyl ether **12** in 94% yield. Palladium-catalyzed coupling (21, 22) of **12** with propargyl alcohol produced the (Z)-enediyne **13** (80%). Protodesilylation of the acetylenic trimethylsilyl group of **13** was accomplished in 89% yield by exposure of **13** to a methanolic sodium hydroxide solution at 0 °C. The resulting alcohol (**14**) was transformed into the allene **15** using methodology previously developed for this purpose (23, 24). Thus, activation of the hydroxyl group of **14** with methane-

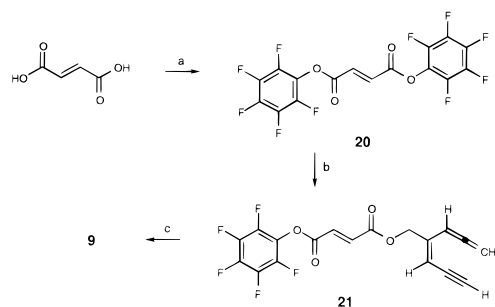
Scheme 2^a

^a Reagents and conditions: (a) TBSCl, Et₃N, DMAP, CH₂Cl₂, 0 → 23 °C, 94%; (b) HC≡CCH₂OH, CH₃CH₂CH₂NH₂, CuI, PdCl₂(PPh₃)₂, THF, 0 → 23 °C, 80%; (c) NaOH, CH₃OH, 0 °C, 89%; (d) CH₃SO₂Cl, Et₃N, CH₂Cl₂, 0 °C; H₂NNH₂, CH₃OH, 0 °C; MTAD, Et₂O, -72 °C, 40%; (e) 3HF·Et₃N, CH₂Cl₂, 23 °C, 76%.



sulfonyl chloride and displacement of the resulting mesylate with hydrazine in methanol afforded a propargylic hydrazine intermediate. Extractive isolation of this intermediate and oxidation of the crude extract with 4-methyl-1,2,4-triazoline-3,5-dione (MTAD) produced the allene **15** in 40% yield from **14**. Cleavage of the *tert*-butyldimethylsilyl ether group of **15** (triethylamine trihydrofluoride) then provided the biradical precursor **10** in 76% yield.

The nitrodistamycin derivative **16** (**14**) has been much used as a precursor for the binding domain of synthetic DNA-cleaving agents and is typically linked to an effector molecule via its hydrogenation product, the air-sensitive amine **17**. Dervan and co-workers (25), as well as others (11), have shown that the proper choice of a linking group can be a critical component in the design of DNA-cleaving agents. We initially chose a succinyl linker to couple the binding and effector domains but soon found that the resulting conjugate **18** was unstable, readily cyclizing to form the imide **19** by expulsion of the alcohol **10**. This problem was avoided by using a fumarate linking group (26). Activation of fumaric acid as its bis(pentafluorophenyl) ester (**20**, Scheme 3) was accomplished in 63% yield by bis-coupling with pentafluorophenol in the presence of 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (EDC). Addition of excess diester **20** (3.0 equiv) to a solution of the alcohol **10** in dichloro-

Scheme 3^a

^a Reagents and conditions: (a) pentafluorophenol, EDC, CH₂Cl₂, 23 °C, 63%; (b) **10**, Et₃N, DMAP, CH₂Cl₂, 23 °C, 60%; (c) **17**, DMF, 23 °C, 36%.

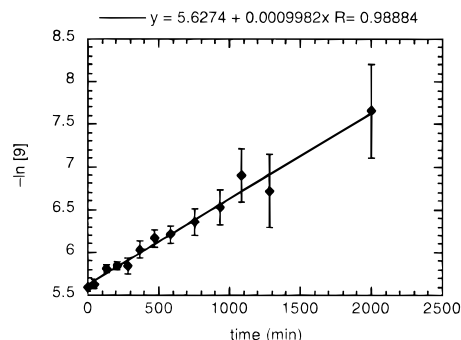
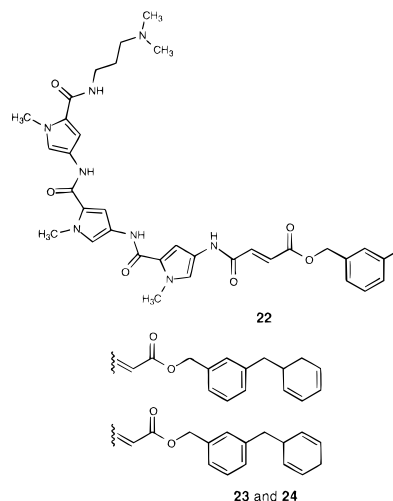


Figure 1. Plot of kinetic data for cyclization of **9** at 37 °C in dimethyl sulfoxide-*d*₆ containing 1,4-cyclohexadiene.

methane containing triethylamine (5.0 equiv) and DMAP (0.01 equiv) gave rise to the activated ester **21** (60%). When this product was stirred with a solution of the amine **17** [freshly prepared by hydrogenation of **16** (**14**)] in *N,N*-dimethylformamide (DMF) at 23 °C, the conjugate **9** was formed in 36% yield (from **16**) after purification on silica gel using 1:1 acetonitrile/methanol containing 1% concentrated aqueous ammonium hydroxide solution as the eluent. Spectroscopic and analytical data were found to be fully consistent with the assigned structure **9**.

Thermal Cyclization of 9. Thermal cyclizations of **9** (3.8 mM) were conducted at 37 °C in dimethyl sulfoxide-*d*₆ as solvent containing 1,4-cyclohexadiene (300 mM) and *N,N*-dimethylformamide (internal standard) in NMR tubes that had been sealed in vacuo. In a typical experiment, run to ~85% completion, the cyclization products **22** (25%) and **23** and **24** (inseparable mixture, 25% combined yield) were isolated by high-performance liquid chromatography (HPLC). A similar distribution of



values due to the presence of calf thymus DNA in these experiments, Table 1). Inspection of the data shows that conjugate **9** binds with higher affinity at the 5'-TTTTT-3' site than at the 5'-AATAA-3' site, the same order of binding preference displayed by distamycin analog **25** (29). Importantly, the data of Table 1 show that **9** binds some 30 times more weakly than the distamycin analog **25** (5'-AATAA-3' site), despite the larger size and greater hydrophobicity of **9**. We believe that this may reflect a poor fit of the allene-ene-yne cleaving domain of **9** into the minor groove of DNA, a feature which might explain, in part, the poor DNA cleavage efficiency of **9** (vide infra). Variation of the linking domain may well lead to improved DNA binding (and cleaving efficiency) in future conjugate molecules.

DNA Cleavage Studies. The ability of **9** to cleave double-stranded B-form DNA was probed using the 3'- and 5'- ^{32} P-labeled 517 bp restriction fragment of plasmid pBR322 described above in footprinting studies. Cleavage reactions employed the conjugate **9** (50–0.5 μ M), calf thymus DNA (100 μ M bp), and 3'- or 5'-radiolabeled restriction fragment ($\sim$ 18 000 cpm) and were performed in association buffer (10 mM NaCl, 25 mM Tris-acetate, pH 7.0) at 37 $^{\circ}$ C for 60–65 h. DNA cleavage was assayed by gel electrophoresis (Figures 4 and 5) using standard phosphor-imaging technology to quantitate cleavage at each base pair site. The gel data are translated into histogram format in Figure 3B. The minimum cleavage efficiency, calculated by integration of all migrating radiolabeled fragments against total radioactivity, was 0.2%. This value contrasts with cleavage efficiencies of 5.7% for dynemicin A (30), 25% for neocarzinostatin chromophore (31), and $\sim$ 90% for calicheamicin γ_1 (32), all members of the "enediyne" class of natural products, and, as discussed above, may be due, in part, to a poor fit of the effector domain into the minor groove of DNA. The observed patterns of cleavage by **9** do not resemble typical patterns produced by diffusible cleaving agents and are site selective, exhibiting a preference for the 3' side of each of the two binding sites defined in the footprinting studies above. This contrasts with the behavior of the affinity cleaving agent distamycin-EDTA (33), which produces a "Gaussian" cleavage pattern typical of diffusible cleaving agents and shows a bias for cleavage toward the 5' side of the 5'-AATAA-3' site. The time course of DNA cleavage by **9** was studied by gel electrophoresis of a parallel series of cleavage reactions conducted as above, but with varied reaction periods (0–60 h). Analysis of the data shows that DNA cleavage increases linearly for the first 20 h of the reaction before slowing. This time course parallels the observed half-life for the cyclization of **9**, as required by a mechanism wherein DNA cleavage is brought about by an α ,3-dehydrotoluene intermediate.

The sequence selectivity of DNA cleavage by **9** exhibits an interesting variation as a function of the concentration of **9** (Figures 4 and 5). At the highest concentration of **9** (50 μ M), the cleavage pattern is clearly shifted versus that produced by a reaction employing 10 μ M **9**. One explanation for this shifting could be that more than one conjugate molecule binds to the DNA-binding site at higher concentrations of conjugate. The binding of distamycin analogs as dimeric species at elevated concentrations has precedent (34–37). Shifting of the protection pattern in footprinting studies of **9** (Figure 2) is also evident at the highest concentration of **9** (50 μ M).

Evaluation of single-stranded versus double-stranded DNA cleavage products from the reaction of **9** with the 3'-radiolabeled 517 bp restriction fragment, as described above, was conducted by nondenaturing gel electrophore-

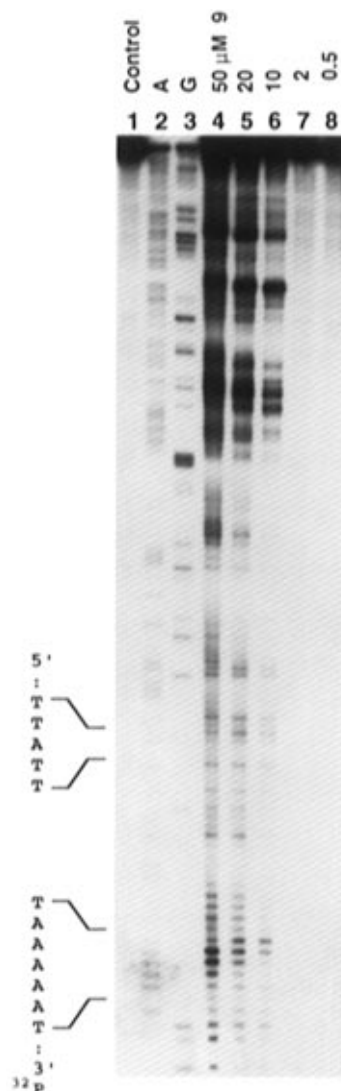


Figure 4. DNA cleavage brought about by thermal reaction (37 $^{\circ}$ C, 60–65 h) with bioconjugate **9**. Autoradiogram of an 8% denaturing polyacrylamide gel. All reaction mixtures contain calf thymus DNA (100 μ M bp), the 3'-labeled 517 bp restriction fragment ($\sim$ 18 000 cpm), and NaCl (10 mM) in Tris-acetate (25 mM, pH 7.0) buffer. Lanes 1–8 contain intact DNA, A reaction mixture (17), Maxam–Gilbert G reaction mixture (16), and 50, 20, 10, 2, and 0.5 μ M substrate **9**, respectively.

sis. There was no evidence of double-stranded DNA cleavage (data not shown). This result is consistent with the idea that DNA cleavage is brought about by an α ,3-dehydrotoluene biradical with a single highly reactive radical site; however, given the poor cleavage efficiency overall, the likelihood of a double-stranded nick is remote from a simple probabilistic view as well.

As a control, the parent (Z)-allene-ene-yne alcohol **10** was tested for DNA-cleaving activity. Cleavage trials were performed over a range of concentrations of **10** (100–10 mM) with the 3'-radiolabeled 517 bp DNA restriction fragment of pBR322 described above and calf thymus DNA (100 μ M bp) in association buffer (10 mM NaCl, 25 mM Tris-acetate, pH 7.0) at 37 $^{\circ}$ C for 60–65 h and were evaluated using gel electrophoresis (Figure 6). DNA cleavage was apparent only at concentrations of **10** 25 mM or greater, or some 2000 times higher than concentrations necessary for observable cleavage by the conjugate **9**. In addition, the cleavage pattern exhibited a preference for cleavage at A and G sites, suggesting a possible change in the cleavage mechanism, e.g., alkylative depurination. The data strongly support the view

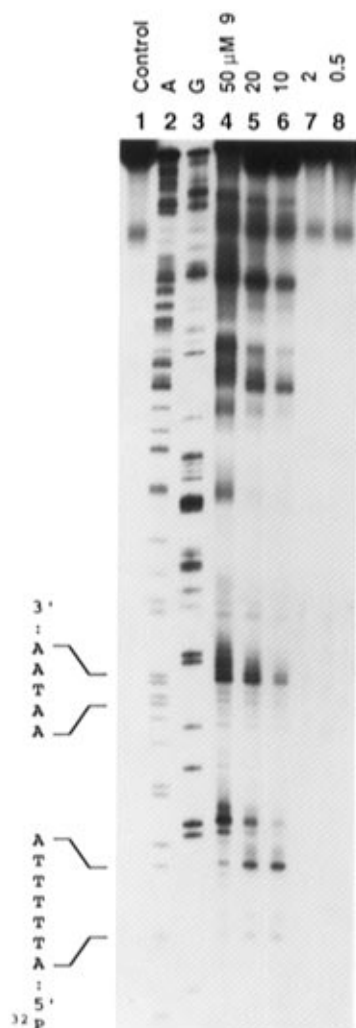


Figure 5. DNA cleavage brought about by thermal reaction (37 °C, 60–65 h) with bioconjugate **9**. Autoradiogram of an 8% denaturing polyacrylamide gel. All reaction mixtures contain calf thymus DNA (100 μM bp), the 5'-labeled 517 bp restriction fragment (~18 000 cpm), and NaCl (10 mM) in Tris-acetate (25 mM, pH 7.0) buffer. Lanes 1–8 contain intact DNA, A reaction mixture (17), Maxam–Gilbert G reaction mixture (16), and 50, 20, 10, 2, and 0.5 μM substrate **9**, respectively.

that the DNA-binding element is critical for the sequence-specific cleavage of DNA by **9** at micromolar concentrations.

CONCLUSIONS

The conjugate **9** has been shown to bind and cleave DNA with sequence selectivity. The binding domain has been shown to localize the allene-ene-yne effector domain for sequence-selective DNA cleavage at micromolar concentrations of conjugate. The time course of DNA cleavage parallels the rate of cyclization of **9** to form an α ,3-dehydrotoluene biradical. The pattern of DNA cleavage by **9** is suggestive of a nondiffusible cleaving agent. Although the data are consistent with the view that DNA cleavage arises from hydrogen atom abstraction by a localized α ,3-dehydrotoluene biradical, the mechanism of DNA cleavage by **9** has by no means been established. Importantly, we have shown that the (Z)-allene-ene-yne functional group is a viable effector domain for DNA cleavage by mild thermal activation. The efficiency of DNA cleavage by **9** is poor and may reflect less than optimum positioning of the effector domain in the minor groove of DNA. This speculation receives some support

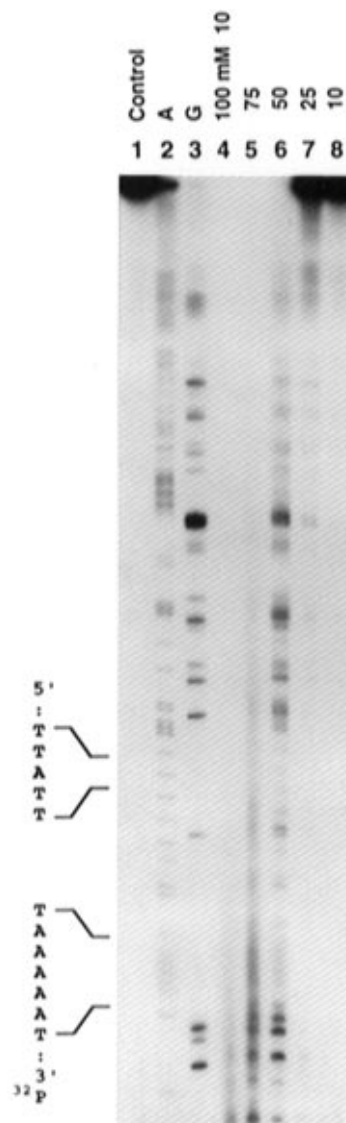


Figure 6. DNA cleavage brought about by thermal reaction (37 °C, 60–65 h) with alcohol **10**. Autoradiogram of an 8% denaturing polyacrylamide gel. All reaction mixtures contain calf thymus DNA (100 μM bp), the 3'-labeled 517 bp restriction fragment (~18 000 cpm), and NaCl (10 mM) in Tris-acetate (25 mM, pH 7.0) buffer. Lanes 1–8 contain intact DNA, A reaction mixture (17), Maxam–Gilbert G reaction mixture (16), and 100, 75, 50, 25, and 10 mM substrate **10**, respectively.

from the fact that the conjugate **9** binds ca. 30 times more weakly to DNA than the smaller, less hydrophobic distamycin analog **25**. This observation also suggests that future studies may benefit from modification of the linking domain.

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LITERATURE CITED

- (1) Myers, A. G., Kuo, E. Y., and Finney, N. S. (1989) Thermal Generation of α ,3-Dehydrotoluene from (Z)-1,2,4-Heptatrien-6-yne. *J. Am. Chem. Soc.* **111**, 8057–8059.

- (2) Myers, A. G., and Dragovich, P. S. (1989) Design and Dynamics of a Chemically Triggered Reaction Cascade Leading to Biradical Formation at Subambient Temperature. *J. Am. Chem. Soc.* **111**, 9130–9132.
- (3) Myers, A. G., Dragovich, P. S., and Kuo, E. Y. (1992) Studies on the Thermal Generation and Reactivity of a Class of (σ,π)-1,4-Biradicals. *J. Am. Chem. Soc.* **114**, 9369–9386.
- (4) Nicolaou, K. C., and Dai, W.-M. (1991) Chemistry and Biology of the Eneidyne Anticancer Antibiotics. *Angew. Chem., Int. Ed. Engl.* **30**, 1387–1530.
- (5) Nicolaou, K. C., Smith, A. L., and Yue, E. W. (1993) Chemistry and biology of natural and designed enediynes. *Proc. Natl. Acad. Sci. U.S.A.* **90**, 5881–5888.
- (6) Dervan, P. B. (1986) Design of Sequence-Specific DNA-Binding Molecules. *Science* **232**, 464–471.
- (7) Baker, B. F., and Dervan, P. B. (1989) Sequence-Specific Cleavage of DNA by *N*-Bromoacetyldistamycin. Product and Kinetic Analyses. *J. Am. Chem. Soc.* **111**, 2700–2712.
- (8) Tokuda, M., Fujiwara, K., Gomibuchi, T., Hiramata, M., Uesugi, M., and Sugiura, Y. (1993) Synthesis of a Hybrid Molecule Containing Neocarzinostatin Chromophore Analogue and Minor Groove Binder. *Tetrahedron Lett.* **34**, 669–672.
- (9) Boger, D. L., and Zhou, J. (1993) CDPI₃-Enediynes and CDPI₃-EDTA Conjugates: A New Class of DNA Cleaving Agents. *J. Org. Chem.* **58**, 3018–3024.
- (10) Bregant, T. M., Groppe, J., and Little, R. D. (1994) New Class of DNA-Cleaving Agents Based on Trimethylene-methane. *J. Am. Chem. Soc.* **116**, 3635–3636.
- (11) Semmelhack, M. F., and Gallagher, J. J. (1994) The Effect on DNA Cleavage Potency of Tethering a Simple Cyclic Enediynes to a Netropsin Analog. *J. Org. Chem.* **59**, 4357–4359.
- (12) Wittman, M. D., Kadow, J. F., Langley, D. R., Vyas, D. M., Rose, W. C., Solomon, W., and Zein, N. (1995) The Synthesis and Biological Activity of Enediynes Minor Groove Binding Hybrids. *Bioorg. Med. Chem.* **5**, 1049–1052.
- (13) Still, W. C., Kahn, M., and Mitra, A. (1978) Rapid Chromatographic Technique for Preparative Separations with Moderate Resolution. *J. Org. Chem.* **43**, 2923–2925.
- (14) Nishiwaki, E., Tanaka, S., Lee, H., and Shibuya, M. (1988) Efficient synthesis of oligo-*N*-methylpyrrolecarboxamides and related compounds. *Heterocycles* **27**, 1945–1952.
- (15) Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- (16) Maxam, A. M., and Gilbert, W. (1980) Sequencing End-Labeled DNA with Base-Specific Chemical Cleavages. *Methods Enzymol.* **65**, 499–560.
- (17) Iverson, B. L., and Dervan, P. B. (1987) Adenine specific DNA chemical sequencing reaction. *Nucleic Acids Res.* **15**, 7823–7830.
- (18) Hertzberg, R. P., and Dervan, P. B. (1984) Cleavage of DNA with Methidiumpropyl-EDTA-Iron(II): Reaction Conditions and Product Analyses. *Biochemistry* **23**, 3934–3945.
- (19) Brenowitz, M., Senear, D. F., Shea, M. A., and Ackers, G. K. (1986) Quantitative DNase Footprint Titration: A Method for Studying Protein-DNA Interactions. *Methods Enzymol.* **130**, 132–181.
- (20) Bevington, P. R. (1969) *Data Reduction and Error Analysis for the Physical Sciences*, pp 204–245, McGraw-Hill, New York.
- (21) Sonogashira, K., Tohda, Y., and Hagihara, N. (1975) A Convenient Synthesis of Acetylenes: Catalytic Substitutions of Acetylenic Hydrogen with Bromoalkenes, Iodoarenes, and Bromopyridines. *Tetrahedron Lett.* **50**, 4467–4470.
- (22) Ratovelomana, V., and Linstrumelle, G. (1981) New Synthesis of the Sex Pheromone of the Egyptian Cotton Leaf-worm, *Spodoptera littoralis*. *Synth. Commun.* **11**, 917–923.
- (23) Myers, A. G., Finney, N. S., and Kuo, E. Y. (1989) Allene Synthesis from 2-Alkyn-1-ols. *Tetrahedron Lett.* **30**, 5747–5750.
- (24) Myers, A. G., and Finney, N. S. (1990) Direct Observation and Retro-Ene Reaction of a Propargylic Diazene. Stereochemical Assignment of Monoalkyl Diazenes. *J. Am. Chem. Soc.* **112**, 9641–9643.
- (25) Oakley, M. G., Turnbull, K. D., and Dervan, P. B. (1994) Synthesis of a Hybrid Protein Containing the Iron-Binding Ligand of Bleomycin and the DNA-Binding Domain of Hin. *Bioconjugate Chem.* **5**, 242–247, and references therein.
- (26) Youngquist, R. S., and Dervan, P. B. (1985) Sequence-Specific Recognition of B DNA by Bis(EDTA-distamycin)-fumaramide. *J. Am. Chem. Soc.* **107**, 5528–5529.
- (27) Wade, W. S., and Dervan, P. B. (1987) Alteration of the Sequence Specificity of Distamycin on DNA by Replacement of an *N*-Methylpyrrolecarboxamide with Pyridine-2-carboxamide. *J. Am. Chem. Soc.* **109**, 1574–1575.
- (28) Schultz, P. G., and Dervan, P. B. (1984) Distamycin and Penta-*N*-Methylpyrrolecarboxamide Binding Sites on Native DNA. A Comparison of Methidiumpropyl-EDTA-Fe(II) Footprinting and DNA Affinity Cleaving. *J. Biomol. Struct. Dyn.* **1**, 1133–1147.
- (29) Wade, W. S., Mrksich, M., and Dervan, P. B. (1993) Binding Affinities of Synthetic Peptides, Pyridine-2-carboxamidonetropsin and 1-Methylimidazole-2-carboxamidonetropsin, That Form 2:1 Complexes in the Minor Groove of Double-Helical DNA. *Biochemistry* **32**, 11385–11389.
- (30) Myers, A. G., Fraley, M. E., Tom, N. J., Cohen, S. B., and Madar, D. J. (1995) Synthesis of (+)-dynemicin A and analogs of wide structural variability: establishment of the absolute configuration of natural dynemicin A. *Chem. Biol.* **2**, 33–43.
- (31) Myers, A. G., Cohen, S. B., and Kwon, B.-M. (1994) DNA Cleavage by Neocarzinostatin Chromophore. Establishing the Intermediacy of Chromophore-Derived Cumulene and Biradical Species and Their Role in Sequence-Specific Cleavage. *J. Am. Chem. Soc.* **116**, 1670–1682.
- (32) Myers, A. G., Cohen, S. B., and Kwon, B.-M. (1994) A Study of the Reaction of Calicheamicin γ_1 with Glutathione in the Presence of Double-Stranded DNA. *J. Am. Chem. Soc.* **116**, 1255–1271.
- (33) Youngquist, R. S., and Dervan, P. B. (1985) Sequence-specific recognition of B-DNA by oligo(*N*-methylpyrrolecarboxamide)s. *Proc. Natl. Acad. Sci. U.S.A.* **82**, 2565–2569.
- (34) Pelton, J. G., and Wemmer, D. E. (1989) Structural characterization of a 2:1 distamycin A·d(CGCAAATTTGGC) complex by two-dimensional NMR. *Proc. Natl. Acad. Sci. U.S.A.* **86**, 5723–5727.
- (35) Pelton, J. G., and Wemmer, D. E. (1990) Binding Modes of Distamycin A with d(CGCAAATTTGCG)₂ Determined by Two-Dimensional NMR. *J. Am. Chem. Soc.* **112**, 1393–1399.
- (36) Wade, W. S., Mrksich, M., and Dervan, P. B. (1992) Design of Peptides That Bind in the Minor Groove of DNA at 5'-(A,T)G(A,T)C(A,T)-3' Sequences by a Dimeric Side-by-Side Motif. *J. Am. Chem. Soc.* **114**, 8783–8794.
- (37) Mrksich, M., Wade, W. S., Dwyer, T. J., Geierstanger, B. H., Wemmer, D. E., and Dervan, P. B. (1992) Antiparallel side-by-side dimeric motif for sequence-specific recognition in the minor groove of DNA by the designed peptide 1-methylimidazole-2-carboxamide netropsin. *Proc. Natl. Acad. Sci. U.S.A.* **89**, 7586–7590.

Synthesis of 17 β -Hydroxyandrost-4-en-3-one-7 α -(Biotinyl-6-*N*-hexylamide), a Conjugate Useful for Affinity Chromatography and for Testosterone Immunoassays

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We describe the synthesis of 17 β -hydroxyandrost-4-en-3-one-7 α -(biotinyl-6-*N*-hexylamide) from 17 β -hydroxyandrost-4-en-3-one (testosterone) via copper-catalyzed 1,6 Michael addition of a 6-(*tert*-butyldimethylsilyloxyhexyl) chain to 6-dehydrotestosterone 17 β -acetate. After chromatographic separation of the 7 α -isomer from the α/β mixture and cleavage of the silyl ether, the alcohol was oxidized to the 6-hexanal side chain and then subjected to reductive amination. The resulting primary amine is easily biotinylated using biotinyl-*N*-hydroxysuccinimide ester. The overall yield for the epimeric 7 α -end product was 30%. The absolute configurations of the epimers were investigated by ¹H NMR studies by the nuclear Overhauser effect. We introduced a biotin label to the testosterone molecule at ring position 7 in compliance with Landsteiner's principle, which states that antibody specificity is directed primarily at that portion of the hapten furthest from the functional group linking it to the carrier protein. Thus, this negligible alteration in comparison to the structure of the respective testosterone hapten used to elicit antibodies offers the feasibility of applying the testosterone derivative as an optimal immunoabsorbent in affinity chromatography. The 7 α -biotinylated testosterone was used to obtain active antitestosterone antibodies from a specific antiserum by affinity chromatography. This was achieved by attaching the biotinylated testosterone to agarose-coupled streptavidin beads. Accordingly, a ³H-testosterone-binding test demonstrated a 20-fold increase in affinity of the purified antibody to the steroid compared to the original antiserum, and a recovery of >80% could be obtained. The antitestosterone antibody, obtained by that method, is an effective component for use in a competitive immunoassay for testosterone in human sera. An assay configuration is conceivable with the same 7 α -biotinylated testosterone employed as tracer in combination with a streptavidin-linked reporter enzyme.

INTRODUCTION

Isotope- or enzyme-labeled steroids can be used as immunochemical probes for a number of different applications. These include immunoassays for the determination of steroids in serum or steroid receptors in the tumor cell cytosol. When steroid probes labeled with prosthetic groups such as biotin are used, the isolation of steroid receptors or antisteroid antibodies via affinity chromatography is also possible.

In the case of the determination of steroids in human serum, analytical sensitivity and specificity of such competitive enzyme immunoassays may be severely limited when steroids have been derivatized at ring positions 3 or 17 (1–6), leading to altered epitopes of the respective tracers. The easily derivatized functional groups at position 3 and 17 are the groups that define the identity of testosterone. Therefore, we have developed a new assay concept that is based on ring core-biotinylated steroid tracers to preserve the epitope structure of the hapten. This leads one to expect high specificity due to a greater conformational similarity to the native steroid. As previously shown for the estrogen estrone (E1) (7), we were able to introduce a biotin

residue as the label in ring B position 6, according to Landsteiner's principle (8) that antibody specificity is directed primarily at that portion of the hapten furthest from the group linking it to the carrier protein. We applied an anti-E1 antibody raised against the hapten E1 conjugated to a carrier protein via its benzylic 6 position using the (*O*-carboxymethyl)oximation method to reach a nearly identical recognition site between the immunogen and the tracer. The biotin residue is attached via a defined spacer group to the steroid to minimize steric hindrances between the biotin moiety and the antiestrogen antibody. Additionally, the chemical structure of the spacer has a relevant influence on the process of antibody recognition: the structure must be similar to the linkage used in the immunization procedure. A competitive, nonradioactive immunoassay for E1 was set up with this new kind of steroid tracer (9), taking advantage of the high biotin affinity to a streptavidin reporter enzyme conjugate.

We have now developed an analogous immunoassay for the determination of 17 β -hydroxyandrost-4-en-3-one (testosterone) by synthesizing the biotinylated tracer 17 β -hydroxyandrost-4-en-3-one-7 α -(biotinyl-6-*N*-hexylamide) (7 α -Bio-T). For this assay it was necessary to isolate the antitestosterone antibodies from the specific antiserum by affinity chromatography. As previously mentioned, the 7 α -biotinylated testosterone derivative with its extended spacer group readily lends itself to this adaptation as an immunoabsorbent for affinity chromatography. Streptavidin-coupled agarose supports are

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stable and are inert matrices with defined porosity and protein content whereby streptavidin binds the biotin moiety of the steroid tracer with the well-known high affinity constant.

Affinity chromatography, based on the principle of specific protein–ligand recognition, is an efficient method for the refinement of proteins and has been successfully used for the purification of antibodies. To acquire pure, active antibodies, immunoabsorbents are widely used and numerous protocols especially for the purification of antisteroid antibodies are found in the literature (10, 11). The use of an appropriate spacer is known to be crucial for successful interaction between ligand and the complementary protein (12), especially in the case of small ligands such as steroids. Immunoabsorbed steroids can also be used for the purification of steroid receptors (13, 14) and hormone-binding globulins, such as sex hormone-binding globulin (15, 16). A number of practical difficulties are involved in the use of steroids as immunoabsorbents, including unintentional modifications of the steroid structure, leading to an ineffective or unselective adsorbance for antibodies or hormone-binding globulins (15, 16), and inappropriate chemical nature and length of the spacer group coupling the steroid to the insoluble support, leading to unstable ligand substitution (13, 14).

Therefore, our aim was to develop an efficient synthesis of 17 β -hydroxyandrost-4-en-3-one–7 α -(biotinyl-6-*N*-hexylamide) as an immunochemical tracer and to establish an affinity chromatography with this steroidal derivative for the extraction of a polyclonal antitestosterone antibody from a specific antiserum.

EXPERIMENTAL PROCEDURES

General Methods. *Apparatus.* Melting points (uncorrected) were determined on a Reichert thermovar. ^1H NMR spectra were recorded in CDCl_3 on a Bruker ARX 400 (400 MHz), using Me_4Si for the reference signal (δ 0.00 ppm). The data are reported in the following form: chemical shift (multiplicity, coupling constant in Hz if available, number of protons, position of proton). IR spectra were obtained, using KBr pellets or a neat solution, on a Beckman 24 spectrophotometer, and only characteristic IR bands are reported (in cm^{-1}). UV spectra were measured in MeOH (CH_3CN for **4a**) on a Hitachi spectrophotometer U 2000. Optical rotation values were gained from a Perkin-Elmer 241 polarimeter, where *c* is given in $\text{g}/100\text{ cm}^{-3}$. MS analyses were performed on a Finigan MAT 90 (FAB) and on a Varian MAT 112S (EI).

The preparative HPLC system consisted of an HPLC pump Knauer 64 (Knauer, Bad Homburg, Germany) and an UV detector ERC 7210 (ERC, Alteglofsheim, Germany). Preparative HPLC columns were from Bischoff Analysentechnik, Leonberg, Germany (Kromasil) and Knauer (LiChrospher). For β -counting we used a 1219 Rackbeta liquid scintillation counter from LKB Wallac (Bromma, Sweden). The chromatography columns Poly-Prep (8 mm diameter, 40 mm length) were from Bio-Rad Laboratories (Hercules, CA) and the Centricon-30 centrifugal concentrators from Amicon (Witten, Germany).

Chemicals. For column chromatography silica gel (grain 0.063–0.200) was used and TLC was performed using silica gel 60 F₂₅₄ (0.2 mm), both materials from E. Merck (Darmstadt, Germany). Testosterone, bovine serum albumin (BSA), charcoal, dextran (MW 500 000), and tris(hydroxymethyl)aminomethane (Tris) were from Sigma (Deisenhofen, Germany); 6-bromohexanol, tetrabutylammonium fluoride, *tert*-butyldimethylchlorosilane, and (+)-biotinyl-*N*-hydroxysuccinimide ester were all from Fluka (Buchs, Switzerland). [1,2,6,7- ^3H]tes-

tosterone (3.7 TBq/mmol) was from Amersham-Buchler, Braunschweig, Germany. The *polyclonal rabbit antitestosterone antibody* (raised against testosterone-7-BSA) was obtained from Accurate Chemical & Scientific Corp. (Westbury, NY), and the microtiter plates, coated with *donkey antirabbit antibody*, were from Johnson & Johnson Clinical Diagnostics (Neckargemünd, Germany). The agarose-coupled streptavidin, 50% in phosphate-buffered saline (ImmunoPure immobilized streptavidin), was obtained from Pierce Europe (Oud-Beijerland, Netherlands). Opti-Fluor LSC as scintillation cocktail was from Packard Instrument Company (Meriden, CT). All other laboratory chemicals were from Merck.

Reagent Solutions. For the affinity chromatography we used a neutral (0.01 M Tris, pH 7.5), an acidic (0.1 M glycine, 0.1 M NaCl, adjusted to pH 2.5 with 0.1 N HCl), and a basic (0.1 M glycine, 0.1 M NaCl, adjusted to pH 11.5 with 0.1 N NaOH) buffer. For the ^3H -testosterone-binding test the working buffer consisted of 0.1 M Tris and 0.15 mM (10 g/L) BSA, pH 7.5. As a washing buffer, we used the original borate buffer (pH 8.4) provided with the Amerlite immunoassay system (Johnson & Johnson Clinical Diagnostics).

Preparation of 17 β -Hydroxyandrost-4-en-3-one–7 α -(Biotinyl-6-*N*-hexylamide) (7 α -Bio-T). 17 β -Hydroxyandrost-4,6-dien-3-one 17 β -Acetate (6-Dehydrotestosterone-17 β -acetate, **1**). **1** was prepared from testosterone according to Bucourt et al. (14) with a yield of 66%. Melting point ($^{\circ}\text{C}$), 140–142 (lit. (17) mp ($^{\circ}\text{C}$), 143–144).

6-(*tert*-Butyldimethylsilyloxyhexyl) Bromide, first reported by Kim et al. (18), was prepared from 6-bromohexanol and *tert*-butyldimethylchlorosilane according to the method of Bowler et al. (19) with a yield of 76% of a colorless oil, d_{20} 1.07. MS (70 eV EI, m/z): 296 (MH^+); IR (neat) (cm^{-1}): 1390, 1360 (*tert*-butyl), 1250 (C–O), 1100, 835, 765 (Si–R).

17 β -Hydroxyandrost-4-en-3-one 17 β -Acetate–7 $\alpha\beta$ -(6-*tert*-Butyldimethylsilyloxyhexane) (**2a/b**). According to Bowler et al. (19), a Grignard reagent was prepared by dripping a solution of 4.41 mL (16 mmol, 4 equiv) of 6-(*tert*-butyldimethylsilyloxyhexyl) bromide in 5 mL of tetrahydrofuran (THF) to 340 mg (14 mmol; 3.5 equiv) of Mg in 3 mL of THF under N_2 . The resulting clear solution was diluted with an additional 25 mL of THF and cooled to -40 to $-30\text{ }^{\circ}\text{C}$. A sample of 1.523 g (8 mmol, 2 equiv) of CuI was added after 30 min, and 15 min later a sample of 1.316 g (4 mmol) of **1** in 25 mL of THF was dripped into the solution within 50 min at -48 to $-35\text{ }^{\circ}\text{C}$. After the solution was stirred for an additional 40 min, the reaction was quenched with 1.5 mL of glacial acetic acid at $-30\text{ }^{\circ}\text{C}$ and then subsequently basified with saturated aqueous NaHCO_3 . Then the solvent was removed and the residue treated with methyl *tert*-butyl ether (MTBE) and aqueous NaHCO_3 . The crude extract was chromatographed with ethyl acetate in CH_2Cl_2 (1:19, v/v) on silica gel, and 1.138 g of **2a** (R_f 0.27) and 0.690 g of **2b** (R_f 0.16) were obtained. Yields: 52% **2a**, 32% **2b**, epimeric excess of 24%. A further purification was achieved by isocratic HPLC. Column: Kromasil SC 100 7 μm , 250 mm, 20 mm diameter with a guard-cartridge, 50 mm, 20 mm diameter, containing the same chromatographic material. Eluent: MeOH/ CH_2Cl_2 (1:99, v/v); flow rate: 9.75 mL/min. For **2a** the retention time was 10.6 min, and for **2b** the retention time was 12.4 min. The following data are for **2a**. MS (70 eV EI, m/z): 543 (0.01%, $\text{M}-\text{H}^+$), 487 (100%, $\text{M}^+-\text{C}_4\text{H}_9$). IR (neat) (cm^{-1}): 1740 (17-OAc), 1675 (3-one), 1620 (olefin), 1100, 835, 775 (Si–R). ^1H NMR: 0.50 (s, 6H, Si(CH_3)₂), 0.84 (s, 3H, 18- CH_3), 0.90 (s, 9H, Si–*tert*-butyl), 1.20 (s, 3H, 19- CH_3), 2.32 (dd, $J_{6\alpha\beta} = 14.2$, $J_{6\alpha\gamma\beta} = 2.5$, 1H, 6 α -

H), 2.40 (dd, $J_{6\alpha 6\beta} = 14.2$, $J_{6\beta 7\beta} = 4.9$, 1H, 6 β -H), 3.59 (t, $J = 6.6$, 2H, R-CH₂-OSi), 5.72 (s, 1H, 4-CH). UV: 240 nm, $\log \epsilon = 4.19$, $[\alpha]_D^{20} = +43^\circ$ (MeOH, $c = 1.32$). Anal. Calcd: C, 72.74; H, 10.36. Found: C, 72.33; H, 10.09. The following data are for **2b**. MS (70 eV EI, m/z): 543 (0.1%, M-H⁺), 487 (100%, M⁺-C₄H₉). IR (KBr) (cm⁻¹): 1740 (17-OAc), 1670 (3-one), 1610 (olefin), 1090, 830, 770 (Si-R). ¹H NMR: 0.49 (s, 6H, Si(CH₃)₂), 0.85 (s, 3H, 18-CH₃), 0.90 (s, 9H, Si-*tert*-butyl), 1.16 (s, 3H, 19-CH₃), 2.18 (ddd, $J_{6\alpha 6\beta} = 14.5$, $J_{6\beta 7\alpha} = 12.2$, $J_{4-CH 6\beta} = 1.9$, 1H, 6 β -H), 2.29 (dd, $J_{6\alpha 6\beta} = 14.5$, $J_{6\alpha 7\alpha} = 4.8$, 1H, 6 α -H), 3.60 (t, $J = 6.6$, 2H, R-CH₂-OSi), 5.71 (s, 1H, 4-CH). UV: 241 nm, $\log \epsilon = 4.12$, $[\alpha]_D^{20} = +29^\circ$ (MeOH, $c = 2.00$). Anal. Calcd: C, 72.74; H, 10.36. Found: C, 72.31; H, 10.62. **2b** was crystallized from MeOH (mp (°C) 63–64), whereas **2a** remained as an oily residue.

17 β -Hydroxyandrost-4-en-3-one 17 β -Acetate-7 α -(6-Hexanol) (3a). To 606 mg (1.11 mmol) of **2a** in 3 mL of THF was added 2.22 mL of 1 M tetrabutylammonium fluoride in THF. After the mixture was stirred for 90 min at room temperature under N₂, 5 mL of a saturated aqueous NaCl solution was added. The reaction mixture was extracted with 2 $\times$ 100 mL MTBE and chromatographed on silica gel with MTBE ($R_f = 0.3$). Yield: 446 mg (1.04 mmol, 94%). A further purification was achieved by isocratic HPLC. Column: Kromasil SC 100 7 μ m, 250 mm, 20 mm diameter, no guard-cartridge. Eluent: MeOH/CH₂Cl₂ (1:19, v/v); flow rate: 16.0 mL/min. For **3a** the retention time was 5.3 min. MS (70 eV EI, m/z): 430 (23%, M⁺), 329 (100%, M⁺-C₆H₁₂OH). HR-MS: calcd, 430.308 31; found 430.308 67. IR (KBr) (cm⁻¹): 3400 (OH). ¹H NMR: 2.32 (dd, $J_{6\alpha 6\beta} = 14.3$, $J_{6\alpha 7\beta} = 2.6$, 1H, 6 α -H), 2.40 (dd, $J_{6\alpha 6\beta} = 14.3$, $J_{6\beta 7\beta} = 4.9$, 1H, 6 β -H), 3.63 (t, $J = 6.6$, 2H, R-CH₂-OH). UV: 235 nm, $\log \epsilon = 3.55$, $[\alpha]_D^{20} = +26^\circ$ (MeOH, $c = 3.24$).

17 β -Hydroxyandrost-4-en-3-one 17 β -Acetate-7 α -(6-Hexanol) (4a). According to a prescription of Piancatelli et al. (20), 80 mg (0.186 mmol) of **3a** and 80 mg (0.372 mmol) of pyridinium chlorochromate were stirred for 65 min in 10 mL of CH₂Cl₂ at room temperature. The brown sludge was chromatographed with MTBE on silica gel ($R_f = 0.43$). A purification using HPLC was performed. Column: LiChrospher 5 μ m, 240 mm, 16 mm diameter without guard-cartridge. Eluent: MeOH/CH₂Cl₂ (1:19, v/v); flow rate: 14.5 mL/min. For **4a** the retention time was 6.7 min, and 71 mg (0.166 mmol, 92%) of the aldehyde **4a** resulted. MS (70 eV EI, m/z): 428 (3%, M⁺), 329 (100%, M⁺-C₅H₁₀CHO). HR-MS: calcd, 428.292 64; found, 428.2955. IR (KBr) (cm⁻¹): 2920 (CHO). ¹H NMR: 2.31 (dd, $J_{6\alpha 6\beta} = 14.2$, $J_{6\alpha 7\beta} = 2.3$, 1H, 6 α -H), 2.40 (dd, $J_{6\alpha 6\beta} = 14.2$, $J_{6\beta 7\beta} = 4.8$, 1H, 6 β -H), 2.43 (td, $J = 1.8$ and 7.3, 2H, -CH₂CHO), 5.72 (s, 1H, 4-CH), 9.77 (t, $J = 1.8$, 1H, -CHO). UV: 236 nm, $\log \epsilon = 3.94$, $[\alpha]_D^{20} = +39^\circ$ (CH₃CN, $c = 0.29$).

17 β -Hydroxyandrost-4-en-3-one 17 β -Acetate-7 α -(6-Hexylamine) (5a). As described previously by Luppa et al. (7), 2 mL of a buffer solution, prepared by dissolving 4.05 g of NH₄OAc in 15 mL of dry MeOH, was added to 325 mg (0.760 mmol) of **4a** and 287 mg (4.560 mmol) of NaCNBH₃ under N₂. The reaction was worked up after 2 h by removing the solvent and extracting the residue with aqueous NaHCO₃ and Et₂O. A sample of 300 mg of the crude amine **5a** was obtained by this procedure. MS (70 eV EI, m/z): 430 (14%, M-H⁺). IR (KBr) (cm⁻¹): 3440 (-NH₂).

17 β -Hydroxyandrost-4-en-3-one-7 α -(6-Hexylamine) (6a). A sample of 36 mg (approximately 0.1 mmol) of the amine **5a** was processed without further purification and stirred for 12 h in a solution made from 3 mL of MeOH, 3 mL of H₂O, and 200 mg of solid NaOH. The solution was

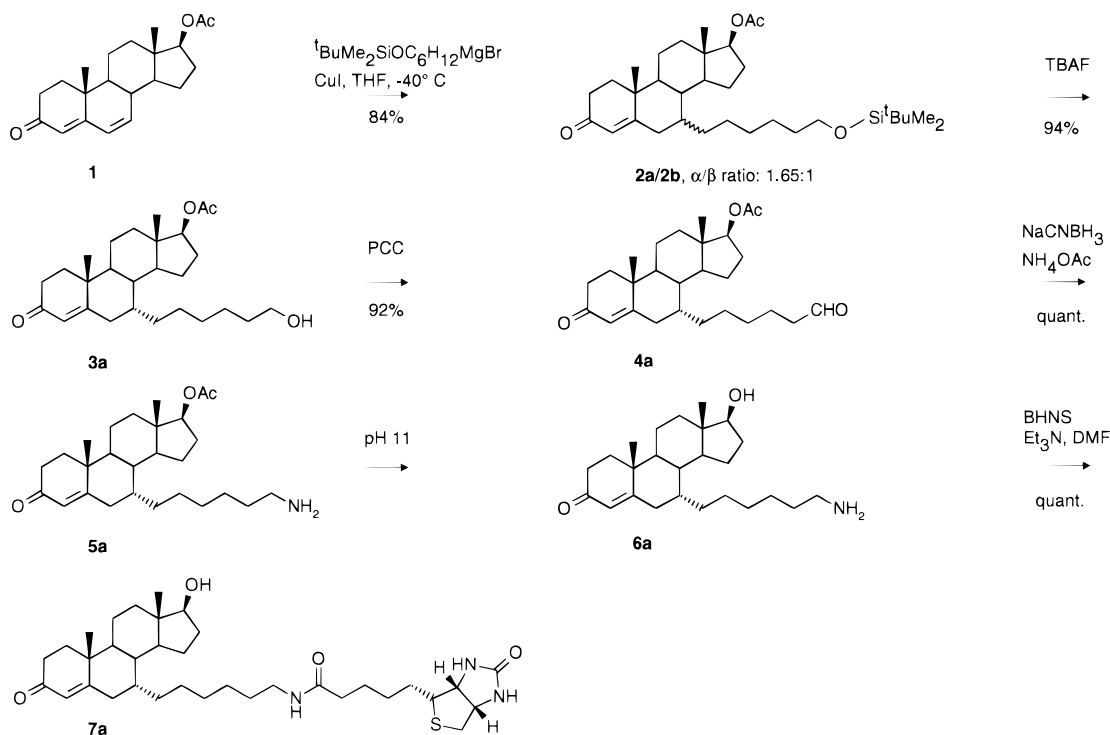
extracted with EtOAc and H₂O to give 29 mg of the deprotected steroid **6a**. IR (KBr) (cm⁻¹): 3400 (-NH₂, -OH).

17 β -Hydroxyandrost-4-en-3-one-7 α -(Biotinyl-6-N-hexylamide) (7a). Twenty-eight mg (0.072 mmol) of **6a**, 25 mg of (+)-biotinyl-N-hydroxysuccinimide ester (0.073 mmol), and 19 μ L Et₃N were stirred for 18 h in 3 mL of DMF under N₂ at room temperature. The solvent was removed and the residue chromatographed with MeOH in CH₂Cl₂ (1:4.88, v/v) to gain 44 mg of the anticipated crude 7 α -biotinylated testosterone **7a** (yield 99%). The overall yield of the epimeric pure product was 30% relative to the dienone **1**. A further purification was performed by isocratic HPLC. Column: Kromasil SC 100 7 μ m, 250 mm, 20 mm diameter, no guard-cartridge. Eluent: MeOH/CH₂Cl₂ (1:6.14, v/v); flow rate: 10.5 mL/min. For **7a** the retention time was 11.8 min. MS (70 eV EI, m/z): 614 (MH⁺). MS (FAB, PI-LISIMS, glycerol/MeOH): calcd for C₃₅H₅₆N₃O₄S (MH⁺), 614.399 15; found, 614.396 61; difference, 4.1 ppm. ¹H NMR: 0.80 (s, 3H, 18-CH₃), 1.21 (s, 3H, 19-CH₃), 2.32 (dd, $J_{6\alpha 6\beta} = 14.3$, $J_{6\alpha 7\beta} = 2.3$, 1H, 6 α -H), 2.42 (dd, $J_{6\alpha 6\beta} = 14.3$, $J_{6\beta 7\beta} = 4.9$, 1H, 6 β -H), 3.66 (t, 1H, 17-CH), 5.72 (s, 1H, 4-CH). UV: 235 nm, $\log \epsilon = 4.03$, $[\alpha]_D^{20} = +44^\circ$ (MeOH, $c = 2.30$). Anal. Calcd: C, 68.52; H, 8.97; N, 6.85. Found: C, 68.89; H, 9.81; N, 5.79.

Affinity Chromatography. Column Preparation. The suspension of streptavidin-coated agarose beads was incubated with a 16-fold molar excess of 7 α -Bio-T in neutral Tris buffer overnight at room temperature to ensure complete binding of the biotinylated immunoadsorbent to streptavidin. Thereafter, the Poly-Prep columns were packed with this suspension and washed according to the following protocol: 20 bed volumes of neutral buffer, 10 volumes of acidic buffer, 10 volumes of neutral buffer, 10 volumes of basic buffer, and neutral buffer until pH 7.5 was reached.

Chromatography. The polyclonal rabbit antitestosterone antiserum was diluted 1:3 (v/v) with neutral buffer and passed slowly (flow rate 0.4 mL/min) through the column to bind the antibody. The specific antitestosterone antibody was then eluted according to the following protocol: 20 bed volumes of neutral buffer, 10 volumes of acidic buffer, 10 volumes of neutral buffer, 10 volumes of basic buffer, and 20 volumes of neutral buffer. The acidic eluates were collected in tubes containing Tris buffer (1 M, pH 10) and the basic eluates in tubes containing 100 μ L Tris buffer (1 M, pH 6). After the antibody content was tested for in the ³H-testosterone-binding test, the antibody-containing fractions were pooled and concentrated by use of centrifugal concentrators. The antibody solution is then stored at -70 °C.

³H-Testosterone-Binding Test. This test was used to check the affinity of the purified rabbit antibody against testosterone after binding to a donkey antirabbit antibody immobilized to a microtiter plate. In the first step, 10 μ L of the antibody stock solution was pipetted into the coated microtiter wells together with 190 μ L of working buffer. After 60 min of incubation at 37 °C in a shaker incubator, the wells were washed four times with washing buffer. In the second step, 50 μ L of ³H-testosterone in working buffer (3.5 nmol/L) was pipetted into the wells together with 150 μ L of working buffer. Again, a 60 min incubation at 37 °C was performed, and the wells were washed four times with washing buffer. In the third step, 200 μ L Triton X-100 (1%, v/v in water) was filled into the wells and a further incubation for 20 min at 37 °C is performed. The percent binding (%B) of ³H-testosterone was measured by counting the Triton solution and the

Scheme 1. Reaction Scheme for the Synthesis of 17 β -Hydroxyandrost-4-en-3-one-7 α -(Biotinyl-6-*N*-hexylamide) (7 α -Bio-T) from 17 β -Hydroxyandrost-4-en-3-one (Testosterone)^a

^a Abbreviations: BNHS, (+)-biotinyl-*N*-hydroxysuccinimide ester; ^tBuMe₂Si, *tert*-butyldimethylsilyl-; PCC, pyridinium chlorochromate

test well itself in a scintillation counter after vigorous suspension in 5 mL of scintillation cocktail.

The binding test can also be used as a radioimmunoassay method for estimating the recovery of purified antibodies. In brief, the percentage binding of tritiated testosterone by various antiserum dilutions gave a calibration curve of % antibody content vs % ³H-testosterone binding. By measurement of the % ³H-testosterone binding of affinity column eluates of the antiserum, the yield of specific antibodies bound to the column could be assessed.

To check the concentration of 7 α -Bio-T in eluates from chromatographic runs, we used the radioimmunoassay technique with tritiated testosterone as the tracer and 7 α -Bio-T as the analyte. The test protocol was as follows. 7 α -Bio-T containing eluates, the specific antiserum (working dilution of 1:100), and ³H-testosterone (working concentration of 3.5 nmol/L, both in working buffer) were incubated for 16 h at 4 °C. Subsequently, separation of bound from free tritiated testosterone was achieved by addition of a dextran-coated charcoal suspension and, after centrifugation, the radioactivity in the supernate with the scintillation counter was counted.

RESULTS

Chemical Synthesis. This paper describes the synthesis of the immunochemical tracer 17 β -hydroxyandrost-4-en-3-one-7 α -(biotinyl-6-*N*-hexylamide) (7 α -Bio-T, **7a**) by attaching a biotin residue via a hexyl spacer arm at the C-7 position of testosterone. The six-step synthesis of **7a** from dihydrotestosterone **1** is given in Scheme 1. All reaction steps reported here for the α -compounds can be applied to the product line **3b**–**7b** with similar results (data not shown). The overall yield for the **7a** end product is 30%.

The absolute configurations of the separated products 17 β -hydroxyandrost-4,6-dien-3-one 17 β -acetate-7 α/β -(6-*tert*-butyldimethylsiloxyhexyl) **2a/2b** were determined by

¹H NMR measurements and nuclear Overhauser effect (NOE) experiments. These experiments established the chemical shifts and the coupling pattern of the AB part of the ABX system of the 6 α -H, 6 β -H, and 7-H. The olefinic proton's signal was saturated at 5.71 ppm, giving rise to positive NOE signals for the 6 α -proton (**2a**: 2.32 ppm, dd, ²*J*_{6 α 6 β} = 14.2 Hz, and ³*J*_{6 α 7 α} = 2.5 Hz. **2b**: 2.29 ppm, dd, ²*J*_{6 α 6 β} = 14.5 Hz, ³*J*_{6 α 7 α} = 4.8 Hz). In the next step we exposed the 6 β -proton's signal by irradiating the 19-CH₃ signal at 1.2 ppm (**2a**: 2.40 ppm, dd, ²*J*_{6 α 6 β} = 14.2 Hz, and ³*J*_{6 β 7 β} = 4.9 Hz. **2b**: 2.18 ppm, dd, ²*J*_{6 α 6 β} = 14.5 Hz, and ³*J*_{6 β 7 α} = 12.2 Hz). This assignment is substantiated by an additional coupling between the 6 β and the allylic 4-CH that is observed for **2b** (⁴*J*_{4-CH6 β} = 1.9 Hz). The nature of this interaction could also be confirmed by an additional coupling experiment: when **2b** was irradiated at 5.71 ppm, the 1.9 Hz coupling constant in the 6 β -signal disappeared (21). The large ³*J*_{axial-axial} coupling constant of 12.2 Hz of **2b** led us to the conclusion that the 7-C of **2b** is β -substituted, and therefore, **2a** must be α -substituted.

Affinity Chromatography. The affinity purification of a polyclonal antitestosterone antibody from a specific antiserum by use of the biotinylated testosterone derivative 7 α -Bio-T was performed according to the protocol described in the Experimental Procedures section.

In Figure 1 the pH-dependent elution profile is depicted, where the line of %B in the ³H-testosterone-binding test represents the elution of the antibody in fraction numbers 30–45. Regarding equivalent dilutions, the purified and concentrated specific antibody had a 20-fold increase in affinity (corresponding to the percentage binding of ³H-testosterone) to the steroid compared to the original antiserum. The recovery of purified antibody, estimated by a ³H-testosterone radioimmunoassay technique described in the Experimental Procedures section, was found to be >80%.

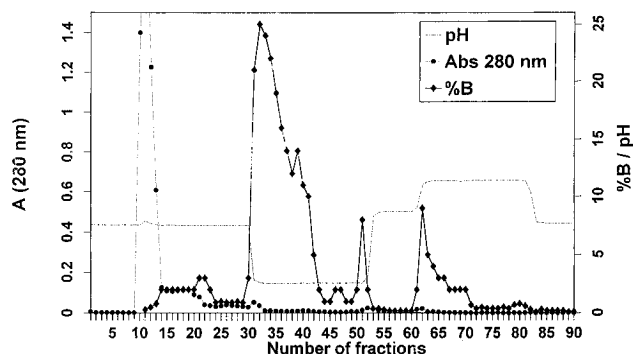


Figure 1. Chromatographic elution patterns of the affinity purification of polyclonal antitestosterone antibodies from a specific antiserum by use of the biotinylated testosterone derivative 7 α -Bio-T: continuous line, percentage of binding (%B) in the ^{3} H-testosterone-binding test; dotted line, UV absorption at 280 nm; dashed line, pH.

A presumed release of the gel-bound steroid adsorbent could be ruled out either by washing the gel slurry after specific adsorption of 7 α -Bio-T with elution and acidic buffer or by deproteinization of the antibody eluates with perchloric acid. A quantity of 0.4% of the total adsorbed 7 α -Bio-T could be determined in these combined eluates by the ^{3} H-testosterone radioimmunoassay technique. This was equivalent to approximately 60 nmol/L (detection limit of this analytical technique for 7 α -Bio-T is 5 nmol/L).

DISCUSSION

By use of biotinylated steroids as stable immunochemical probes in connection with streptavidin-conjugated reporter enzymes, it is possible to develop a series of competitive immunoassays for the determination of individual steroid concentrations in biological fluids (9). In the process of establishing such an assay for total testosterone, we synthesized a 7 α -biotinylated testosterone derivative (7 α -Bio-T). This immunochemical probe is used both as a biospecific ligand for affinity purification of an antitestosterone antibody from a specific antiserum and as a specific tracer molecule in a competitive immunoassay. Data concerning performance characteristics of this immunoassay will be given elsewhere. The ability of a labeled steroid molecule to fulfill these two functions in a specific manner is largely dependent upon the attachment point of the label to the steroid ring, as well as upon the alteration of the key structural determinants of the steroid and the chemical nature and orientation of the spacer linkage. These crucial points must be considered when inserting a prosthetic group into a steroidal structure, prior to the subsequent addition of enzyme reporter groups or linkers to insoluble chromatography matrices. A minimized alteration of the labeled steroid leads to an improved specificity of the respective immunoassay and to a superior ligand quality, whereas optimal structure design and orientation of the bridge are associated with exposed ligand-binding sites fitting into the binding pocket of the antibody, improving the assay's sensitivity and the efficiency of the affinity chromatography.

The purpose for the synthesis of a new testosterone tracer was therefore to insert a biotin moiety via a defined spacer group into position C-7 of the B-ring, which is furthest from the critical positions 3 and 17. Additionally, the axial α -position of the 7 α -Bio-T promises an optimal orientation of a nearly unchanged steroid structure in the solid-phase agarose of the chromatographic matrix.

Synthesis. The approach of the six-step synthesis of 17 β -hydroxyandrost-4-en-3-one-7 α -(biotinyl-6-*N*-hexylamide) is based on the work of Bowler et al. (19), who alkylated 6-dehydro-19-nortestosterone by the use of copper(I)-catalyzed 1,6 Michael addition of prefunctionalized Grignard compounds at the C-7 position. Similar 1,6 additions were carried out by French et al. (22). We adapted and optimized the reaction procedure for the addition of a 6-(*tert*-butyldimethylsilyloxyhexyl) C₆-chain to dehydrotestosterone **1** to achieve yields of 52% **2a** and 32% **2b**. We found that only the silylated products **2a** and **2b** are separable by simple liquid chromatography. It is essential that separation of the α/β epimers is carried out at this stage, since the separation of the α/β mixtures of products **3–7** is not practicable. From these advantageous precursors we proceeded to get convenient high yield steps for the transformation of the alcohol **3a** to the amine **6a**, which is easily biotinylated. This is achieved by the oxidation of **3a** to the corresponding aldehyde **4a**, which is then converted to the amine **5a** by reductive amination followed by the cleavage of the 17 β -acetoxy protective group.

The convergent synthetic pathway offers good accessibility and gives a stereochemical pure product with an overall yield of 30%. The proposed 1,6 Michael addition of prefunctionalized Grignard compounds at the C-7 position offers the possibility of inserting CH₂ groups in various lengths to dehydrotestosterone. This is advantageous for modeling different immunochemical tracers.

The absolute configurations of the 7 α/β derivatives were confirmed by ^{1} H NMR spectroscopy including NOE experiments for both silyl ethers **2a** and **2b**. Now that the absolute configuration of the β -silyl ether has been established, it has become easier to define structural details of the α -epimeric end product 7 α -Bio-T and to demonstrate the postulated negligible alteration of the tracer's structure in comparison to underivatized testosterone.

Affinity Chromatography. The effectiveness of the method is determined by the following features: *First*, the biotinylated steroid bound to agarose-linked streptavidin was not released from the gel because of the exceptionally strong noncovalent binding of biotin to streptavidin. This is in contrast to related affinity chromatography adsorbents, which were developed for the purification of steroid receptors (13, 14). These reports emphasized difficulties of washing adsorbents sufficiently well to remove free, unbound steroid hormone. *Second*, the structure of the biotinylated testosterone derivative as an adsorbent for affinity chromatography is suitable because of the substitution at the 7 α -position together with the utilization of a long spacer chain. Bucourt et al. (14) found that estradiol derivatives as biospecific adsorbents for the estradiol receptor are best when substituted at the 7 α -position with a 14 atom long spacer chain. According to these characteristics, their most biospecific adsorbent had low impairment of affinity compared to estradiol.

In summary, the affinity chromatography of a polyclonal antitestosterone antibody by use of a 7 α -biotinylated testosterone derivative offers a convenient, single step purification that results in a highly purified antibody. Thus, the procedure described in this report has overcome some difficulties in the purification of antibodies, which are related to the binding of the appropriate biospecific ligand to the insoluble matrix by the application of the streptavidin-bound biotinylated ligand.

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LITERATURE CITED

- (1) Folan, J., Gosling, J. P., and Fottrell, P. F. (1988) Solid-phase enzymeimmunoassay of estrone in serum. *Clin. Chem.* 34, 1843–1846.
- (2) Folan, J., Gosling, J. P., Finn, M. F., and Fottrell, P. F. (1989) Solid-phase enzymeimmunoassay of estrone in saliva. *Clin. Chem.* 35, 569–572.
- (3) Elder, P. A., Manley, L., and Lewis, J. G. (1990) Use of a monoclonal antibody to estrone-3-glucuronide in an enzyme-linked immunosorbent assay (ELISA). *J. Steroid Biochem.* 36, 439–443.
- (4) Barnard, G., Kohen, F., Mikola, H., and Lövgren, T. (1989) Measurement of estrone-3-glucuronide in urine by rapid, homogeneous time-resolved fluoroimmunoassay. *Clin. Chem.* 35, 555–559.
- (5) Kim, J. B., Barnard, G. J., Collins, W. P., Kohen, F., Lindner, H. R., and Eshhar, Z. (1982) Measurement of plasma estradiol-17 β by solid-phase chemiluminescence immunoassay. *Clin. Chem.* 28, 1120–1124.
- (6) De Boever, J., Kohen, F., Usanachitt, C., Vandekerckhove, D., Leyseele, D., and Vandewalle, L. (1986) Direct chemiluminescence immunoassay for estradiol in serum. *Clin. Chem.* 32, 1895–1900.
- (7) Lippa, P., Birkmayer, C., and Hauptmann, H. (1994) Synthesis of estra-1,3,5(10)-trien-3-ol-17-one and estra-1,3,5(10)-triene-3,17 β -diol 6 α -N-(ϵ -biotinyl)aminocaproamide, tracer substances for developing immunoassays for estrone and estradiol. *Bioconjugate Chem.* 5, 167–171.
- (8) Landsteiner, K. (1962) *The Specificity of Serological Reactions*, 240–266, Dover Publications, New York.
- (9) Lippa, P., Hauck, S., Schwab, I., Birkmayer, C., and Hauptmann, H. (1995) 6 α -biotinylated estrone, novel tracer in competitive chemiluminescence immunoassay for estrone in serum. *Clin. Chem.* 41, 564–570.
- (10) Lowe, C. R., and Dean, P. D. G. (1974) The Principles of Affinity Chromatography, in *Affinity Chromatography*, 12–89, Wiley and Sons, London.
- (11) Hermanson, G. T., Mallia, A. K., and Smith, P. K. (1992) Immobilization of Ligands, in *Immobilized Affinity Ligand Techniques*, 137–427, Academic Press, San Diego.
- (12) Cuatrecasas, P. (1970) Protein purification by affinity chromatography. *J. Biol. Chem.* 245, 3059–3065.
- (13) Sica, V., Parikh, I., Nola, E., Puca, G. A., and Cuatrecasas, P. (1993) Affinity chromatography and the purification of estrone receptors. *J. Biol. Chem.* 248, 6543–6558.
- (14) Bucourt, R., Wignau, M., Torelli, V., Richard-Foyh, H., Geynet, C., Secco-Millet, C., Redeuilh, G., and Baulieu, E. (1978) New biospecific adsorbants for the purification of estradiol receptor. *J. Biol. Chem.* 253, 8221–8228.
- (15) Mickelson, K. E., and Pétra, P. H. (1975) Purification of the sex steroid binding protein from human serum. *Biochemistry* 14, 957–963.
- (16) Rosner, W., and Smith, R. N. (1975) Isolation and characterization of the testosterone–estradiol-binding globulin from human plasma. Use of a novel affinity column. *Biochemistry* 14, 4813–4820.
- (17) Hill, R. A., Kirk, D. N., Makin, H. L. J., and Murphy, G. M. (1991) *Dictionary of Steroids, Chemical Data, Structures and Biographies*, Chapman and Hall, London.
- (18) Kim, S., and Kee, I. S. (1990) A novel method for direct conversion of tetrahydropyranyl ethers into *t*-butyldimethylsilyl ethers with *t*-butyldimethylsilyl triflate and dimethyl sulfide. *Tetrahedron Lett.* 31, 2899–2900.
- (19) Bowler, J., Lilley, T. J., Pittam, J. D., and Wakeling, A. E. (1989) Novel steroidal pure antiestrogens. *Steroids* 54, 71–99.
- (20) Piancatelli, G., Scettri, A., and D'Auria, M. (1982) Pyridinium chlorochromate: a versatile oxidant in organic synthesis. *Synthesis*, 245–258.
- (21) Kirk, D. N., Toms, H. C., Douglas, C., and White, K. A. (1990) A survey of the high-field ^1H NMR spectra of the steroid hormones, their hydroxylated derivatives and related compounds. *J. Chem. Soc., Perkin Trans. 2*, 1567–1594.
- (22) French, A. N., Wilson, S. R., Welch, M. J., and Katzenellenbogen, J. A. (1993) A synthesis of 7 α -substituted estradiols: synthesis and biological evaluation of a 7 α -pentyl-substituted BODIPY fluorescent conjugate and a fluorine-18-labeled 7 α -pentylestradiol analog. *Steroids* 58, 157–169.

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Presentation of Peptide Antigens as Albumin Conjugates for Use in Detection of Serum Antibodies by Enzyme-Linked Immunosorbent Assay

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The use of linear peptides as antigens for detection of serum antibodies has been studied using a sequence of the *Borrelia burgdorferi* protein, flagellin, and Lyme disease sera as a model. It was found that a novel presentation of the peptide as a hapten on the carrier protein, bovine serum albumin, in the enzyme-linked immunosorbent assay format can be successfully applied to distinguish between Lyme disease and control sera.

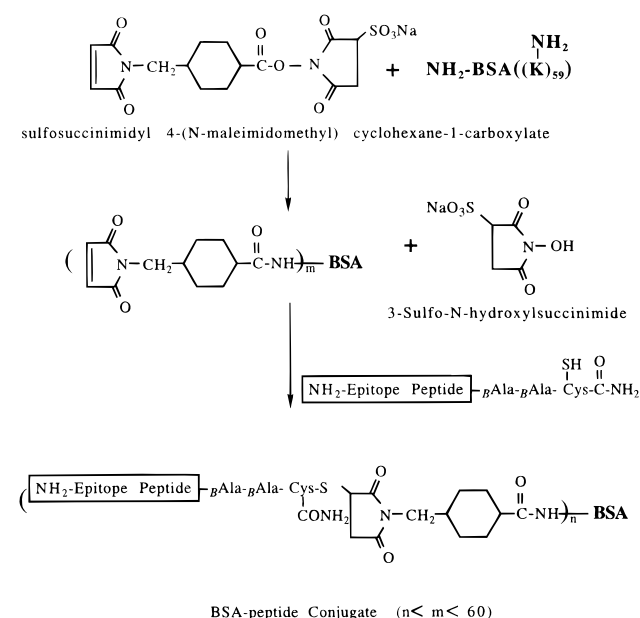
INTRODUCTION

Diagnosis of many infectious diseases depends on detection of serum antibodies against determinants of the pathogenic microorganism. Our interest is in the serologic confirmation of the diagnosis of Lyme disease, which is caused by infection with the spirochete, *Borrelia burgdorferi* (*Bb*), transmitted by the deer tick (1). The most common Lyme disease diagnostic test is the enzyme-linked immunosorbent assay (ELISA) (2), in which a bacterial lysate is adsorbed to the wells of a microtiter plate. Theoretically, in order to increase specificity (that is, decrease the number of false positives), it would be preferable to use defined peptide epitopes without adjacent sequences rather than entire bacterial proteins, which may be recognized by antibodies to proteins from other microorganisms. In some individuals without Lyme disease, such nonspecific binding may produce a false positive test result due to the presence of antibodies which cross-react with *Bb* proteins. Similarly, increased sensitivity (that is, fewer false negatives) might be secured by the use of more densely presented immunoreactive epitopes, rather than the entire proteins (sparsely populated epitopes). Although only a fraction of the antibodies elicited in an immune response to a protein may bind to a continuous epitope, as represented by a linear peptide, this may be sufficient for a diagnostic immunoassay (3). In furthering this technology, we present a generally applicable method for presentation of peptide antigens on ELISA plates as conjugates of bovine serum albumin.

EXPERIMENTAL PROCEDURES

Antigen Preparation. Linear monomeric peptides and branched peptides were synthesized on PAL resin (Milligen/Bioscience, Woburn, MA) and Tentagel SRAM

Scheme 1. Conjugation of Cys-Containing Peptide to Bovine Serum Albumin



resin (Applied Biosystem, Foster City, CA), respectively, by Fmoc/t-butyl strategy, using a Model 9400 Excell instrument (Milligen/Bioscience, Milford, MA) or a Model 430A instrument (Applied Biosystems, Foster City, CA). Monomeric peptides were purified by high-performance liquid chromatography (HPLC) on a 2.2 × 25 cm C₁₈ reverse phase column (Vydac 218TP1022, 10 μm, 300 Å pore size), using a linear gradient from 0.1% trifluoroacetic acid (TFA) (buffer A) to 100% buffer B (70% acetonitrile and 20% 2-propanol in buffer A) within 50 min, at a flow rate of 4.0 mL/min, whereas branched peptides were desalted by gel filtration chromatography (Sephadex G-15). Peptide structure and homogeneity were confirmed by laser desorption mass spectrometry, amino acid analysis, and/or Edman degradation on a Model 477 microsequencer (Applied Biosystems).

Synthetic peptides were coupled to bovine serum albumin (BSA, Sigma, St. Louis, MO) as shown in Scheme 1. The BSA (2.5 mg/mL) was first activated by

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reaction with sulfosuccinimidyl 4-(*N*-maleimidomethyl)-cyclohexane-1-carboxylate (sulfo-SMCC, Pierce Chemical Co., Rockford, IL) (2.5 mg/mL) at 4 °C for 2 h in 20 mM sodium phosphate buffer (pH 7.0) containing 0.15 M sodium chloride and then freed of excess reagent by passage through a column of Sephadex G-75. The activated BSA was then reacted with the cysteine-containing peptide at 4 °C overnight, in 0.1 M sodium phosphate buffer (pH 7.0) containing 0.15 M sodium chloride and 1 mM ethylenediaminetetraacetic acid (EDTA). The resulting peptide–BSA conjugate was freed of excess peptide by passage through a column of G-75. Conjugates were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) on 10% polyacrylamide gels (Novex, San Diego, CA) under reducing conditions and by amino acid analysis of aliquots hydrolyzed for 24 h in HCl vapor.

ELISA. For coating the ELISA plate (Immulon 4, Dynatech Labs, Chantilly, VA), 100 μ L of each antigen, at 25 μ g/mL (unless otherwise indicated) in 0.1 M sodium phosphate buffer (pH 7.0) containing 0.15 M sodium chloride (PBS buffer), was added to each well in triplicate, and the wells were incubated overnight at room temperature. The wells were washed three times with PBS and then blocked for 1 h with 200 μ L/well of 5% nonfat dry milk in PBS. For the assay, 100 μ L of each serum sample, diluted 1:100 (unless otherwise indicated) in blocking buffer, was added per well and the wells were incubated for 2 h at room temperature. The plate was washed with PBS and incubated for 1 h with 100 μ L/well of peroxidase-conjugated goat anti-human IgG/IgM/IgA antiserum (Cappel, Durham, NC) that had been diluted 1:4000 with a solution of 1% goat serum and 0.1% casein in PBS. After thorough washing with PBS, color was developed for 1 h (unless otherwise indicated) using 100 μ L/well of 0.5 mg/mL ABTS substrate (Sigma), and absorbance was read on an MR 600 (Dynatech) plate reader at 410 nm.

RESULTS

Epitope Selection. The first step in the development of a peptide-based ELISA is selection of the epitope sequences. This can be accomplished using a technology in which a series of peptides, comprising the sequence of an antigenic protein, are synthesized in a sequence-overlapping manner on noncleavable pins (4). In a previous study using a small number of Lyme disease and control sera (5), antibody specificity to the immunodominant *Bb* protein, flagellin, was observed with peptides corresponding to a central region of the protein. This epitope was confirmed in an approach using fusion proteins, as well as the soluble peptide, EGVQGEAQQA (6). A region in the N-terminal portion of *Bb* flagellin, although reactive with Lyme disease sera, was cross-reactive with control sera (5). We synthesized a series of epitope-related peptides on pins varying in length from 6 to 13 residues and varying in the position of the N-terminal starting point. By testing nine individual Lyme sera, we found the optimal peptide sequence for detecting Lyme sera to be VQEGVQGEAQQA.

Peptide Presentation Formats. Different approaches for presentation of the flagellin epitope sequence on ELISA plates were investigated. A solution of each peptide (N-terminal-acetylated monomer, tandem repeat, 4-branched and 8-branched) was serially diluted, allowed to adsorb passively to the ELISA plate overnight, and then tested with a pool of nine flagellin positive Lyme disease sera and a pool of 10 control (non-Lyme disease) sera. No response was observed for the monomer, tandem repeat peptide, or 4-branched multiple antigenic

peptide (MAP); only a minimal response was obtained for the 8-branched MAP, but it was not considered to be strong enough to distinguish between Lyme disease and normal sera (data not shown). The lack of response with the monomeric and branched peptides might be due to either poor retention on the plate or masking of the epitope.

To improve peptide immobilization, the plate was first treated with glutaraldehyde, as previously reported (7). An N-terminal free MAP-4 peptide gave a dose-dependent response (Figure 1). At the same condition, N-terminal-acetylated MAP-8 peptide still showed a minimal response (data not shown), demonstrating that the primary amine groups are involved in peptide immobilization. Furthermore, when 0.1 M glycine was added to the blocking reagent, the signal in the test with MAP-4 was totally quenched (data not shown), suggesting that the immobilization through glutaraldehyde is reversible.

Since peptides are usually not immunogenic, they are covalently conjugated to carrier proteins for generation of antibodies (8). Accordingly, the presentation on an ELISA plate of peptide epitopes as haptens on a carrier protein was investigated. A facile approach is to use thiol-specific chemistry (Scheme 1). A cysteine residue, followed by two β -alanine residues (spacer), was incorporated at the C-terminus of the flagellin peptide during solid phase synthesis. In the first reaction of the conjugation protocol, the amino groups of bovine serum albumin (BSA), which was chosen as the carrier protein, were converted to thiol-reactive maleimido groups using the reagent sulfo-SMCC. The activated BSA was purified by gel filtration chromatography. In the second part of the protocol, the activated BSA was reacted with the cysteine-containing flagellin peptide, which had previously been purified by reverse phase HPLC.

When the activation reaction was done with a 150:1 molar excess of SMCC to BSA (which is a 2.5-fold equivalent excess, based on the 60 primary amino groups in BSA), a coupling ratio of 13 molecules of peptide per molecule of protein was obtained, as determined by amino acid analysis (9) (Table 1). The calculation is based on quantitation of β -Ala in the peptide, and Tyr or Phe in the BSA. Thus, the peptide accounts for about one-fourth of the mass of the conjugate. When the first reaction was performed with a 60:1, 24:1, or 9.6:1 molar excess of sulfo-SMCC to BSA, peptide/protein coupling ratios of 8, 4, and 2, respectively, were obtained (Table 1). In each second reaction, the molar ratio of peptide, which was measured by quantitative Ellman's assay (10), to (activated) BSA was always 120:1 (2.0-fold equivalent excess). The peptide reagent was in excess, as confirmed in separate experiments in which increasing the amount of peptide in the second reaction did not change the final coupling ratio (data not shown). Analysis by polyacrylamide gel electrophoresis showed broad bands with an average molecular mass for each conjugate comparable to that calculated from the coupling ratio (Figure 2).

The ELISA response for each peptide–BSA conjugate at different dilutions was tested using pooled Lyme disease and control sera, and a dose-dependent response was obtained (Figure 1). The maximal level of protein adsorption, which was reached when 2.5 μ g per 100 μ L was added to a well, was about 70 ng/well, as determined by bicinchoninic acid protein assay (11). This value is close to previous findings (12). Similar to the dependence of ELISA response on the quantity of conjugate adsorbed to the well, an increase in response with the relative content of antigenic peptide in the conjugate was observed (Figure 1). In separate experiments, the use of longer spacers, namely four or eight residues of γ -amino-

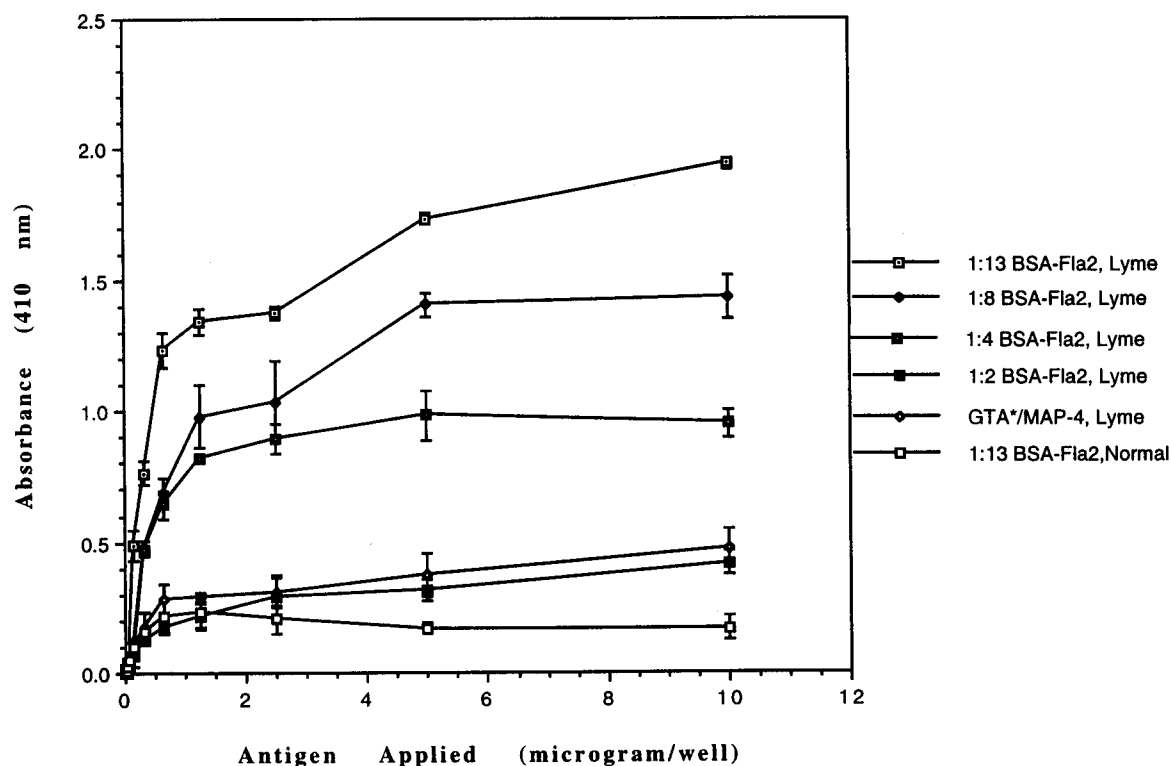


Figure 1. Comparison of different antigen presentations. The amount of BSA-peptide conjugate or MAP peptide applied per well is given. Each point represents the average of six determinations using a pool of nine Lyme disease sera or a pool of ten normal sera diluted 1:100 in blocking buffer and using a substrate development time of 25 min. Standard error bars are shown. GTA* indicates pretreatment of the plate with glutaraldehyde. The list is in the same order as the curves.

Table 1. Determination of Coupling Ratios by Amino Acid Analysis

BSA conjugate	amino acid	amount (pmol)	coupling ratio ^a	average ratio
9.6:1 sulfoSMCC:BSA	Phe	571	2.2	2.3
	Tyr	369	2.4	
	β -Ala	93		
24:1 sulfoSMCC:BSA	Phe	701	3.8	4.1
	Tyr	457	4.3	
	β -Ala	197		
60:1 sulfoSMCC:BSA	Phe	757	7.1	7.6
	Tyr	473	8.0	
	β -Ala	398		
150:1 sulfoSMCC:BSA	Phe	918	12.2	12.5
	Tyr	613	12.8	
	β -Ala	830		

^a Coupling ratio = $(\beta\text{-Ala}/2)/(\text{Tyr}/19)$ or $(\beta\text{-Ala}/2)/(\text{Phe}/27)$.

butyric acid or a polyethylene glycol heterobifunctional linker of 2 kDa (Shearwater Polymers, Huntsville, AL), resulted in progressively weaker ELISA responses with Lyme disease sera (data not shown). A lower ELISA response was also observed when the two β -alanine residues were not included (data not shown).

The conjugate having the highest relative content of flagellin peptide (1:13 BSA:Fla) was added to each well at a level of 2.5 μg in 100 μL . The ELISA response, determined at a wide range of serum dilutions, was found to easily distinguish individual Lyme sera from normal controls (Figure 3). Since the readings for control sera also increased with concentration, the optimal discrimination between Lyme disease and non-Lyme disease sera may not necessarily be achieved at a high serum concentration. Another variable is the time of substrate incubation. Color development is essentially completed within 1 h (data not shown). On the basis of these results, a well loading of 2.5 μg of conjugate, a serum dilution of 1:100, and an incubation time of 60 min were

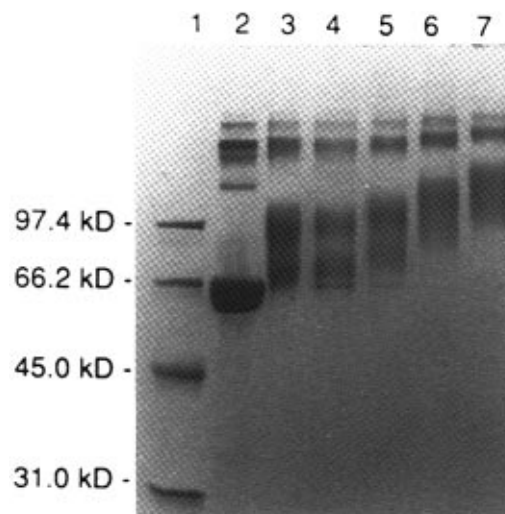


Figure 2. SDS-PAGE of BSA-peptide conjugates: lane 1, molecular mass standard; lane 2, BSA; lane 3, activated BSA; lanes 4–7, BSA-flagellin conjugates with activation ratios (sulfo-SMCC:BSA) of 9.6:1, 24:1, 60:1, and 150:1, respectively.

used to evaluate the flagellin epitope peptide with serum samples.

Preliminary Studies on Serodiagnosis. Forty-one sera (Figure 4A), in which 16 were clinically diagnosed as Lyme disease, were tested with 1:13 BSA:Fla as the antigen. An additional 10 normal sera were used to establish a cutoff value as follows. The average absorbance reading of the ten control sera and the standard deviation from this average were calculated. The cutoff was set as the average plus three standard deviations. Any reading above this cutoff value was considered positive for Lyme disease. As seen in Figure 4A, 12 positive responses were obtained, 11 of which corresponded to Lyme disease sera, giving a sensitivity of

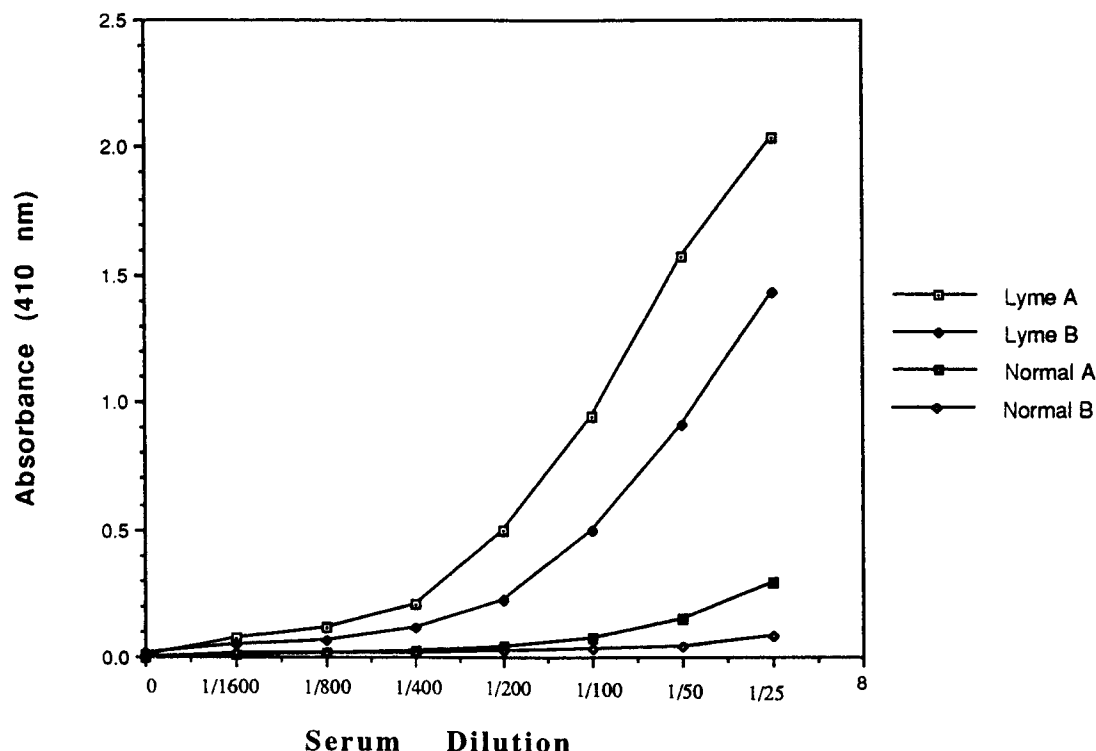


Figure 3. Serum dilution curves. Serum was diluted into blocking buffer. Analysis was with 2.5 μ g of the BSA conjugate of flagellin peptide (1:13). Each point represents the average of triplicate assays using a suboptimal substrate development time of 10 min to keep all points on scale. The list is in the same order as the curves.

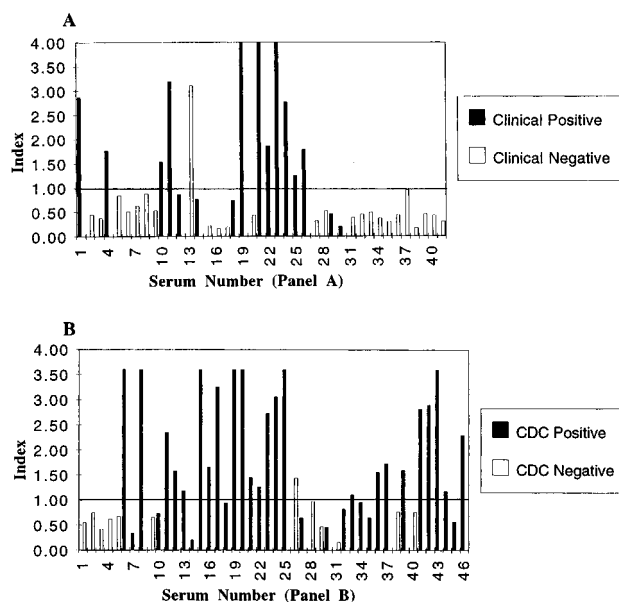


Figure 4. Preliminary serodiagnosis with BSA–flagellin (1:13) as the antigen. Clinically diagnosed Lyme sera or CDC positive sera are represented by filled bars, whereas clinically normal sera or CDC negative sera are represented by empty bars. In our peptide ELISA, the index value of each serum was obtained by division of its absorbance by the cutoff value (0.51), which is the average (0.30) plus three standard deviations (3×0.07) of a group of ten normal controls (not shown).

69% (11/16). One false positive was observed among 25 normal samples, leading to a specificity of 96% (24/25).

Another 46 sera (panel B), obtained from the Centers for Disease Control and Prevention (CDC), were also tested using the same BSA–flagellin peptide conjugate (Figure 4B). On the basis of the composite results of three CDC tests (Western blotting for IgG and for IgM and flagellin protein ELISA), 24 out of 35 Lyme sera were identified in our assay, giving a sensitivity of 69%. One

CDC negative serum (no. 26) was positive in our peptide ELISA (91% specificity). Clinically, this patient had the rash typical for Lyme disease, so the true nature of this serum is unclear.

DISCUSSION

The use of small peptides as antigens for capturing antibodies in the serodiagnostic ELISA has the potential to limit false positive responses. In addition to the advantage of epitope specificity, synthetic peptide antigens can be carefully characterized from batch to batch, as opposed to cell lysates which may vary with passage of the bacteria. An obstacle to application of this approach, which is the subject of this article, is to devise a facile and general method for presentation of small peptides on the ELISA plate. Although monomeric peptides (13, 14) and multiple-branched peptides (15, 16) have been coated directly on the ELISA plate as antigens, their use in the present application was unsuccessful. The physical adsorption of antigen to the ELISA plate depends mainly on hydrophobic interaction. With small peptides, the force is frequently too weak to permit detection of the final antigen–antibody complex. Besides, passive coating of antigen often masks the epitope and underestimates the antibody level. This masking phenomenon has even been observed with a protein, unless presented as part of a fusion protein (17).

Numerous methods have been developed to immobilize peptides to the polystyrene surface. The plate can be activated with UV or γ irradiation and then treated with glutaraldehyde. Peptides can then be linked to the plate through primary amino groups (18, 19). Peptides can also be immobilized as haptens on a carrier, such as BSA or polylysine (20, 21). Previously, these conjugations were prepared through carboxyl or amino groups under harsh reaction conditions, such as *N*-hydroxysuccinimide/dicyclohexylcarbodiimide, glutaraldehyde, and bis-diazotized benzidine. Coupling with these reagents may completely abolish the antigenicity of the peptides (22),

especially when Asp, Glu, or Lys residues are present. Therefore, these methods are not generally applicable.

By synthesis of a cysteine-containing epitope peptide, conjugation to a carrier protein can be done in a two step reaction. Since the free thiol group preferentially reacts with maleimide, other residues in the peptide are not affected. Thus, this conjugation method is gentle and dependable. None of the epitope sequences identified in our pin-peptide scanning of several *Bb* protein sequences contained a residue of cysteine (data not shown), making the extra C-terminal cysteine residue a unique site for conjugation. Since many copies of the epitope peptide are present on each molecule of conjugate, several haptens will always be sterically available for antibody interaction after immobilization. In addition, several copies of peptides on the same carrier protein molecule may mediate multivalent interaction with antibody, potentially leading to enhanced avidity of interaction and greater reactivity in the immunoassay.

BSA was chosen as the carrier, since it is a major component of serum and is essentially inert with respect to antibody cross-reactivity. Other advantages are its strong binding to the polystyrene microtiter plate surface, its availability in high purity, and its ease of handling. To prevent steric hindrance from the carrier protein, the coupling site was positioned away from the antigenic site with two spacer residues of β -alanine. Similarly, an optimal number of glycine residue spacers had been reported for branched peptide antigens (15).

In our preliminary serological test for Lyme disease, using a single epitope from *Bb* flagellin as the antigen, a sensitivity of 69% and a specificity of 96 or 91% were obtained for panels A and B, respectively. It should be noted that a similar flagellin epitope sequence was presented as a glutathione transferase fusion protein, and a similar sensitivity for detection of Lyme disease was reported (23). A parallel comparison of this set of serum samples with a commercial ELISA kit (MarDx Diagnostics, Carlsbad, CA), using *Bb* whole cell lysate as the antigen, gave a higher sensitivity (81 and 88%) but a lower specificity (68 and 71%). This comparison demonstrates that a peptide ELISA may be useful for elimination of false positives in the serological confirmation of Lyme disease. We have identified some epitope sequences from several other immunodominant *Bb* proteins. These epitope peptides were synthesized, conjugated to BSA, and used to screen the two serum panels reported here. It was concluded that, if more epitopes from other *Bb* antigens are utilized, a higher sensitivity can be achieved (to be published).

ACKNOWLEDGMENT

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LITERATURE CITED

- (1) Barbour, A. G., and Fish, D. (1993) The biological and social phenomenon of Lyme disease. *Science* 260, 1610–1616.
- (2) Karlsson, M. (1990) Western Immunoblot and flagellin enzyme-linked immunosorbent assay for serodiagnosis of Lyme disease. *J. Clin. Microbiol.* 18, 2148–2150.
- (3) Van Regenmortel, H. V., and Pellequer, J. L. (1994) Predicting antigenic determinants in proteins: Looking for uni-dimensional solutions to a three-dimensional problems? *Pept. Res.* 7, 224–228.
- (4) Geysen, H. M., Meloan, R. H., and Barteling, S. J. (1984) Use of peptide synthesis to probe viral antigens for epitopes to a resolution of a single amino acid. *Proc. Natl. Acad. Sci. U.S.A.* 81, 3998–4002.
- (5) Schneider, T., Lange, R., Ronspeck, W., Weigelt, W., and Kolmel, H. W. (1992) Prognostic B-cell epitopes on the flagellar protein of *Borrelia burgdorferi*. *Infect. Immun.* 60, 316–319.
- (6) Fikrig, E., Berland, R., Chen, M., Williams, S., Sigal, L. H., and Flavell, R. A. (1993) Serological response to the *Borrelia burgdorferi* flagellin demonstrates an epitope common to a neuroblastoma cell line. *Proc. Natl. Acad. Sci. U.S.A.* 90, 183–187.
- (7) Ordonneau, P., Abdullah, L. H., and Petrusz, P. (1991) An efficient enzyme immunoassay for glutamate using glutaraldehyde coupling of the hapten to microtiter plates. *J. Immunol. Methods* 142, 169–176.
- (8) Harlow, E., and Lane, D. (1988) *Antibodies: A Laboratory Manual. Immunizations*, pp 53–135, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- (9) Tsao, J., Lin, X., Lackland, H., Tous, G., Wu, Y., and Stein, S. (1991) Internally standardized amino acid analysis for determining peptide/carrier protein coupling ratio. *Anal. Biochem.* 197, 137–142.
- (10) Riddles, P. W., Blakeley, R. L., and Zerner, B. (1979) Ellman's reagent: 5,5'-Dithiobis(2-nitrobenzoic acid)-a re-examination. *Anal. Biochem.* 94, 75–81.
- (11) Wiechelman, K., Braun, R., and Fitzpatrick, J. (1988) Investigation of the bicinchoninic acid protein assay: identification of the groups responsible for color formation. *Anal. Biochem.* 175, 231–237.
- (12) Stevens, P. W., Hansberry, M. R., and Kelso, D. M. (1995) Assessment of adsorption and adhesion of proteins to polystyrene microwells by sequential enzyme-linked immunosorbent assay analysis. *Anal. Biochem.* 225, 197–205.
- (13) Peralta, J. M., Teixeira, M. G. M., Shreffler, W. G., Pereira, J. B., Burns, J. M., Jr., Sleath, P. R., and Reed, S. G. (1994) Serodiagnosis of Chagas' disease by enzyme-linked immunosorbent assay using two synthetic peptides as antigens. *J. Clin. Microbiol.* 32 (4), 971–974.
- (14) Gnann, J. W., Jr., McCormick, J. B., Nelson, J. A., Mitchell, S., Nelson, J. A., and Oldstone, M. B. A. (1987) Synthetic peptide immunoassay distinguishes HIV type 1 and HIV type 2 infections. *Science* 237, 1346–1349.
- (15) Marsden, H. S., Owsianka, A. M., Graham, S., Mclean, G. W., Robertson, C. A., and Subak-Sharpe, J. H. (1992) Advantages of branched peptides in serodiagnosis. Detection of HIV-specific antibodies and the use of glycine spacers to increase sensitivity. *J. Immunol. Methods* 147, 65–72.
- (16) Tam, J., and Zavala, F. (1989) Multiple antigen peptide: A novel approach to increase detection sensitivity of synthetic peptides in solid-phase immunoassays. *J. Immunol. Methods* 124, 53–61.
- (17) Cartwright, G. A., Rothel, J. S., and Lightowlers, M. W. (1995) Conventional immunoassays underestimate anti-GST antibody titre. *J. Immunol. Methods* 179, 31–35.
- (18) Dagenais, P., Desprez, B., Albert, J., and Escher, E. (1994) Direct covalent attachment of small peptide antigens to enzyme-linked immunosorbent assay plates using radiation and carbodiimide activation. *Anal. Biochem.* 222, 149–155.
- (19) Boudet, F., Theze, J., and Zouali, M. (1991) UV-treated polystyrene microtitre plates for use in an ELISA to measure antibodies against synthetic peptides. *J. Immunol. Methods* 142, 73–82.
- (20) Price, M. R., Sekowski, M., Hoo, D. S. W., Durrant, L. G., Hudecz, F., and Tendler, S. J. B. (1993) Measurement of antibody binding to antigenic peptides conjugated in situ to albumin-coated microtitre plates. *J. Immunol. Methods* 159, 277–281.
- (21) Ball, J. M., Henry, N. L., Montelaro, R. C., and Newman, M. J. (1994) A versatile synthetic peptide-bound ELISA for identifying antibody epitopes. *J. Immunol. Methods* 171, 37–44.
- (22) Briand, J. P., Muller, S., and Van Regenmortel, M. H. V. (1985) Synthetic peptides as antigens: pitfalls of conjugation methods. *J. Immunol. Methods* 78, 59–69.
- (23) Cretella, S., Gordon, S., Flavell, R. A., and Fikrig, E. (1995) Evaluation of a Lyme disease enzyme immunoassay using the 41-G fragment of flagellin. *Eur. J. Clin. Microbiol. Infect. Dis.* 14, 609–613.

Synthesis of Azidoaniline Derivatives of Oligonucleotides and Investigation of Their Photochemical Behavior

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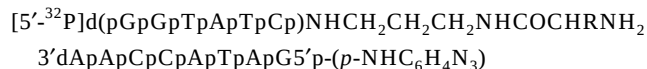
A series of aryl azides, $p\text{-N}_3\text{C}_6\text{H}_4\text{NH}(\text{CH}_2)_n\text{NH}_2$ with $n = 2\text{--}6$, have been synthesized and used to prepare oligonucleotide derivatives carrying photoreactive the p -azidoaniline residue. Reactive moieties have been coupled to the 5'-terminal phosphate of d(pGATACCAA) [compounds **IV(b)**, **IV(c)**, and **IV(e)** with $n = 3, 4$, and 6 , respectively] and of d(pGCC) [compound **V(b)** with $n = 3$] via a phosphoamide bond. Irradiation at wavelengths over >300 nm of **IV(b)** and **V(b)** ($n = 3$) resulted in cleavage of the P–N bond. However, under the same reaction conditions, the P–N bond remained intact for compounds containing longer spacers [**IV(c)** and **IV(e)**]. Intraduplex reaction of the latter derivatives with d(GGTATCp)NH(CH₂)₆NH₂ resulted in cross-linking dependent on the presence of an aliphatic amino group. The results obtained have demonstrated that the azidoaniline derivatives of oligonucleotides capable of the affinity modification of a specific target can be prepared. However, the sufficiently long aliphatic spacer group is necessary to prevent P–N bond cleavage within the photoreactive oligonucleotide.

INTRODUCTION

Previously, derivatives of the nucleoside 5'-triphosphates (NTP)¹ carrying a p -azidoanilide residue attached to the γ -phosphate were proposed and used for modification of a number of the NTP binding proteins (1–4). It has been shown that under irradiation p -azidoanilide derivatives can be converted to quinonediimine derivatives via isomerization of the intermediate nitrene (5, 6). The reagent formed turned out to be a strong electrophile capable of the efficient modification in aqueous solution of a number of compounds containing aliphatic amino groups, imidazole rings, and thiol residues (6, 7). Therefore, these derivatives may be considered as strong electrophiles capable of being switched on under irradiation.

It seemed interesting to prepare similar derivatives of oligonucleotides for the photoaffinity modification of the nucleic acid binding proteins. However, with the [$\gamma\text{-}^{32}\text{P}$]-dTTP- p -azidoanilide, it was found that the modification of the Klenow fragment of DNA polymerase I was not accompanied by equivalent incorporation of ^{32}P labeling of the protein (3). A similar result was recently obtained for the oligonucleotide derivative containing a p -azidoaniline residue directly bound to the 5'-terminal phos-

phate which was subjected to irradiation within the complex:



where R is either the (CH₂)₄NH₂ residue or CH₂C₆H₅ (8). Although modification of the amino acid-carrying fragment of the target definitely took place under irradiation, the oligonucleotide moiety of the reagent was absent from the modification product. This indicated that the P–N bond cleavage took place.

Therefore, prior to design of the oligonucleotide derivatives with the photoswitchable reactive group, on the basis of the properties of p -azidoaniline, it seemed reasonable to change the way of attachment of p -azidoaniline to oligonucleotide. This could be achieved by introduction of the aliphatic spacer between p -azidoaniline and the 5'-terminal phosphate of the oligonucleotide. In the present paper, we describe the preparation of the derivatives containing the (CH₂) _{n} NH spacer separating the p -azidoaniline residue and the terminal phosphate of the oligonucleotide. It has been found that the presence of the sufficiently long spacer ($n > 3$) permits retention of the P–N bond within the irradiated reagent.

EXPERIMENTAL PROCEDURES

General Methods. Melting points were recorded on an electrochemical capillary melting point apparatus and are reported uncorrected. ¹H NMR spectra were recorded on a Bruker WP-200 SY (200 MHz) spectrometer. All ¹H chemical shifts are reported relative to tetramethylsilane (TMS) as an internal standard. ³¹P NMR spectra were recorded on a Bruker AC-200 (81.015 MHz) spectrometer, and the ³¹P chemical shifts are reported relative to H₃PO₄ as an external standard. Infrared spectra were recorded on a Specord M60 instrument (Carl Zeiss, Jena). Mass spectra and exact masses were obtained on a Finnigat-matt-2000. Absorption spectra were recorded

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¹ Abbreviations: NTP, nucleoside 5'-triphosphates; Ph₃P, triphenylphosphine; (PyS)₂, 2,2'-dipyridyl disulfide; DMAP, (*N,N*-dimethylamino)pyridine.

on a Specord M40 instrument (Carl Zeiss, Yena) in 0.15 M NaCl and 0.02 M Na₂HPO₄ at pH 9.02. Molar extinction coefficients at 260 nm of unmodified oligonucleotides **IV** and **V** were estimated to be 89 900 and 30 600 M⁻¹ cm⁻¹ according to ref 9. The coefficients of modified oligonucleotides **IV(b,c,e)** and **V(b)** were estimated as a sum of the oligonucleotide and the aryl azide ($\lambda = 260$ nm, $\epsilon = 11\,000$, pH 9) molar extinction coefficients.

Oligonucleotides were prepared as described by ref 10. The aryl azide-oligonucleotide conjugates containing the amino spacer and an aliphatic amino group containing oligodeoxynucleotides were prepared as described in ref 11. To obtain 3'-phosphorylated oligonucleotides, a solid support containing [[2-[O-(4,4'-dimethoxytrityl)oxy]ethyl]-sulfonyl]ethanol moiety was synthesized (12).

Isolation and purification of the oligonucleotide derivatives were performed by reversed-phase high-performance liquid chromatography using a LiChrosorb RP-18, 10 μ m (Merck, FRG), 4.6 $\times$ 250 mm column, a Waters 600E chromatograph, and a Waters 484 tunable absorbance detector (U.S.A.). A linear gradient (2 mL/min) from 0 to 20% of solvent B (solvent A = 0.05 M LiClO₄, solvent B = acetonitrile) was used. Analytical anion-exchange microcolumn chromatography was performed on the Milichrom chromatograph (Russia) using a Polisil-SA (Russia), 2.5 $\times$ 30 mm column. A linear gradient (flow rate = 50 μ L/min) from 0 to 1 M NaCl in 7 M urea and 0.01 M Na₂HPO₄ (pH 9) was employed.

Materials. *N,N*-Dimethylformamide (DMF), dimethyl sulfoxide (DMSO), acetone, and acetonitrile were purified and dried according to standard procedures. 4-(*N,N*-Dimethylamino)pyridine (DMAP) was purchased from Bergkamen (Berlin), and triphenylphosphine (Ph₃P), 2,2'-dipyridyl disulfide (PyS)₂, and polymethylenediamines were obtained from Fluka AG (Switzerland). *N*-Cetyl-*N,N,N*-trimethylammonium bromide and TLC aluminum sheets (silica gel 60 F₂₅₄) were purchased from Merck (Germany).

Synthesis of *N*-(*p*-Nitrophenyl) Derivatives of Diamines **I(a-e).** A mixture of 0.01 mol of *p*-chloronitrobenzene and 0.1 mol respective diamine was heated in an oil bath at 135–140 °C for 1 h. The excess of diamine was evaporated under reduced pressure. The yellow liquid residue was dissolved in boiling 10% hydrochloric acid and filtered to remove any insoluble material (bis derivative). The solution was adjusted to pH 10–11 with concentrated sodium hydroxide, and the precipitate was filtered. The resulting solid was washed with 20 mL of water and crystallized from 35–50 mL of ethanol/water (1:2, v:v).

The yield of *N*-(*p*-nitrophenyl)-1,2-diaminoethane **I(a)** was 74%, mp 140–141 °C [lit. 139–141 °C (13)].

The yield of *N*-(*p*-nitrophenyl)-1,3-diaminopropane **I(b)** was 81%, mp 112–113 °C [lit. 111 °C (14)].

The yield of *N*-(*p*-nitrophenyl)-1,4-diaminobutane **I(c)** was 72%, mp 103.2–103.7 °C [lit. 101 °C (15)].

The yield of *N*-(*p*-nitrophenyl)diaminopentane **I(d)** was 48%, mp 102.2–102.5 °C [lit. 97 °C (16)].

The yield of *N*-(*p*-nitrophenyl)-1,6-diaminohexane **I(e)** was 86%, mp 98.5–99.0 °C. Anal. Found: C, 60.8; H, 8.07; N, 17.7. Calcd for C₁₂H₁₉N₃O₂: C, 60.7; H, 8.15; N, 17.7.

Synthesis of *N*-(*p*-Azidophenyl) Derivatives of the Protected Diamines **II(a-e) (Scheme 1).** A mixture of the *N*-(*p*-nitrophenyl) derivative of diamine (1 mmol), trifluoroacetic anhydride (3 mmol), and *N*-ethyl-diisopropylamine (3 mmol) in 10 mL of dry chloroform was stirred for 30 min at room temperature. The solvent and the excess of *N*-ethyl-diisopropylamine were evaporated under

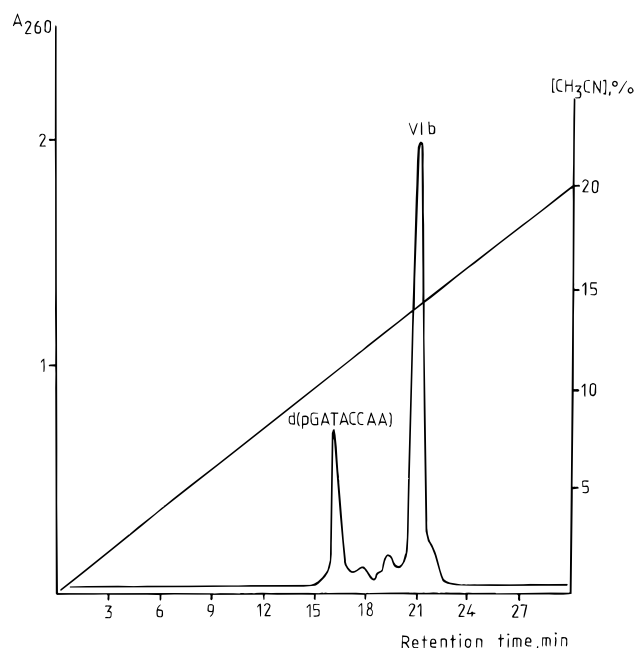


Figure 1. Reversed-phase chromatography of the reaction mixture for synthesis of *p*-azidoaniline derivatives of oligonucleotide **IV(b)**.

reduced pressure. The resulting residue was washed with 1 N hydrochloric acid. The reaction progress was monitored by TLC [CHCl₃/MeOH, 10:1, v:v]. The solid residue was dissolved in 10 mL of ethanol, and compounds were catalytically hydrogenated for 2 h at room temperature using 0.1 g of 10% Pd/C. The reaction mixture was filtered, the solvent was removed by evaporation, and the residue was dissolved in 20 mL of 2 N H₂SO₄. The reaction mixture was cooled to 0 °C, and 1 mmol of sodium nitrite in 1 mL of water was added. The mixture was stirred for 30 min at 0 °C, and 3 mmol of sodium azide was added in the dark. After 1 h, compounds **II(a-e)** were extracted with chloroform and the solvent was removed. Compounds **II(a,c)** were crystallized from 10–30 mL of benzene/hexane (1:5, v:v).

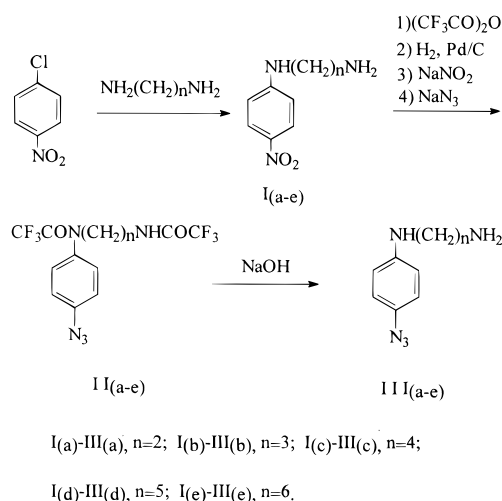
The yield of *N,N'*-bis(trifluoroacetyl)-*N*-(*p*-azidophenyl)-1,2-diaminoethane **II(a)** was 67%; mp 87–90 °C dec; *R*_f 0.48–0.52 (ethanol/CHCl₃, 1:10, v:v; UV (ethanol) λ_{max} 254 nm ($\epsilon = 14\,200$); IR (CHCl₃) 2128–2104 d.b. (N₃), 1728 and 1696 cm⁻¹ (C=O); ¹H NMR (CDCl₃) δ 3.64 (dd, 2H, *J* = 6 Hz), 3.95 (t, 2H, *J* = 6 Hz), 7.08 (d, 2H, *J* = 9 Hz), 7.23 (d, 2H, *J* = 9 Hz); mass spectrum *m/z* ($\pm 1 \times 10^{-4}$) found 369.0660, calcd for C₁₂H₉F₆N₅O₆ 369.0660.

The yield of *N,N'*-bis(trifluoroacetyl)-*N*-(*p*-azidophenyl)-1,3-diaminopropane **II(b)** was 54%; *R*_f 0.52–0.56 (ethanol/CHCl₃, 1:10, v:v; UV (ethanol) λ_{max} 254 nm ($\epsilon = 14\,000$); IR (CHCl₃) 2128–2104 d.b. (N₃), 1724 and 1688 cm⁻¹ (C=O); ¹H NMR (CDCl₃) δ 1.83 (m, 2H), 3.47 (dd, 2H, *J* = 6, 5 Hz), 3.90 (t, 2H, *J* = 6, 5 Hz), 7.06 (d, 2H, *J* = 9 Hz), 7.21 (d, 2H, *J* = 9 Hz); mass spectrum *m/z* ($\pm 1 \times 10^{-4}$) found 383.0817, calcd for C₁₃H₁₁F₆N₅O₂ 383.0817.

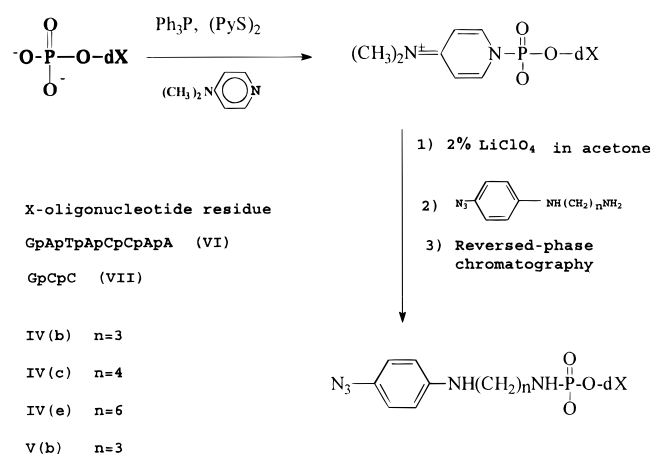
The yield of *N,N'*-bis(trifluoroacetyl)-*N*-(*p*-azidophenyl)-1,4-diaminobutane **II(c)** was 58%; mp 80–82 °C; *R*_f 0.58–0.62 (ethanol/CHCl₃, 1:10, v:v; UV (ethanol) λ_{max} 254 nm ($\epsilon = 14\,000$); IR (CHCl₃) 2128–2104 d.b. (N₃), 1728 and 1696 cm⁻¹ (C=O); ¹H NMR (CDCl₃) δ 1.61 (m, 4H), 3.39 (dd, 2H, *J* = 7 Hz), 3.72 (t, 2H, *J* = 7 Hz), 7.04 (d, 2H, *J* = 9 Hz), 7.17 (d, 2H, *J* = 9 Hz); mass spectrum *m/z* ($\pm 1 \times 10^{-4}$) found 397.0972, calcd for C₁₄H₁₃F₆N₅O₂ 397.0973.

The yield of *N,N'*-bis(trifluoroacetyl)-*N*-(*p*-azidophenyl)-1,5-diaminopentane **II(d)** was 61%; *R*_f 0.61–0.63 (etha-

Scheme 1



Scheme 2



mol/ CHCl_3 , 1:10, v:v; UV (ethanol) λ_{max} 254 nm ($\epsilon = 14\,000$); IR (CHCl_3) 2128–2104 d.b. (N_3), 1726 and 1696 cm^{-1} (C=O); $^1\text{H NMR}$ (CDCl_3) δ 1.45 (m, 4H), 1.63 (m, 2H), 3.33 (dd, 2H, $J = 7$ Hz), 3.70 (t, 2H, $J = 7$ Hz), 7.05 (d, 2H, $J = 9$ Hz), 7.17 (d, 2H, $J = 9$ Hz); mass spectrum m/z ($\pm 1 \times 10^{-4}$) found 411.1129, calcd for $\text{C}_{15}\text{H}_{15}\text{F}_6\text{N}_5\text{O}_2$ 411.1130.

The yield of N,N' -bis(trifluoroacetyl)- N -(p -azidophenyl)-1,6-diaminohexane (**II(e)**) was 68%: R_f 0.61–0.63 (ethanol/ CHCl_3 , 1:10, v:v; UV (ethanol) λ_{max} 254 nm ($\epsilon = 14\,000$); IR (CHCl_3) 2128–2104 d.b. (N_3), 1728 and 1698 cm^{-1} (C=O); $^1\text{H NMR}$ (CDCl_3) δ 1.34 (m, 4H), 1.56 (m, 4H), 3.33 (dd, 2H, $J = 7$ Hz), 3.69 (t, 2H, $J = 7$ Hz), 7.05 (d, 2H, $J = 9$ Hz), 7.16 (d, 2H, $J = 9$ Hz); mass spectrum m/z ($\pm 1 \times 10^{-4}$) found 425.1284, calcd for $\text{C}_{16}\text{H}_{17}\text{F}_6\text{N}_5\text{O}_2$ 425.1284.

Synthesis of N -(p -Azidophenyl) Derivatives of Diamines [III(a–e)]. Each of the protected derivatives **II(a–e)** (0.1 mmol) were dissolved in 5 mL of ethanol in the dark. Five milliliters of 1 N sodium hydroxide was added, and the mixture was stirred for 15 min at room temperature. The excess of ethanol was evaporated under reduced pressure, and the products **III(a–e)** were crystallized from water or were extracted with ethyl acetate, followed by subsequent removal of the solvent: UV (ethanol/water, 1:2, v:v, pH 8) λ_{max} 274 nm ($\epsilon = 16\,000$); IR (CHCl_3) 2112 cm^{-1} (N_3).

Synthesis of Aryl Azide Oligonucleotide Derivatives [IV(b,c,e) and V(b)]. The reaction mixture consisting of 0.05 μmol of the N -cetyl- N,N,N -trimethylammonium salt of the oligonucleotide, 15 μmol of Ph_3P ,

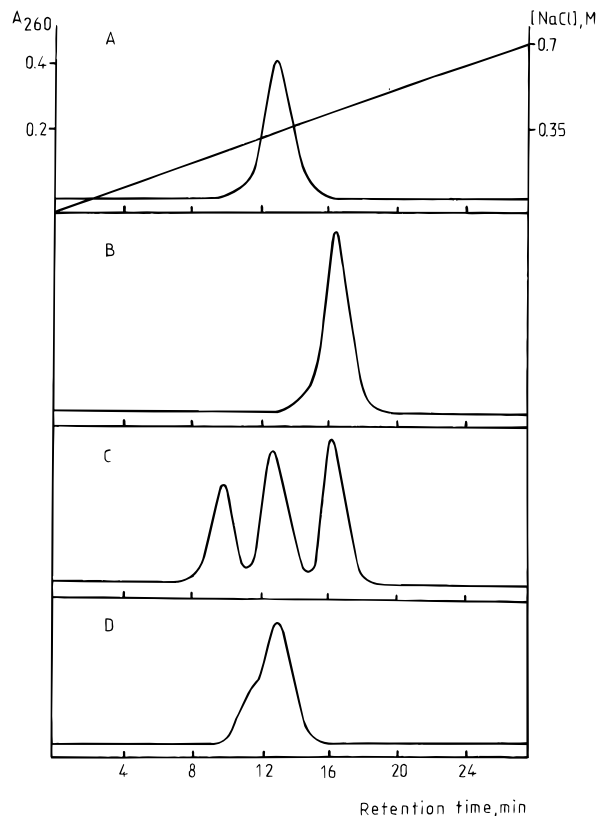


Figure 2. Profile of analytical anion-exchange microcolumn chromatography of compounds **IV(b)** (A) and d(pGATACCA) (B) and of the reaction mixture for photolysis of p -azidoaniline derivatives of oligonucleotides **IV(b)** (C) and **IV(e)** (D).

15 μmol of $(\text{PyS})_2$, and 30 μmol of DMAP in 0.1 mL of DMF was incubated for 12 min at room temperature. The 4-(N,N -dimethylamino)pyridine derivative of the oligonucleotide was precipitated with 2% LiClO_4 in acetone. Each aryl azide **III(b,c,e)** (300 μmol) in 50 μL of 50% aqueous DMF was added to the derivatized oligonucleotide precipitate, and the solution was incubated in the dark for 12 h. The aryl azide oligodeoxyribonucleotide derivatives formed were isolated by reversed-phase high-performance liquid chromatography (Figure 1). The retention times for compounds **IV(b,c,e)** were found to be 20–25 min. Products **IV(b,c,e)** were precipitated by 2% LiClO_4 in acetone. The yield was 70–90%.

Studies of the Photochemical Behavior of Aryl Azide Oligodeoxyribonucleotide Derivatives. A 5×10^{-5} M solution (20 μL) of photoreagent **V(b)**, **IV(b)**, **IV(c)**, or **IV(e)** in 0.15 M NaCl and 0.02 M Na_2HPO_4 (pH 9) were pipetted into a 96-well polystyrene plate at 4 $^\circ\text{C}$. Irradiation was carried out by exposing the plate for 5 min to filtered light (303–365 nm) emitted by a high-pressure mercury lamp with an intensity of 10^{15} quanta $\text{cm}^{-2} \text{s}^{-1}$. Photoreagents **IV(c)** and **IV(e)** were also combined with 5×10^{-5} M complementary aliphatic amino containing oligodeoxynucleotides. Following irradiation, the reaction mixtures were subjected to ion-exchange chromatography.

RESULTS AND DISCUSSION

(A) Synthesis of Aryl Azides. Syntheses of azides were accomplished as shown in Scheme 1. Compounds **I(a–c)** were obtained using the method described earlier for compound **I(b)** (15), with yields ranging from 48 to 86%. The reaction of **I(a–e)** with trifluoroacetic anhydride in dry CHCl_3 and in the presence of N -ethyl-diiso-

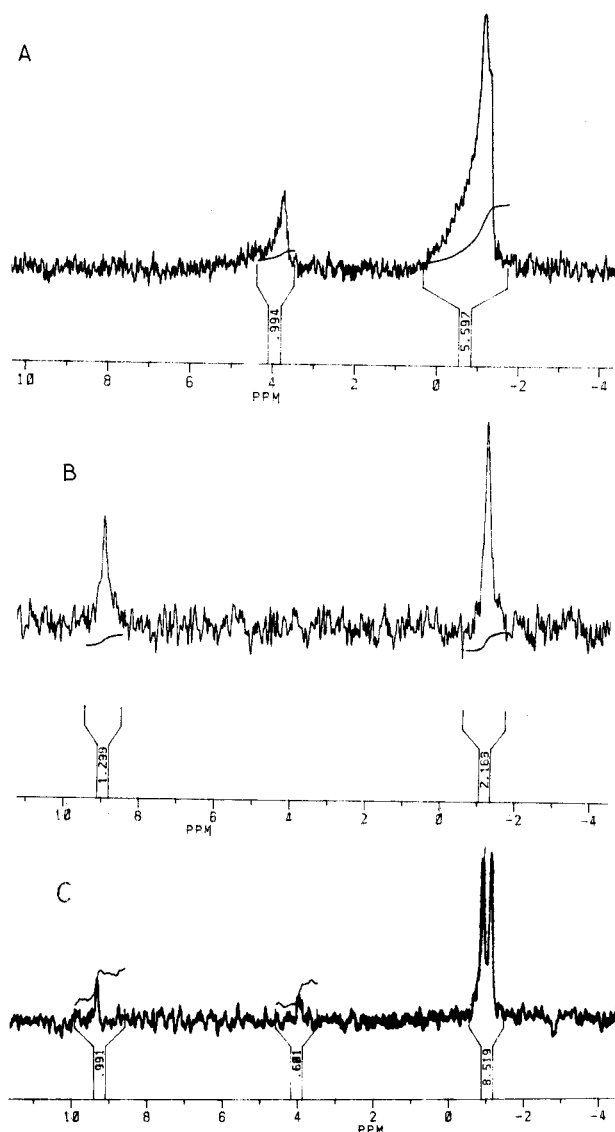
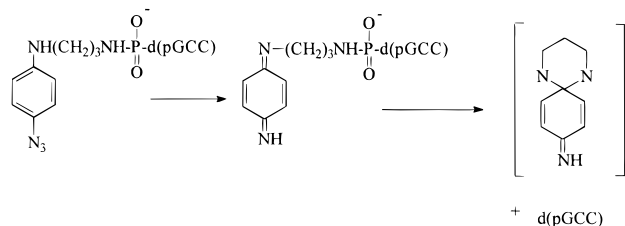


Figure 3. ^{31}P NMR spectra in 0.15 M NaCl (pH 9) of 5×10^{-3} M d(pGCC) (A) and **V(b)** before (B) and after irradiation (C).

Scheme 3



propylamine leads to the formation of the corresponding protected diamines. The reduction of the NO_2 group and subsequent conversion of the amino group to the azido were carried out without isolation of intermediates (Scheme 1). The trifluoroacetylated azides **II(a–e)** were stored at -10°C and deprotected just prior to the preparation of the oligonucleotide derivatives.

(B) Synthesis of Aryl Azide Oligodeoxynucleotide Derivatives. The photoreactive oligonucleotide derivatives **IV(b,c,e)** and **V(b)** were obtained by phosphorylation of the amino groups of **III(b,c,e)** with the activated 4-(*N,N*-dimethylamino)pyridinium derivatives of the respective oligodeoxyribonucleotides (Scheme 2). The activated oligonucleotides were prepared by reaction of the phosphate group with a mixture of triphenylphosphine

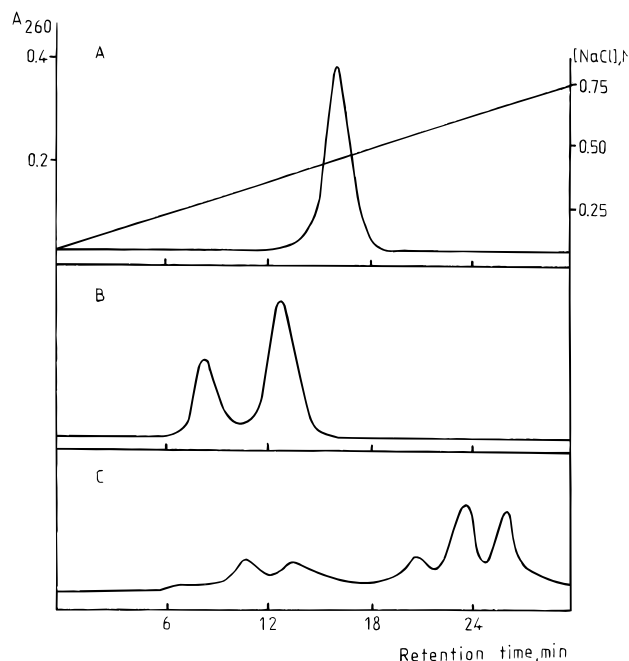


Figure 4. Profile of analytical anion-exchange microcolumn chromatography of the compound d(pGATACCA) (A), of the duplex **IV(e)**·d(GGTATCp)NH(CH₂)₆NH₂ without irradiation (B), and of the reaction mixture of irradiated duplex **IV(e)**·d(GGTATCp)NH(CH₂)₆NH₂ (C). Concentrations of the reagents were 5×10^{-5} M. Reaction mixture was irradiated and incubated at 4°C for 12 h in the dark prior to analysis.

and 2,2'-dipyridyl disulfide in the presence of 4-(*N,N*-dimethylamino)pyridine (11). These derivatives were isolated prior to coupling to compound **III** in order to avoid reaction with triphenylphosphine.

The products obtained were isolated by reversed-phase chromatography (Figure 1). The derivatized oligonucleotides were observed to elute after the underivatized oligonucleotide. The isolated compounds were homogeneous by anion-exchange chromatography. In addition, the photoreactive oligonucleotides eluted as compounds having one charge less than the parent oligonucleotide. The yields of **IV(b,c,e)** and **V(b)** were at least 70%. IR absorption spectra of the obtained compounds revealed the presence of a band at 2100 cm^{-1} , characteristic of the azido group. UV absorption spectra of the compounds had shown λ_{max} to be 260 nm, characteristic of the oligonucleotide component. Anion-exchange chromatography and ^{31}P NMR spectroscopy (the presence of the peak with $\delta = 8.7\text{ ppm}$, D_2O) proved that the oligonucleotide and *p*-azidoaniline residues were connected by a phosphoamide bond.

(C) Photochemical Behavior of the Aryl Azide Oligodeoxyribonucleotide Derivatives.

The photolytic behavior of four representatives of oligodeoxyribonucleotide derivatives **IV(b,c,e)** and **V(b)** was investigated. The solution of photoreagents ($5 \times 10^{-5}\text{ M}$) in 0.15 M NaCl and 0.02 M Na_2HPO_4 (pH 9) was subjected to photolysis for 5 min and was analyzed by anion-exchange chromatography. The irradiation time was chosen to be 10-fold greater than the half-life of photolysis. The kinetics of photolysis was studied by recording the IR spectra ($2000\text{--}3000\text{ cm}^{-1}$) of the samples irradiated for definite time intervals.

As shown in Figure 2C, reagent **IV(b)** undergoes a transformation leading to three products differing in their retention times with the anion-exchange column. The retention time of the main product is greater as compared with that of the parent azide derivative and coincides

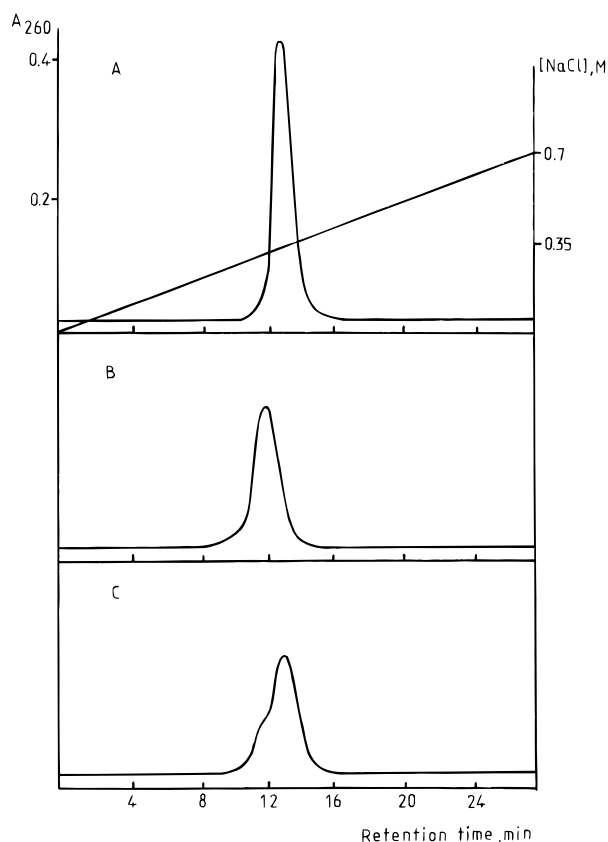


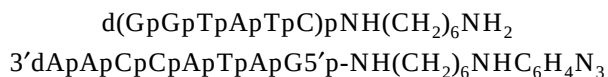
Figure 5. Profile of analytical anion-exchange microcolumn chromatography of compound **IV(e)** (A), of the duplex **IV(e)**·d(GGTATCp) without irradiation (B), and of the reaction mixture obtained by photolysis of the duplex **IV(e)**·d(GGTATCp) (C). Concentrations of the reagents were 5×10^{-5} M. Reaction mixture was irradiated and incubated at 4 °C for 12 h in the dark and then analyzed.

with that of the parent oligonucleotide. This suggested that the P–N bond is cleaved under irradiation with liberation of the 5'-terminal phosphate. To prove this statement, a similar experiment has been carried out with **V(b)** with fewer phosphate residues, and reaction mixture was subjected to ^{31}P NMR investigation. As shown in Figure 3, the resonance typical of an aliphatic phosphoamide group (8.7 ppm) disappears after irradiation and a new resonance at 3.7 ppm arises. The new resonance corresponds to the phospho monoester group. To explain these results, we propose the tentative reaction as shown in Scheme 3. The scheme is based on the suggestion that the N atom forming the P–N bond attacks the strongly electrophilic C atom, the second C in the polyene structure of intermediate quinonediimine. This reaction seems to be facilitated by the formation of the geometrically favorable six-membered cycle.

If this is the case, the use of longer spacers, $(\text{CH}_2)_n\text{NH}$, should hinder cyclization and prevent the P–N bond scission. Therefore, we have investigated the irradiation behavior of derivatives **IV(c)** and **IV(e)** (with $n = 4$ and 6). In Figure 2D, the result of the ion-exchange chromatography of the irradiated solution of **IV(e)** is presented. As shown, the appearance of the chromatographic profile differs strongly from that for **IV(b)**. No peak corresponding to the parent oligonucleotide was observed. This indicates that no P–N bond scission had taken place. The same results were obtained for **IV(c)**.

These results permitted one to expect that derivatives **IV(c)** and **IV(e)** should be capable of intraduplex reaction with the nucleophilic groups of the complementary oligonucleotide target without the loss of the addressing

oligonucleotide moiety. To check this possibility, we have subjected the below duplex to irradiation.



In Figure 4C, the result of the anion-exchange chromatography of the irradiated solution of the duplex is presented. As shown by the chromatographic profile, the peaks appear with greater retention time as compared with the retention time of the parent oligonucleotides. This indicates that the formation of the cross-linking products has taken place. In the same time, no cross-linking was observed in the irradiated duplex containing the parent oligonucleotide lacking an aliphatic amino group (Figure 5C). The same data were obtained for the duplex formed by **IV(c)**. The structure of the cross-linked products is now under investigation and will be discussed in a separate communication.

Thus, the results of the present investigation have demonstrated that the azidoaniline derivatives of the oligonucleotides are capable of affinity modification of the specific target without the loss of the modifying moiety. However, the aliphatic spacer group is necessary to prevent splitting of the address, and this group should be sufficiently long.

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LITERATURE CITED

- (1) Knorre, D. G., and Vlassov, V. V. (1989) Photoaffinity Modification. *Affinity Modification of Biopolymers* pp 111–113, CRC Press Inc., Boca Raton, FL.
- (2) Grachev, M. A., Knorre, D. G., and Lavrik, O. I. (1981) Interaction of *E. coli* Phe-RSase with photoreactive ATP γ -amides. Photoaffinity modification of some *E. coli* ARSase by ATP γ -azidoanilide. *γ -Derivatives of nucleoside-5'-triphosphates as probes for enzyme and protein factor active sites*, pp 125–127, Harwood Academic, New York.
- (3) Kudryashova, N. V., Shamanina, M. Yu., Godovikova, T. S., Anan'ko, E. A., Akhmadieva, F. F., and Romashchenko, A. G. (1993) Specific effects of dTTP γ -p-azidoanilide on *Escherichia coli* DNA polymerase 1 and its Klenow fragment. *Biokhimiya* 58, 224–223.
- (4) Rasenick, M. M., Talluri, M., and Dunn, W. J., III (1994) Photoaffinity Guanosine 5'-Triphosphate Analogs as a Tool for the Study of GTP-Binding Proteins. *Methods Enzymol.* 237, 100–110.
- (5) Gall, T. S., Griezian, N. P., Myzina, S. D., Bazhin, N. M., and Nemzeva, E. V. (1983) *p*-Benzenequinonediimine reactive intermediate of photolysis *p*-azidoaniline in aqueous solutions. *Dokl. AS USSR* 273, 883–887.
- (6) Badashkeyeva, A. G., Gall, T. S., Efimova, E. V., Knorre, D. G., Lebedev, A. V., and Myzina, S. D. (1983) Reactive derivative of adenosine-5'-triphosphate formed by irradiation of ATP- γ -p-azidoanilide. *FEBS Lett.* 155, 263–266.
- (7) Badashkeyeva, A. G., Gall, T. S., Knorre, D. G., Lebedev, A. V., Efimova, E. V., and Myzina, S. D. (1984) A study on photoconversion products of nucleoside 5'-triphosphate γ -p-azidoanilides in aqueous solution. *Bioorg. Khim.* 10, 696–701.
- (8) Godovikova, T. S., Berezovskye, M. V., and Knorre, D. G. (1995) Photoaffinity modification of aminoacid derivatives of oligonucleotides in the complimentary complex. *Bioorg. Khim.* 21, 858–867.

- (9) Cantor, C. R., and Tinoco, I. (1965) Absorption and optical rotatory dispersion of seven trinucleoside diphosphates. *J. Mol. Biol.* 13, 65–77.
- (10) Gryaznov, S. M., Gorn, V. V., Zarytova, V. F., Kumarev, V. P., Levina, A. S., Polistchuk, A. S., Potapov, V. K., Potemkin, G. A., Sredin, Ju. G., and Shabarova, Z. A. (1987) Automatic synthesis of oligodeoxyribonucleotides using phosphoramidite method on "Victoria 4M". *Izv. Sib. Otd. Akad. Nauk SSSR* 2, 119–123.
- (11) Godovikova, T. S., Zarytova, V. F., Mal'tzeva, T. S., and Khalimskaya, L. M. (1989) Reactive derivatives of oligonucleotides with a zwitterionic terminal phosphate group for affinity reagents and probe construction. *Bioorg. Khim.* 15, 1246–1252.
- (12) Horn, T., and Urdea, M. S. (1986) A chemical 5'-phosphorylation of oligodeoxyribonucleotides that can be monitored by trityl cation release. *Tetrahedron Lett.* 27, 4705–4708.
- (13) Chung, T. E., Wu, Y. M., and Chang, C. H. (1984) Reduction of aromatic nitro compounds by ethylenediamine. A new selective reagent for the synthesis of symmetric azo compounds. *J. Org. Chem.* 49, 1215–1217.
- (14) Berkner, K. L., and Folk, W. R. (1977) Polynucleotide kinase exchange reaction. *J. Biol. Chem.* 252, 3176–3184.
- (15) Perillo, I., and Lamdan, S. (1973) Synthesis of 1,2-disubstituted 1,4,5,6-tetrahydropyridines. *J. Heterocycl. Chem.* 10, 915–923.
- (16) Perillo, I., Fernandez, B., and Lamdan, S. J. (1977) Synthesis and properties of 1,2-diaryl-4,5,6,7-tetrahydro-1H-1,3-diazepines and 1,2-diaryl-1,4,5,6,7,8-hexahydro-1,3-diazocines. Comparison with five- and six-membered homologues. *J. Chem. Soc., Perkin Trans. 2*, 2068–2072.

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Synthesis and Properties of an Oligonucleotide Modified with an Acridine Derivative at the Artificial Abasic Site

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The synthesis of an oligodeoxynucleotide (ODN) modified with 2-methoxy-6-chloro-9-aminoacridine (Acr) at an abasic site is described. A stereochemically defined aminodiol, L-threoninol, was used to serve as artificial abasic nucleoside. The molecule was modified so as to be suitable for the standard phosphoramidite method and was incorporated into the interior of an ODN. In addition, *N*-hydroxysuccinimidyl *N*-[9-(6-chloro-2-methoxy)acridinyl]-6-aminocaproate has been synthesized for postsynthetic modification of the amino substrate of the L-threoninol moiety in the ODN. By using absorption spectroscopy, it is shown that oligo(dA) conjugated with acridine binds with complementary strand in a 1:1 ratio. The melting temperature showed that the nonmodified (abasic) duplex is destabilized as a result of lacking in base at the abasic site, but the covalently linked acridine ring compensates for the destabilization effect. The fluorescence quantum yield of the acridine ring was enhanced by connection to oligo(dA) and, further, by formation of a double-strand with the complementary ODN. The quantum yield is larger than that of intermolecular intercalation. The excitation spectra of Acr-ODN in the duplex is quite similar to the absorption spectra. The results indicate that the covalently linked acridine ring is selectively intercalated into the adjacent abasic site.

INTRODUCTION

Oligonucleotides covalently linked with functional molecules have been attracting current interest because of their widespread biological usage as antisense (1) and DNA probes (2) and so on. For example, Hélène et al. (3) have reported that oligodeoxynucleotide (ODN) attached to a phenanthroline-copper chelate at the 5'-termini cleaves DNA by triple-stranded formation showing high specificity. Miller et al. (4) have shown that ODN methylphosphonates covalently linked to psoralen and targeted single-stranded DNA give cross-linked duplexes by cycloaddition reaction under photoirradiation. In these investigations, functional molecules are connected to the oligonucleotide or the derivative so that its functions are selectively directed toward the target position of DNA or RNA. Therefore, it is necessary for improving efficiency and specificity that the molecule should be correctly anchored to the target position. There are important factors for such molecular design: (i) the number and species of linker atoms connecting the functional molecule and ODN; (ii) the linking position of the ODN. It has been previously reported that ODN can covalently link to functional molecules through linker atoms at the 5' or 3' terminus (3–10), base (11–13), internal phosphate (14, 15), sugar 2' (16–20), and non-nucleotide (21–29). The 5' or 3' terminus brings about difficulties for anchoring the molecule at the desired position; the base may interfere with properties such as base pairing and generate at least two interaction sites neighboring the base; the internal phosphate loses the

negative charge of the phosphodiester and forms diastereoisomers, which will give at least two possible structures as a result of duplex formation; and although the sugar 2' may have some advantages for the purpose, derivatives of four nucleosides must be synthesized to accommodate an automated DNA synthesizer. Therefore, one ought to select the non-nucleoside that has no base. It has been indicated that some aromatic rings strongly interact with an abasic site in DNA (30).

In this paper, we describe the synthesis of a stereochemically defined non-nucleoside suitable for the incorporation of a reactive alkyl amine into an oligonucleotide as illustrated in Figure 1. We also report the synthesis of a 9-amino-6-chloro-2-methoxyacridine reagent activated as the succinimidyl ester for postsynthetic modification of the amino substrate. The acridine derivative covalently linked to ODN (Acr-ODN) through a linker was prepared with these reagents. We will also describe the interaction between the covalently linked intercalating agent and the adjacent artificial abasic site in double-stranded DNA using spectroscopic and thermodynamic measurements.

EXPERIMENTAL PROCEDURES

¹H NMR and ³¹P NMR spectra were obtained on a JEOL EX-90 spectrometer, using tetramethylsilane and 85% H₃PO₄ as internal and external standards, respectively. High-performance liquid chromatography (HPLC) was performed on a Shimadzu Model LC/CTO-6A equipped with a Waters 991J 3D-UV detector, using a reversed-phase COSMOSIL AR-300 column (4.6 × 150 mm). Absorption spectra were recorded on a Shimadzu UV-2200 spectrophotometer with a thermoelectrical cell holder. Fluorescence spectra were recorded on a Shimadzu RF-503A spectrofluorometer. Fluorescence lifetimes were determined by a single-photon-counting method on a Horiba NAES-550 with a flash lamp filled with hydrogen. The fluorescence decay curves were analyzed by a deconvolution program.

Synthesis of O¹-(4'-Monomethoxytrityl)-N-[(fluoren-9-ylmethoxycarbonyl)-L-threoninol (4)]. Prepa-

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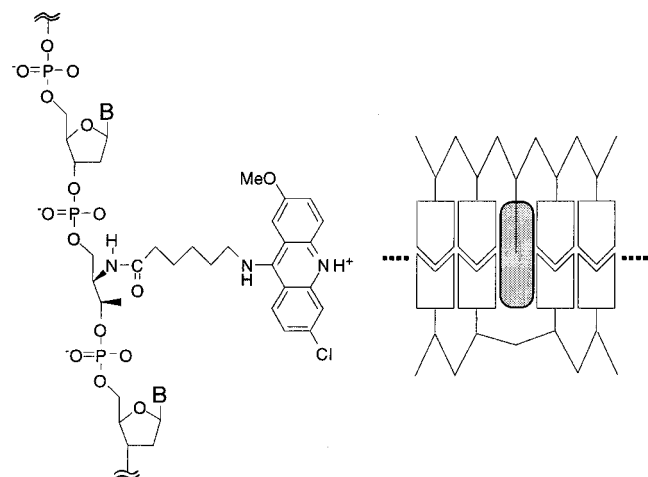


Figure 1. Oligonucleotide containing L-threoninol backbone modified with acridine derivative (left) and schematic representation of selective intercalation into the abasic site (right).

ration of *N*-[(fluoren-9-ylmethoxy)carbonyl]-L-threoninol (**3**) was described elsewhere (28). *N*-[(Fluoren-9-ylmethoxy)carbonyl]-L-threoninol (1.5 g, 4.58 mmol) was co-evaporated with dry pyridine (3 × 15 mL) and then dissolved into dry pyridine (10 mL). Monomethoxytrityl chloride (1.49 g, 4.81 mmol) was added with stirring, and the reaction was allowed to proceed at room temperature for 4 h. Then the reaction was quenched with methanol (1 mL), and stirring was continued for 10 min. The solvent was removed under reduced pressure, and the residue was dissolved into chloroform (50 mL) and washed with 5% sodium hydrogen carbonate solution (3 × 30 mL). The organic layer was dried over anhydrous sodium sulfate and filtered. After filtration and removal of the solvent, the product was purified with silica gel column chromatography (25 × 3.6 cm) eluting with chloroform/hexane (6:4) (2.63 g, 96% yield): ^1H NMR (CDCl_3) δ 1.18 (m, 3H, CH_3), 2.72 (s, 1H, OH), 3.48–3.47 (m, 7H, OCH_2 , NCH, OCH, OCH_3 singlet at 3.76), 4.11–4.46 (m, 3H, CH, CH_2), 5.53 (d, 1H, NH), 6.57–7.79 (m, 2H, Ar).

Synthesis of *O*¹-(4'-Monomethoxytrityl)-*N*-[(fluoren-9-ylmethoxy)carbonyl]-*O*³-[2-cyanoethyl-*N,N*-diisopropylaminophosphinyl]-L-threoninol (5**).** *O*¹-(4'-Monomethoxytrityl)-*N*-[(fluoren-9-ylmethoxy)carbonyl]-L-threoninol (**4**) (1.99 g, 3.32 mmol) was coevaporated with dry acetonitrile (10 mL) and dissolved into dry acetonitrile (10 mL), and then tetrazole (232 mg, 3.32 mmol) and 2-cyanoethylbis(diisopropylamino) phosphite (1.00 g, 3.32 mmol) were added; the mixture was reacted for an hour at room temperature. The reaction mixture was extracted with ethyl acetate (150 mL). The organic layer was washed with saturated sodium hydrogen carbonate solution (150 mL) and dried over anhydrous sodium sulfate. The solution was filtered. The solvent was evaporated, and the product was coevaporated with dry acetonitrile (3 × 10 mL) for removing a small amount of water. The product was used in the DNA synthesizer without further purification (2.41 g, 91% yield): ^1H NMR (CDCl_3) δ 0.97–1.30 (m, 15H, CH_3), 2.27–2.80 (m, 2H, CH_2CN), 3.16–3.76 (m, 11H, POCH_2 , PNCH, OCH_2 , OCH, OCH_3 singlet at 3.76), 4.04–4.47 (m, 3H, CH, CH_2 of Fmoc), 5.53 (d, 1H, NH), 6.75–7.79 (m, 22H, Ar); ^{31}P NMR (CDCl_3) 147.9, 148.2. The only impurities observed were ca. 1% of dimer [(MMTr-Fmoc-L-threoninol)₂POCH₂-CH₂CN, δ_p 140.4] and two hydrolysis products [ca. 1% of MMTr-Fmoc-L-threoninol-P(OH)OCH₂CH₂CN, δ_p 7.5 and ca. 5% of NCCH₂CH₂OP(OH)NiPr₂, δ_p 14.0] assigned from the literature (31).

Synthesis of *N*-[9-(6-Chloro-2-methoxy)acridinyl]-6-aminocaproic Acid Hydrochloride (7**).** 6,9-Dichloro-2-methoxyacridine (**6**) (1 g, 3.60 mmol) and phenol (5 g) were stirred at 100 °C for 40 min, and then 6-aminocaproic acid (500 mg, 3.81 mmol) was added. The reaction mixture was allowed to react at 100 °C for 2 h. The reaction mixture was cooled and dropped slowly to 300 mL of diethyl ether. The precipitate was filtrated, washed with diethyl ether, and then dried in vacuo. The resulting bright yellow solid was recrystallized from diluted HCl solution to give the product as HCl salt (1.24 g, 81% yield): ^1H NMR ($\text{DMSO}-d_6$) δ 1.43–1.87 (m, 6H, CH_2), 2.41–2.28 (t, 2H, CH_2COO), 3.96 (m, NCH₂, OCH_3 singlet at 3.96), 7.41–8.51 (m, 6H, Ar).

Synthesis of *N*-Hydroxysuccinimidyl *N*-[9-(6-Chloro-2-methoxy)acridinyl]-6-aminocaproate Hydrochloride (8**).** *N*-[9-(6-Chloro-2-methoxy)acridinyl]-6-aminocaproic acid hydrochloride (**7**) (500 mg, 1.17 mmol) and *N*-hydroxysuccinimide (183 mg, 1.59 mmol) were suspended in DMF (15 mL). The reaction mixture was cooled at 0 °C, and then 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride (EDC) (304 mg, 1.59 mmol) was added. The reaction mixture was stirred for an hour at 0 °C and reacted at room temperature. After about 2 h, the suspension became a yellow solution. The solution was allowed to react overnight. Acetic acid (2 mL) was added and stirred for 10 min to quench the small excess of EDC. The solvent was removed under reduced pressure, and the reaction mixture was partitioned between chloroform (50 mL) and water (30 mL)/acetic acid (20:1). The organic layer was washed with 3 × 30 mL of water/acetic acid (20:1), dried over anhydrous sodium sulfate, and evaporated to dryness. Purification was performed on silica gel column chromatography (6 × 25 cm) eluting with chloroform/methanol/acetic acid (80:20:3). The resulting gummy residue was dissolved in a minimum amount of chloroform and then dropped in 150 mL of cold ethyl acetate/hexane (1:2). The product was precipitated as a bright yellow solid. The reagent was stored in a desiccator to prevent hydrolysis with water (373 mg, 61% yield): ^1H NMR (CDCl_3) δ 1.72–2.13 (m, 10H, CH_2 , CH_3 of acetic acid), 2.58–2.96 (m, 6H, NCH₂, NCOCH₂), 4.45 (m, 5H, NH, OCH_3), 7.05–8.09 (m, 6H, Ar).

Oligonucleotide Synthesis. *O*¹-(4'-Monomethoxytrityl)-*N*-[(fluoren-9-ylmethoxy)carbonyl]-*O*³-[2-cyanoethyl-*N,N*-diisopropylaminophosphinyl]-L-threoninol (**5**) was coupled using a concentration of 0.15 M on Applied Biosystems Model 391 according to the protocols supplied by the manufacturer. The oligonucleotide was synthesized on a 1.0 μmol scale. The coupling time was set for 5 min. The coupling efficiency was determined by measuring the deprotected dimethoxytrityl cation concentration. The average coupling efficiency was 80%. The 5'-DMTr protecting group of synthesized ODN was left at the end of each synthesis to facilitate purification. Cleavage from the solid support and deprotection of the base, phosphate, and amino substrate of L-threoninol was accomplished with concentrated ammonium hydroxide according to the manufacturer's recommendations. The tritylated oligonucleotide was purified by reversed-phase HPLC on a COSMOSIL AR-300 column (4.6 × 150 mm) eluted with a linear gradient of 6–60% acetonitrile in 0.1 M triethylammonium acetate (pH 7) over 30 min (flow rate 0.8 mL/min). Appropriate fractions were combined and concentrated to dryness. The residue was detritylated in 80% acetic acid (at room temperature, 15 min). Figure 2A shows the analytical HPLC chromatogram for the purified ODN containing L-threoninol backbone with the sequence dA7[]dA7[] ([] means L-threoninol). Split-

Scheme 1

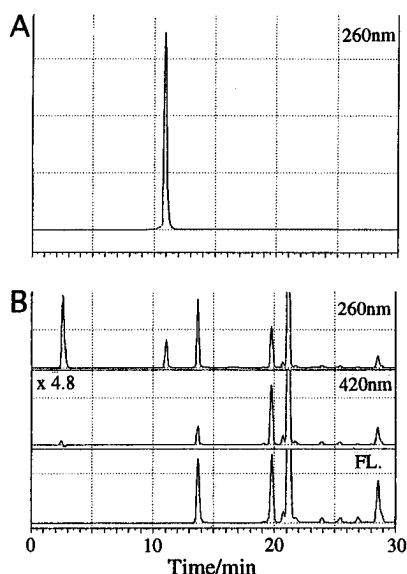
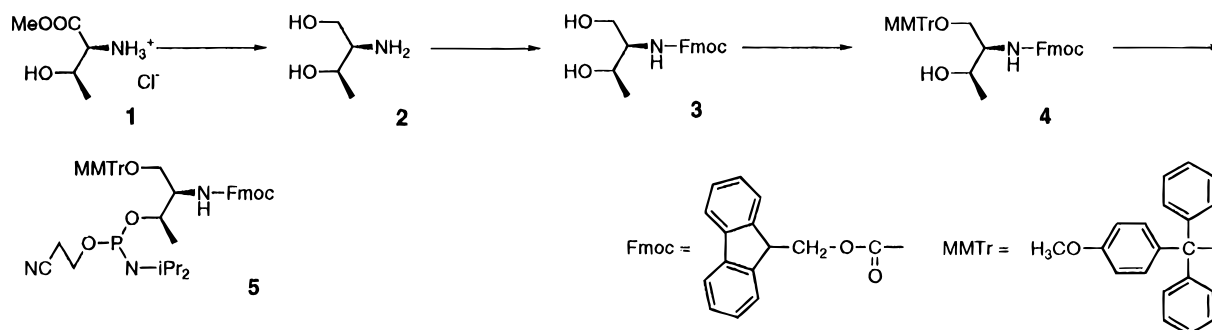


Figure 2. Reversed-phase HPLC chromatograms: (A) analytical HPLC for ODN containing artificial abasic site (da7[]da7); (B) analytical HPLC chromatogram for the reaction mixture containing the ODN and **8** (see text). The HPLC system used a 150×4.5 mm C_{18} column, and the flow rate was 0.8 mL/min; for both HPLC systems a gradient of 6–60% solvent B over 30 min [solvent A = 0.1 M triethylammonium acetate (pH 7.0); solvent B = acetonitrile] was used. Detection was by UV absorbance at 260 and 420 nm and emission (excited at 420 nm, detected at 510 nm).

ting of the peak assigned to optical isomers was not detected. This means that the stereochemistry of L-threoninol is not rearranged during DNA synthesis cycles. The following ODNs were synthesized according to the above method: dT5[]dT5, dT7[]dT7, dA5[]dA5, da7[]da7.

Acridine Modification of the Oligonucleotides. The ODN product was dissolved into 300 μL of 0.15 M HEPES buffer (pH 8) and 270 μL of dimethyl sulfoxide. A 30 μL sample of *N*-hydroxysuccinimidyl *N*-[9-(6-chloro-2-methoxy)acridinyl]-6-aminocaproate hydrochloride (**8**) in dimethyl sulfoxide (ca. 0.5 mg/90 μL) was added to the ODN solution every 2 h for three times. Moreover, the mixture was vortexed at room temperature for 10 h in the dark. The crude acridine-modified ODN was then freed from **8** and a large excess of its hydrolysis product using Sep-Pak C_{18} cartridges. The reaction mixture was dissolved into 1 mL of water and applied to the Sep-Pak cartridge. The cartridge was washed with 4 mL of water, and the product oligomers were eluted with 1.5 mL of 20% acetonitrile in water. The products were further purified by reversed-phase HPLC in the same manner as described above. Figure 2B shows the analytical HPLC chromatogram for the reaction mixture containing the ODN (da7[]da7) and **8** after an hour. The peaks in

both chromatograms at 2.5 and 11 min correspond to *N*-hydroxysuccinimide and unreacted ODN, respectively. The peak at 13.5 min corresponds to ODN modified with **8** (da7[Ac]da7). The peaks at 28.5 and 21.5 min were assigned to **8** and the hydrolysis product **7**, respectively, which was confirmed by coinjections of the authentic samples. It is apparent that the reagent **8** was almost decomposed after an hour in pH 8 buffer solution. Absorption spectra of 21.5 and 28.5 min peaks coincide with those of **7**. On the other hand, the absorption spectrum of Ac-ODN is consistent with the presence of a single acridine ring tethered to the ODN, and the absorption around 430 nm, which is attributed to the absorption of the acridine ring, showed a red shift. The result suggests that the acridine ring appreciably contacts with the neighboring adenosine base even in a single-stranded DNA.

RESULTS

Synthesis of L-Threoninol Phosphoramidite. The synthetic scheme for preparing *O*¹-(4'-monomethoxytrityl)-*N*-[(fluoren-9-ylmethoxy)carbonyl]-*O*³-[2-cyanoethyl-*N,N*-diisopropylaminophosphinyl]-L-threoninol (**5**) is shown in Scheme 1. The starting material for the preparation of Fmoc-L-threoninol (**3**) has been described elsewhere (28). For the purpose of selective protection of the primary alcohol, 4,4'-dimethoxytrityl (DMTr) substrate is usually used, but this substrate was not stable for the primary alcohol even at -5°C . Thus, in the present study, 4-monomethoxytrityl (MMTr) substrate was employed for protecting the primary alcohol. The MMTr substrate was sufficiently stable for at least 6 months at room temperature and could be completely removed by following the standard protocol in DNA synthesizer. Finally, the secondary hydroxyl group was activated with bis(diisopropylamino)-2-cyanoethylphosphine. In the purification of such a product, the solvent containing triethylamine is usually used in silica gel column chromatography to prevent decomposition of the product. The Fmoc groups, however, are slowly cleaved by triethylamine used in chromatography solvent (32). Therefore, the product was coevaporated with dry acetonitrile and used in DNA synthesis without further purification. Purity of the product was estimated with the use of ^1H NMR and ^{31}P NMR.

Acridine Modification Reagent. For the acridine modification of the ODN-containing L-threoninol backbone, *N*-hydroxysuccinimidyl *N*-[9-(6-chloro-2-methoxy)acridinyl]-6-aminocaproate hydrochloride (**8**) was synthesized (Scheme 2). The carboxylic acid and *N*-hydroxysuccinimide was condensed with EDC and then purified with column chromatography on silica gel. In aqueous solvent the succinimidyl ester is hydrolyzed to the carboxylic acid according to the basicity of the solvent (33). On the other hand, in organic solvent, the succinimidyl ester is not hydrolyzed and does not react with

Scheme 2

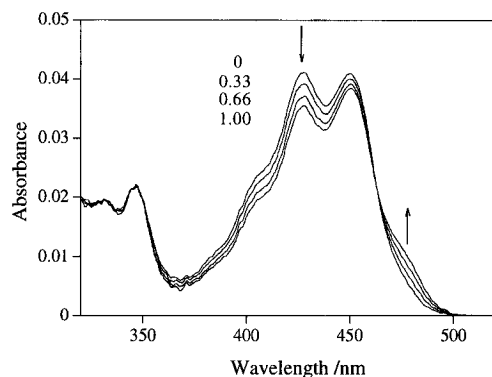
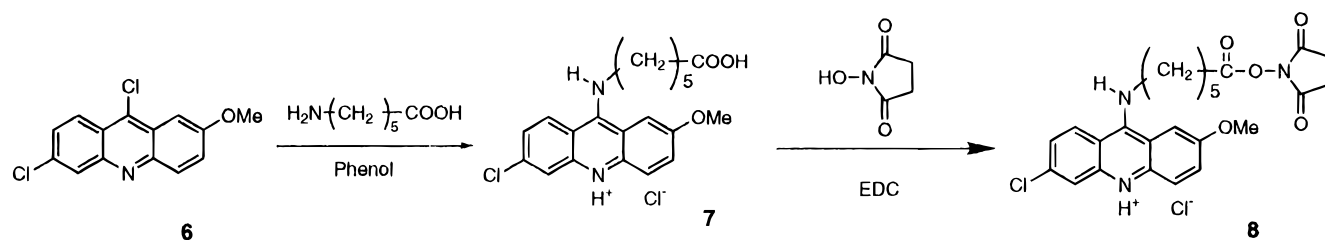


Figure 3. Change in absorption spectra of dA7[Acridine]dA7 upon the addition of dT14. The numbers indicated on the figures indicate the ratio of dT14 to dA7[Acridine]dA7. Measurements were carried out at 0 °C in 10 mM phosphate buffer/150 mM sodium chloride (pH 7.2). A cell of 1 cm path length was used.

alcohol (34). However, **8** was slowly hydrolyzed under the circumstances of humidity and also reacted with methanol during column chromatography, which prevented complete purification. It can be assumed that these reactions are catalyzed by the *N*-pyridyl of the acridine ring. It was found that the use of an eluent for column chromatography containing a few percent of acetic acid suppresses these side reactions. The modified yield estimated from the intensity ratio of the HPLC chart was about 80%. The resulting acridine-modified ODN was separated from impurities with a Sep-Pak C₁₈ cartridge and then with reversed-phase HPLC.

Absorption Spectra. The change in the absorption spectrum of dA7[Acridine]dA7 upon addition of complementary ODN, dT14, is shown in Figure 3. As the concentration of the complementary ODN increased, the absorption at 425 and 450 nm decreased linearly and the new absorption peak at 475 nm appeared. This behavior is quite similar to that of the 5'-tethered acridine ODN (**7**). The decrease in the extinction coefficient definitely ceased at a specific concentration of the complementary ODN. The decrease in the extinction coefficient of the base moiety (260 nm) also ceased at the same concentration of the complementary ODN. The result indicates that Acridine-ODN binds to the complementary ODN in the ratio 1:1 and the acridine ring is selectively intercalated into the double-stranded DNA. The nonmodified (abasic) ODN, dA7[]dA7, also bound to the complementary sequence in the same manner. On the other hand, acridine tethered to the oligo(dT) strand bound to the complementary ODN, oligo(dA), in the ratio at 2:1 at room temperature, suggesting the formation of triple-stranded DNA (35, 36). The degree of hypochromicity at 425 nm was 21%. This value is lower than that of quinacrine and poly(dA-dT) or 5'-tethered acridine (**7**). However, there are adenosine bases adjacent to the acridine ring even in single-stranded DNA, and thus red shift of the absorbance was observed in the single-strand compared with free acridine ring. Therefore, stronger

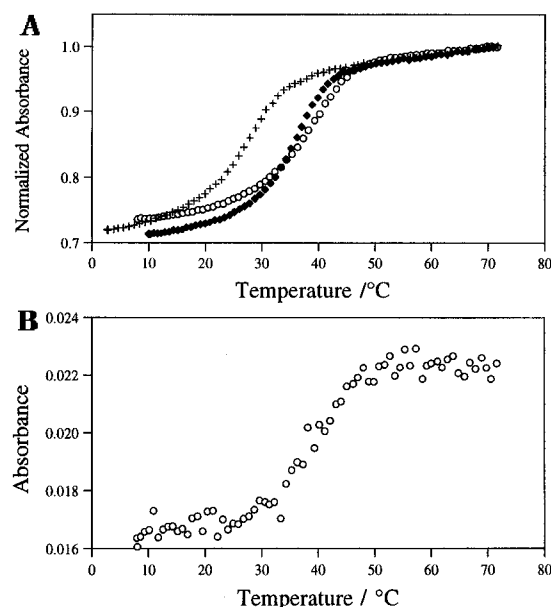


Figure 4. Melting curves measured at 260 nm (A) and 425 nm (B) for complexes of dA7[]dA7 (+), dA7[Acridine]dA7 (O), and dA14 (◆) with dT14. The buffer was the same as that used in Figure 3.

hypochromicity would be obtained if compared with the free acridine ring.

Thermodynamical Properties. A thermal denaturation experiment was conducted to evaluate the effect of the acridine ring incorporated into ODN on the hybridization characteristics (Figure 4). All of the oligonucleotides showed a clear sigmoid. Melting temperatures (*T_m*) of the native, abasic, and Acridine-ODN duplex were 35.6, 26.7, and 37.9 °C, respectively. The *T_m* of nonmodified ODN was lower than that of native DNA. These results are considered to be attributable to the lack of π - π stacking. Acridine-ODN, however, showed a higher *T_m* than that of abasic DNA and native DNA. Figure 4B shows melting curves detected at 425 nm characteristic of the acridine ring. Although there was some noise due to the low absorbance of the acridine ring, the thermodynamic behaviors at 425 and 260 nm were nearly the same. Therefore, it is clearly demonstrated that double-strand formation and acridine intercalation are cooperative. The acridine ring compensates the destabilization resulting from the lack of base.

Photophysical Properties. Figure 5 shows the absorption and fluorescence excitation spectra of the Acridine-ODN duplex detected at 510 nm. These spectra were quite similar, and the shape of the fluorescence excitation is independent of the detected wavelength over 480–540 nm. The results indicate that the acridine ring experiences a single environment in double-stranded DNA.

Fluorescence properties of acridine derivatives, single-stranded Acridine-ODN, and double-stranded Acridine-ODN were determined (Figure 6). The shapes of the spectra are similar, but the intensity strongly depends on the

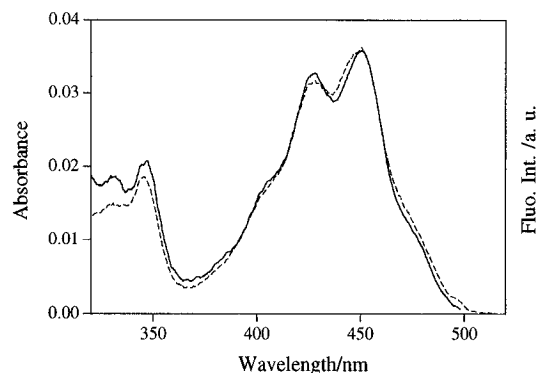


Figure 5. Absorption spectra (solid line) and fluorescence excitation spectra (broken line) of dA7[Acridine]dA7 and dT14 duplex. Excitation was recorded for an emission wavelength of 510 nm. The condition was the same as that used in Figure 3.

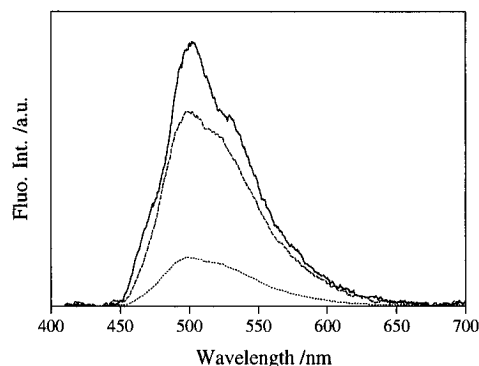


Figure 6. Fluorescence emission spectra of quinacrine (dotted line) and dA7[Acridine]dA7 free of (broken line) and bound to dT14 (solid line). Excitation wavelength was 427 nm. The condition was the same as that used in Figure 3.

chemical environments around the acridine ring. The quantum yields of acridine in single- and double-stranded DNA were 0.375 and 0.276, respectively, although that of the free acridine **7** was only 0.07 at 0 °C. Furthermore, the quantum yield of acridine in double-stranded ODN was higher than that of intermolecular intercalation [quinacrine and poly (dA-dT)] showing 0.27 (in our preliminary check). The increase in emission intensity is consistent with the fact that the acridine ring covalently linked to ODN is effectively intercalated into the double-stranded ODN and, to a significant extent, even in the single strand. In these strands, the acridine ring experiences a hydrophobic environment due to the adenine bases at both ends.

The photophysical and thermodynamical behavior of another Acr-ODN, dA5[Acridine]dA5, was very similar to that above.

DISCUSSION

Many kinds of molecules have been used as handles of DNA at 5', 3', or internucleotide position at which a functional molecule such as pyrene or biotin is linked after DNA synthesis. Nelson et al. were the first to use an "aminodiol" (1-amino-2,3-propanediol) backbone for this purpose (21, 22). They also synthesized the 1,3-propanediol backbone systems to avoid steric hindrance and to mimic the traditional ribose moiety (26). Reed et al. have used an optically pure L-pyrrolidinol backbone derived from the natural amino acid L-proline to prevent the appearance of several optical isomers which necessarily occur from connection of the amino substrate to the alkyldiol backbone (23). Therefore, the backbone should be optically pure if the reagent is used as therapeutic tool or biochemical probe.

The L-threoninol backbone was employed to mimic the spacing of the traditional ribose moiety, which has three carbons between the primary and secondary alcohols, and to suppress the appearance of several optical isomers in the incorporated DNA (Figure 1). The backbone has already been synthesized and used by Reed et al. as non-nucleotide backbone in methylphosphonate (28). The backbone contains a secondary amine which can be modified through the amide linkage. The amino substrate of L-threoninol, (2R,3R)-2-amino-1,3-butanediol, is directed to the major groove, thereby reducing steric hindrance. Then the linker chain could lie along the major groove between the handles and the acridine ring. The backbone is also considered as an artificial abasic nucleoside. An intercalator such as acridine is inserted into DNA by spreading the stacked base (37). At the abasic site, the spreading energy is not required, although the energy would be required on the target strand. Therefore, the abasic site is considered as a "pocket" appropriate to hold the intercalator, where the spreading is not required on the abasic strand. The intercalator covalently linked to the abasic site is widely useful as a pseudobase, which can intercalate to the abasic site selectively and compensate for the lack of base stacking.

9-Amino-6-chloro-2-methoxyacridine has been extensively studied as an intercalating agent in DNA or RNA (38). The optical properties of aminoacridine derivatives are strongly perturbed upon intercalation (39, 40). These properties make the acridines effective as DNA probes and also able to construct electron-transfer (41) or energy-transfer systems in DNA matrix (42). Upon intercalation, the acridine ring lies so as to maximize the stacking area with the bases above and under the acridine ring (37). Hélène et al. have systematically investigated the chain length of oligo(dT) covalently linked to 9-amino-6-chloro-2-methoxyacridine at the 3'-phosphate group. It has been indicated that the chain length of pentamethylene gives the most stable complex (7). To satisfy this condition, the linker arm has to connect the 9-amino substrate of acridine with the amino substrate of the backbone. From the CPK model, we predict that the length of tetramethylene or pentamethylene is suitable for the linker. Acridine caproic acid was synthesized on the basis of this assumption and activated as the succinimidyl ester. Although a phosphoramidite connecting 9-amino-6-chloro-2-methoxyacridine has been reported (43), the acridine modification reagent **8** can modify not only the L-threoninol moiety but also other alkylamines tethered to oligonucleotides previously synthesized (5, 6, 11–13, 16, 17, 21–23, 26).

Both Acr-ODN and nonmodified (abasic) ODN bind to the complementary oligo(dT) with the ratio 1:1. The abasic ODN was strongly destabilized compared with native duplex, which comes from the lack of π - π stacking. This destabilization brought about decrease of T_m by -8.9 °C. This large value is not consistent with the results of Reynolds et al. (28). The discrepancy is possibly due to the difference of double-stranded DNA consisting of, in their case, RNA and methylphosphonate. The results, however, show coincidence with those of Bertrand et al. (44), who used ODNs containing the true abasic site. Linking of the acridine ring at the abasic site increased T_m by 11.2 °C compared with abasic ODN. This increase is comparable with the 14 °C increase reported for a 3'-acridine tailed dT12 ODN (7). In Figure 5, the excitation spectrum of the Acr-ODN as duplex is quite similar to the absorption spectrum. This similarity indicates that the acridine ring is under the influence of

only one kind of environment, although the linker is rather long (7).

The fluorescence quantum yield of the Acr-ODN-forming duplex was larger than that of quinacrine plus poly(dA-dT) as seen in Figure 6. The result indicates that the acridine ring is strongly intercalated. The ratio of the absorption intensities of the peaks at 425 and 450 nm (I_{450}/I_{425}) increases upon the concentration of the complementary ODN. This behavior was different from that of quinacrine plus poly(dA-dT) (45). This difference is considered to be caused by the binding mode. Quinacrine is intercalated through the minor groove of the DNA. From the CPK model, the linker of the Acr-ODN is directed to the major groove. Hence, the acridine ring would be intercalated through the major groove. The acridine ring binding to the duplex with only one mode will give a single-exponential decay in emission. The acridine, however, shows biexponential decays of 26 (96%) and 8 ns (4%) (excited at 450 nm and detected at 510 nm). The lifetime of Acr-ODN was nearly identical with that of quinacrine plus poly(dA-dT), but its ratio of long lifetime components was smaller than that of Acr-ODN. However, the lifetime does not necessarily reflect multiplicity of the binding mode, because 9-amino-6-chloro-2-methoxyacridine alone shows a complex lifetime of emission (46). Therefore, for the investigation of strict coordination of acridine ring, two-dimensional NMR or X-ray crystallography will be required. Moreover, systematic investigations about the chain length will be useful to afford stronger interaction between the acridine ring and DNA. Such studies are now in progress and will be reported in a forthcoming paper.

LITERATURE CITED

- (1) Cohen, J. S., Ed. (1989) *Oligodeoxynucleotides: Antisense Inhibitors of Gene Expression*, CRC Press, Boca Raton, FL.
- (2) Symon, R. H., Ed. (1989) *Nucleic Acid Probes*, CRC Press, Boca Raton, FL.
- (3) François, J.-C., Saison-Behmoaras, T., Barbier, C., Chassignol, M., Thuong, N. T., and Hélène, C. (1989) Sequence-specific recognition and cleavage of duplex DNA via triple-helix formation by oligonucleotides covalently linked to a phenanthroline-copper chelate. *Proc. Natl. Acad. Sci. U.S.A.* 86, 9702–9706.
- (4) Lee, B. L., Murakami, A., Blake, K. R., Lin, S.-B., and Miller, P. S. (1988) Interaction of psoralen-derivatized oligodeoxyribonucleoside methylphosphonates with single-stranded DNA. *Biochemistry* 27, 3197–3203.
- (5) Connolly, B. A. (1987) The synthesis of oligonucleotides containing a primary amino group at the 5'-terminus. *Nucleic Acids Res.* 15, 3131–3139.
- (6) Sinha, N. D., and Cook, R. M. (1988) The preparation and application of functionalised synthetic oligonucleotides: III. Use of H-phosphonate derivatives of protected amino-hexanol and mercapto-propanol or -hexanol. *Nucleic Acids Res.* 16, 2659–2669.
- (7) Asseline, U., Delarue, M., Lancelot, G., Toulmé, F., Thuong, N. T., Montenay-Garestier, T., and Hélène, C. (1984) Nucleic acid-binding molecules with high affinity and base sequence specificity: Intercalating agents covalently linked to oligodeoxynucleotides. *Proc. Natl. Acad. Sci. U.S.A.* 81, 3297–3301.
- (8) Mann, J. S., Shibata, Y., and Meehan, T. (1992) Synthesis and properties of an oligodeoxynucleotide modified with a pyrene derivative at 5'-phosphate. *Bioconjugate Chem.* 3, 554–558.
- (9) Doan, T. L., Perrouault, L., Helene, C., Chassignol, M., and Thuong, N. T. (1986) Targeted cleavage of polynucleotides by complementary oligonucleotides covalently linked to iron-porphyrins. *Biochemistry* 25, 6736–6739.
- (10) Chen, J.-K., Carlson, D. V., Weith, H. L., O'Brien, J. A., Goldman, M. E., and Cushman, M. (1992) Synthesis of an oligonucleotide-intercalator conjugate in which the linker chain is attached via the phenolic hydroxyl group of fagarone. *Tetrahedron Lett.* 33, 2275–2278.
- (11) Roget, A., Bazin, H., and Teoule, R. (1989) Synthesis and use of labelled nucleoside phosphoramidite building blocks bearing a reporter group: biotinyl, dinitrophenyl, pyrenyl and dansyl. *Nucleic Acids Res.* 17, 7643–7651.
- (12) Telser, J., Cruickshank, K. A., Morrison, L. E., and Netzel, T. L. (1989) Synthesis and characterization of DNA oligomers and duplexes containing covalently attached molecular labels: comparison of biotin, fluorescein, and pyrene labels by thermodynamic and optical spectroscopic measurements. *J. Am. Chem. Soc.* 111, 6966–6976.
- (13) Casale, R., and McLaughlin, L. W. (1990) Synthesis and properties of an oligodeoxynucleotide containing a polycyclic aromatic hydrocarbon site specifically bound to the N² amino group of a 2'-deoxyguanosine residue. *J. Am. Chem. Soc.* 112, 5264–5271.
- (14) Letsinger, R. L., and Scott, M. E. (1981) Selectivity in binding a phenanthridinium-dinucleotide derivative to homopolynucleotides. *J. Am. Chem. Soc.* 103, 7394–7396.
- (15) Hodges, R. R., Conway, N. E., and McLaughlin, L. W. (1989) "Post-assay" covalent labeling of phosphorothioate-containing nucleic acids with multiple fluorescent markers. *Biochemistry* 28, 261–267.
- (16) Aurup, H., Tuschl, T., Benseler, F., Ludwig, J., and Eckstein, F. (1994) Oligonucleotide duplexes containing 2'-amino-2'-deoxycytidines: thermal stability and chemical reactivity. *Nucleic Acids Res.* 22, 20–24.
- (17) Manoharan, M., Guinasso, C. J., and Cook, P. D. (1991) Novel functionalization of the sugar moiety of nucleic acids for multiple labeling in the minor groove. *Tetrahedron Lett.* 32, 7171–7174.
- (18) Yamana, K., Nishijima, Y., Ikeda, T., Gokota, T., Ozaki, H., Nakano, H., Sangen, O., and Shimidzu, T. (1990) Synthesis and interactive properties of an oligonucleotide with anthraquinone at the sugar fragment. *Bioconjugate Chem.* 1, 319–324.
- (19) Yamana, K., Ohashi, Y., Nunota, K., Kitamura, M., Sangen, O., Nakano, H., and Shimidzu, T. (1991) Synthesis of oligonucleotide derivatives with pyrene group at sugar fragment. *Tetrahedron Lett.* 32, 6347–6350.
- (20) Divakar, K. J., Mottoh, A., Reese, C. B., and Sanghvi, Y. S. (1990) Approaches to the synthesis of 2'-thio analogues of pyrimidine ribosides. *J. Chem. Soc., Perkin Trans. 1*, 969–974.
- (21) Nelson, P. S., Sherman-Gold, R., and Leon, R. (1989) A new and versatile reagent for incorporating multiple primary aliphatic amines into synthetic oligonucleotides. *Nucleic Acids Res.* 17, 7179–7186.
- (22) Nelson, P. S., Frye, R. A., and Liu, E. (1989) Bifunctional oligonucleotide probes synthesized using a novel CPG support are able to detect single base pair mutations. *Nucleic Acids Res.* 17, 7187–7194.
- (23) Reed, M. W., Adams, A. D., Nelson, J. S., and Mayer, Rich B., Jr. (1991) Acridine- and cholesterol-derivatized solid supports for improved synthesis of 3'-modified oligonucleotides. *Bioconjugate Chem.* 2, 217–225.
- (24) Korshun, V. A., Pestov, N. B., Birikh, K. R., and Berlin, Y. A. (1992) Reagent for introducing pyrene residues in oligonucleotides. *Bioconjugate Chem.* 3, 559–562.
- (25) Theisen, P., McCollum, C., Upadhyaya, K., Jacobson, K., Vu, H., and Andrus, A. (1992) Fluorescent dye phosphoramidite labelling of oligonucleotides. *Tetrahedron Lett.* 33, 5033–5036.
- (26) Nelson, P. S., Kent, M., and Muthini, S. (1992) Oligonucleotide labeling methods 3. Direct labeling of oligonucleotides employing a novel, non-nucleosidic, 2-aminobutyl-1,3-propanediol backbone. *Nucleic Acids Res.* 20, 6253–6259.
- (27) Misiura, K., Durrant, I., Evans, M. R., and Gait, M. J. (1990) Biotinyl and phosphotyrosinyl phosphoramidite derivatives useful in the incorporation of multiple reporter groups on synthetic oligonucleotides. *Nucleic Acids Res.* 18, 4345–4354.
- (28) Reynolds, M. A., Beck, T. A., Hogrefe, R. I., McCaffrey, Arnold, L. J., Jr., and Vaghefi, M. M. (1992) A non-nucleotide-based linking method for the preparation of psoralen-deriva-

- tized methylphosphonate oligonucleotides. *Bioconjugate Chem.* 3, 366–374.
- (29) Gamper, H. B., Reed, M. W., Cox, T., Virosco, J. S., Adams, A. D., Gall, A. A., Scholler, J. K., and Mayer, R. B., Jr. (1993) Facile preparation of nuclease resistant 3' modified oligodeoxynucleotides. *Nucleic Acids Res.* 21, 145–150.
- (30) Letellier, R., Taillandier, E., Bertrand, J. R., and Malvy, C. (1991) Molecular modelling of 9-aminoellipticine interactions with abasic oligonucleotides. *J. Biomol. Struct. Dyn.* 9, 579–597.
- (31) Nielsen, J., Taagaard, M., Marugg, J. E., Boom, J. H. v., and Dahl, O. (1986) Application of 2-cyanoethyl *N,N,N',N'*-tetraisopropylphosphorodiamidite for in situ preparation of deoxyribonucleoside phosphoramidites and their use in polymer-supported synthesis of ligodeoxyribonucleotides. *Nucleic Acids Res.* 14, 7391–7403.
- (32) Hébert, N., Davis, P. W., DeBaets, E. L., and Acevedo, O. L. (1994) Synthesis of N-substituted hydroxyprolinol phosphoramidites for the preparation of combinatorial libraries. *Tetrahedron Lett.* 35, 9509–9512.
- (33) Lomants, A. J., and Fairbanks, G. (1976) Chemical probes of extended biological structures: synthesis and properties of the cleavable protein cross-linking reagent [³⁵S]dithiobis(succinimidyl propionate). *J. Mol. Biol.* 104, 243.
- (34) Brinkley, M. (1992) A brief survey of methods for preparing protein conjugates with dyes, haptens, and cross-linking reagents. *Bioconjugate Chem.* 3, 2–13.
- (35) Doan, T. L., Perrouault, L., Praseuth, D., Habhou, N., Decout, J.-L., Thuong, N. T., Lhomme, J., and Hélène, C. (1987) Sequence-specific recognition, photo-cross-linking and cleavage of the DNA double helix by an oligo-[α]-thymidylate covalently linked to an azidoproflavine derivative. *Nucleic Acids Res.* 15, 7749–7760.
- (36) Asseline, U., Hau, J.-F., Czernecki, S., Diguarher, T. L., Perlat, M.-C., Valery, J.-M., and Thuong, N. T. (1991) Synthesis and physicochemical properties of oligonucleotides built with either α -L or β -L nucleotides units and covalently linked to an acridine derivative. *Nucleic Acids Res.* 19, 4067–4074.
- (37) Sarma, R. H., Ed. (1979) *Nucleic Acid Geometry and Dynamics*, pp 289–323, Pergamon Press, Oxford, U.K.
- (38) Steck, E. A. (1972) *Chemotherapy of Protozoan Disease*, pp 77–123, U.S. Government Printing Office, Washington, DC.
- (39) Kubota, Y., Motoda, Y., Shigemune, Y., and Fujisaki, Y. (1979) Fluorescence quenching of 10-methylacridinium chloride by nucleotides. *Photochem. Photobiol.* 29, 1099–1106.
- (40) Georgiou, S. (1977) Interaction of acridine drugs with DNA and nucleotides. *Photochem. Photobiol.* 26, 59–68.
- (41) Murphy, C. J., Arkin, M. R., Jenkins, Y., Ghatlia, N. D., Bossmann, S. H., Turro, N. J., and Barton, J. K. (1993) Long-range photoinduced electron transfer through a DNA helix. *Science* 262, 1025–1029.
- (42) Brun, A. M., and Harriman, A. (1994) Energy- and electron-transfer processes involving palladium porphyrins bound to DNA. *J. Am. Chem. Soc.* 116, 10383–10393.
- (43) Thuong, N. T., and Chassignol, M. (1988) Solid phase synthesis of oligo- α - and oligo- β -deoxynucleotides covalently linked to an acridine. *Tetrahedron Lett.* 29, 5905–5908.
- (44) Bertrand, J.-R., Vasseur, J.-J., Rayner, B., Imbach, J.-L., Paoletti, J., Paoletti, C., and Malvy, C. (1989) Synthesis, thermal stability and reactivity toward 9-aminoellipticine of double-stranded oligonucleotides containing a true abasic site. *Nucleic Acids Res.* 17, 10307–10319.
- (45) Baldini, G., Doglia, S., Dolci, S., and Sassi, G. (1981) *Biophys. J.* 36, 465–477.
- (46) Sun, J.-S., Rougée, M., Delarue, M., Montenay-Garestier, T., and Hélène, C. (1990) Solvent relaxation around excited 2-methoxy-6-chloro-9-aminoacridine in aqueous solvent. *J. Phys. Chem.* 94, 968–977.

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Cyanine-Labeling Reagents: Sulfo benzindocyanine Succinimidyl Esters

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A synthetic method for shifting the absorption and emission wavelengths of cyanine dye labels by 15–30 nm to the red has been developed. This step significantly increases the potential for preparing fluorescent probes for multiparameter analysis in cytometry and diagnostics. The new sulfo benzindocyanine dyes contain succinimidyl esters as reactive groups and can be readily conjugated to antibodies, avidin, modified DNA, and other amino group-containing materials. The labeling reagents are water soluble, and their fluorescence is not sensitive to pH. One of the reagents, Cy3.205, can be optimally excited with the 568 nm Kr laser line and is useful for confocal microscopy and flow cytometry. Another dye, Cy5.205, can be excited with the 633 He-Ne or 647 nm Kr laser lines available with many flow cytometers and laser-scanning microscopes. New laser diodes emitting near 660–690 nm should also be excellent excitation sources for Cy5.205.

INTRODUCTION

Bright and water soluble fluorescent labeling reagents are important for sensitive detection with fluorescent antibodies and DNA probes. A group of such probes that emit in different regions of the spectrum, and can be independently detected, provide a powerful tool for correlating multiple antigenic or genetic parameters in individual cells and tissues.

Cyanine dyes have an advantage for designing multi-color probes because they can be tuned to desired wavelengths by altering the heterocyclic nucleus and the number of double bonds in the polymethine chain. So far, we have focused our development on the dyes using indolenine as a heterocyclic base, but this approach has left several gaps in the visible and near-infrared regions of the spectrum (1–4). The photographic industry literature describes cyanine dyes containing benzindolenine base that absorb 30 nm toward longer wavelength than the corresponding cyanine dyes of the indolenine base (5). While our investigation was in progress, Narayanan and Patonay (6) published the syntheses and use of benzindolenine-based heptamethinecyanines as near-infrared (NIR) fluorescent labels. However, they lack water solubility required to avoid dye–dye interaction and nonspecific binding. Using the successful sulfoindocyanine labels as models, we have prepared benzindolenine cyanine active esters with two or four aryl sulfonate groups at various positions on the heterocyclic rings to increase water solubility.

EXPERIMENTAL PROCEDURES

Chemicals, Antibodies, and Media. 6-Amino-2-naphthalenesulfonic acid, 5-amino-1-naphthalenesulfonic acid, and 6-amino-1,3-naphthalenedisulfonic acid disodium salt were purchased from TCI American, Portland,

OR. Sheep γ -globulin (IgG) was purchased from Sigma Chemical Co., St. Louis, MO. Dowex-50 (8% cross-link, 100–200 mesh, moisture content of 50%), disuccinimidyl carbonate (DSC), triethyl orthoformate, 6-bromohexanoic acid, and dry solvents diethyl ether and ethyl acetate were purchased from Aldrich Chemical Co., Milwaukee, WI. Trimethoxypropene was purchased from Eastman Kodak, Rochester, NY. Texas Red was purchased from Molecular Probes, Eugene, OR. All other solvents were purchased from Fisher Scientific, Pittsburgh, PA. Monoclonal antibodies were purchased from Becton Dickinson, Mountainview, CA. Hank's balanced salt solution (HBSS) buffer solution was purchased from Gibco, Long Island, NY.

Purification and Spectroscopic Analysis. Purification of the dyes for spectroscopic analysis was performed on a Spectra-Physics model SP8700 analytical high-performance liquid chromatography (HPLC) unit equipped with a C8-RP column. Purification could also be achieved by conventional or flash column chromatography on a C18-RP powder (Analtech, Newark, DE). Water/methanol mixtures were used for elution in all experiments. Dyes were recovered from the fractions on a rotary evaporator at 60–70 °C without appreciable loss. Although these purification procedures isolate the fluorophore from various intermediates and precursors, these multi-ionic dyes typically associate with alkali metal cations, halide anions, and water of hydration in non-stoichiometric amounts. Therefore, it is essential to obtain the final product in a form that is chemically well-defined for elemental and mass spectrometric analysis. This was accomplished by passing the dye with unknown counterion composition through a Dowex-50W hydrogen, strongly acidic cation exchange column that had been previously washed with 0.1 N sulfuric acid and then distilled water. For example, a pure sample of Cy5.205. OH (**X**) was obtained by ion-exchange chromatography for molecular weight determination. Mass spectral determination was made at the Midwest Center for Mass Spectrometry at the University of Nebraska—Lincoln, Lincoln, NE, with partial support from NSF Grant DIR9017262.

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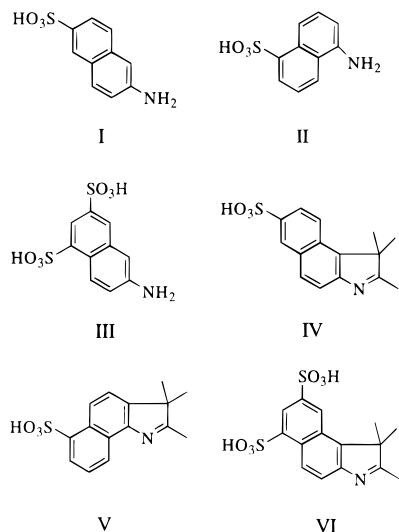


Figure 1. Structures of sulfonaphthylamines and 1,1,2-trimethylsulfobenzindoles used in the cyanine dye synthesis.

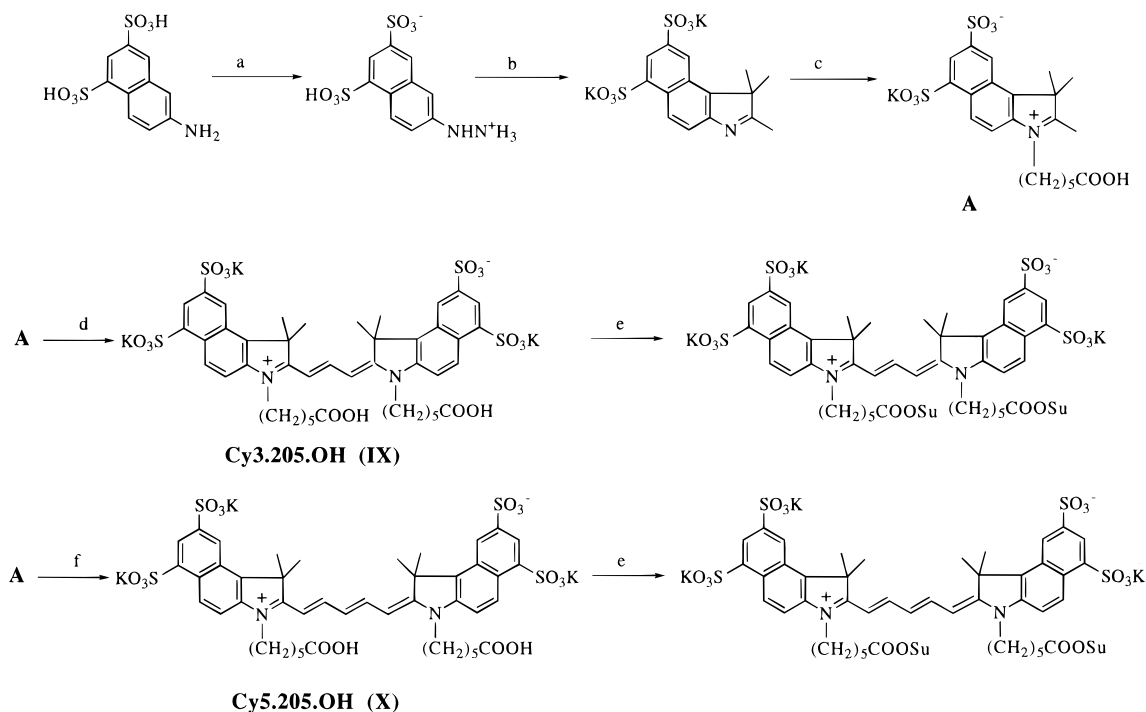
Ultraviolet–visible spectra were measured on a Hewlett-Packard HP 8452 diode array spectrophotometer. The proton NMR spectra were obtained on an IBM 300 FT-NMR spectrometer. NMR signals are described by use of s for singlet, d for doublet, t for triplet, q for quartet, and m for multiplet and are expressed in δ with TMS as internal standard. Fluorescence measurements were performed using a Spex Fluorolog 2 system. Quantum yields were determined as previously described (3, 4). Extinction coefficients in Table 2 were determined from absorbance values of HPLC-purified, ion-exchange-purified, weighted samples of dried material and their molecular weights. Flow cytometry was performed on a dual laser FACS 440 instrument (Becton Dickinson) equipped with an Ar⁺ and a Kr⁺ laser.

General Syntheses. Three new pentamethine cyanines (**VII**, **VIII**, and **X**) and one trimethine cyanine (**IX**)

were synthesized using sulfonated naphthylamines (**I–III**, Figure 1) as starting materials. The general synthesis of the reactive dye involves five steps with slight modifications of methods described previously (1–4). Following are the representative procedures for the syntheses of disuccinimidyl esters, Cy3.205.OSu (**IX**), and Cy5.205.OSu (**X**). The reaction sequence and the conversion of carboxylic acids to succinimidyl esters are shown in Figure 2.

(a) *Preparation of 6-Hydrazinonaphthalene 1,3-Disulfonate.* A suspension of naphthylamine (**III**) (10 g, 0.033 mol) in 200 mL of 50% hydrochloric acid was stirred and maintained at 0–5 °C. A solution of sodium nitrite (2.5 g, 0.036 mol in 10–20 mL of water) was added dropwise. The rate of addition was controlled to keep the temperature from rising above 5 °C. Cooling was continued while a solution of stannous chloride (8.2 g, 0.036 mol in 10 mL of concentrated HCl) was added dropwise to the stirred mixture at a rate to keep the temperature at or below 5 °C. After the addition of stannous chloride, stirring was continued for 1 h. The product was concentrated on a rotary evaporator and triturated with hot 2-propanol until free powder was obtained. λ_{max} (water): 279, 353 nm. ¹H NMR (DMSO-*d*₆): δ 8.72 (d, 1H, *J* = 9.2 Hz, 8-H), 8.1 (d, *J* = 1.2 Hz, 1H, 4-H), 7.95 (s, 1H, 2-H), 7.3 (s, 1H, 5-H), 7.15 (d, 1H, *J* = 9.2 Hz, 7-H). *R*_f: 0.83 (RP-C18, water).

The disodium salt of 6-amino-1,3-naphthalenedisulfonic acid (**III**) has a very poor solubility in concentrated hydrochloric acid. Sodium free base was obtained by ion-exchange chromatography. Cation-exchange resin, Dowex-50 (8% cross-link, 100–200 mesh, moisture content of 50%), was washed in a beaker with 0.1 M sulfuric acid (3 × 350 mL), packed in a glass column (25 × 1.5 cm), and eluted with distilled water (approximately 1000 mL) until eluent was neutral. 6-Amino-1,3-naphthalenedisulfonic acid disodium salt (25 g in 200 mL of water) was loaded onto the column and eluted with distilled water (450 mL) at the rate of 2 mL/min. The first fraction of



a) NaNO₂/HCl, 0–5 °C; b) CH₃COCH(CH₂)₂/KOAc/CH₃COOH, 100 °C; c) Br(CH₂)₅COOH/C₆H₄Cl₂, 110 °C; d) CH(OC₂H₅)₃/pyridine, 80 °C; e) DSC/pyridine 55–60 °C; f) CH₃O-CH=CH-CH=CH(OCH₃)₂/pyridine, 80 °C

Figure 2. Scheme showing the structures and methods of preparation of disuccinimidyl esters of sulfonated benzindocyanines.

100 mL was discarded (colorless neutral solution). The second fraction (300 mL, yellow color, strongly acidic) was collected and dried on a rotary evaporator, yielding a pale yellow solid of the free acid almost quantitatively.

(b) *Preparation of 1,1,2-Trimethylbenzindoleninium 1,3-Disulfonate (VI)*. A mixture containing the hydrazine (6.2 g, 0.02 mol), methyl isopropyl ketone (6.4 mL, 0.06 mol), potassium acetate (5.88 g, 0.06 mol), and glacial acetic acid (35 mL) was refluxed at 135 °C for 24 h. Acetic acid was removed on a rotary evaporator under vacuum and the solid stirred with 2-propanol until free powder was obtained. Gray powder was filtered off and dried (1.95 g, 21%). $\lambda_{\max}$ (water): 254 nm. ^1H NMR (D_2O): δ 8.8 (d, 1H, $J = 7$ Hz, 4-H), 8.7 (s, 1H, 9-H), 8.45 (s, 1H, 7-H), 7.9 (d, 1H, $J = 7$ Hz, 5-H), 2.4 (s, 3H, CH_3), 1.5 (s, 6H, $(\text{CH}_3)_2$). R_f : 0.46 (RP-C18, water).

(c) *Preparation of N-(γ -Carboxypentynyl)-1,1,3-Trimethylbenzindoleninium 6,8-Disulfonate (Structure A in Figure 2)*. The dipotassium salt of 1,1,2-trimethylbenzindoleninium-6,8-disulfonic acid (VI) (4.6 g, 0.01 mol) and 6-bromohexanoic acid (4.9 g, 0.025 mol) in 1,2-dichlorobenzene was heated at 145 °C for 24 h in a nitrogen atmosphere. The mixture was cooled, 1,2-dichlorobenzene was decanted, and the solid was washed with ether, until free powder was obtained (3.91 g, 75%). $\lambda_{\max}$ (water): 202, 226, 252 nm. ^1H NMR (D_2O): δ 8.9 (s, 1H, 9-H), 8.8 (d, 1H, $J = 7$ Hz, 4-H), 8.45 (s, 1H, 7-H), 7.7 (d, 1H, $J = 7$ Hz, 5-H), 4.2 (t, 2H, $J = 7.5$ Hz, $\alpha\text{-CH}_2$), 2.25 (t, 2H, $J = 7.5$ Hz, $\epsilon\text{-CH}_2$), 1.99 (m, 2H, $\beta\text{-CH}_2$), 1.35–1.66 (m, 10H, $\gamma\text{-CH}_2$, $\delta\text{-CH}_2$ with a sharp singlet at 1.61 for $(\text{CH}_3)_2$). R_f : 0.60 (RP-C18, 10% methanol in water).

(d) *Preparation of Cy3.205.OH (IX)*. A mixture of quaternized product A (2 g, 3.8 mmol) and triethyl orthoformate (0.3 g, 2 mmol) in 5 mL of pyridine was heated to reflux for 2 h. The mixture was cooled and diluted with diethyl ether (100 mL). The precipitated product was filtered and dried (1.9 g). The crude product was dissolved in water (10 mL) and chromatographed on a reversed-phase C18 column (30 g) using a 5 to 20% gradient of methanol in water to yield 0.34 g (16%) of IX. The reversed-phase (C18) TLC showed a single spot. R_f : 0.6 (17% methanol in water). $\lambda_{\max}$ (water): 586 nm ($\epsilon = 120\,000\text{ L mol}^{-1}\text{ cm}^{-1}$). $\lambda_{\max}$ (PBS): 578 nm ($\epsilon = 125\,000\text{ L mol}^{-1}\text{ cm}^{-1}$). ^1H NMR of the ion-exchange purified dye was recorded in deuterated water: δ 8.6–8.9 (m, 5H, 9-H, 9'-H, 4-H, 4'-H, $\beta\text{-H}$ of the bridge), 8.45 (s, 2H, 7-H, 7'-H), 7.8 (d, 2H, $J = 7$ Hz, 5-H, 5'-H), 6.2 (d, 2H, $J = 7$ Hz, $\alpha\text{-H}$, $\alpha'\text{-H}$ of the bridge), 4.2 (m, 4H, N-CH_2 , N'-CH_2), 1.3–2.5 (singlet at 2.2 for two $(\text{CH}_3)_2$ is merged in a broad multiplet for eight CH_2 groups).

Preparation of Cy5.205.OH (X). A mixture of quaternized product A (8.0 g, 0.015 mol) and pyridine (10 mL) was heated to reflux. 1,3,3-Trimethoxypropene (1 mL) was added every 30 min over 2.5 h (total of 4 mL). The mixture was cooled and diluted with diethyl ether. The precipitated product was filtered and dried at reduced pressure to yield the dye (4.8 g). The crude product was dissolved in water (25 mL) and chromatographed on a reversed-phase C18 column (100 g) using a 5 to 20% gradient of methanol in water to yield 0.6 g (7%) of X. The reversed-phase TLC showed a single spot, R_f : 0.74 (3/1 water/methanol). Low-resolution FAB (LRFAB) gave m/e of 1003 ($\text{C}_{45}\text{H}_{50}\text{N}_2\text{O}_{16}\text{S}_4$ requires 1003.13) using triethanolamine as matrix. $\lambda_{\max}$ (PBS): 676 nm ($\epsilon = 190\,000\text{ L mol}^{-1}\text{ cm}^{-1}$). $\lambda_{\max}$ (methanol): 680 nm ($\epsilon = 195\,000\text{ L mol}^{-1}\text{ cm}^{-1}$). The absorption and emission spectra of the dye remained unchanged in 0.1 N HCl and 0.1 N NaOH solutions, indicating the dye is insensitive to pH. ^1H NMR (D_2O): δ 8.85 (d, 2H, $J = 7$ Hz, 4-H, 4'-H), 8.7 (s, 2H, 9-H, 9'-H), 8.35 (s, 2H, 7-H, 7'-H), 8.2 (t,

2H, $J = 13$ Hz, $\beta\text{-H}$, $\beta'\text{-H}$ of the bridge), 7.7 (d, 2H, $J = 7$ Hz, 5-H, 5'-H), 6.15 (t, 1H, $J = 13$ Hz, $\gamma\text{-H}$ of the bridge), 6.1 (d, 2H, $J = 13$ Hz, $\alpha\text{-H}$, $\alpha'\text{-H}$ of the bridge), 4.2 (broad triplet, 4H, N-CH_2 , N'-CH_2), 2.25 (broad m, 4H, $\epsilon\text{-CH}_2$, $\epsilon'\text{-CH}_2$), 1.99 (m, 4H, $\beta\text{-CH}_2$, $\beta'\text{-CH}_2$), 1.3–2.2 (m, 24H, six CH_2 and two $(\text{CH}_3)_2$).

(e) *Preparation of Disuccinimidyl Ester*. In a typical experiment, a dye was dissolved in a mixture of dry DMF (2 mL per 100 mg of dye) and dry pyridine (0.1 mL per 100 mg of dye). Disuccinimidyl carbonate (DSC) (3 equiv) was added, and the mixture was stirred at 55–60 °C for 90 min, under a nitrogen atmosphere. After dilution of the mixture with dry ethyl ether, the supernatant was decanted. The product was washed repeatedly with dry ether, filtered under a nitrogen atmosphere, and dried. Nearly quantitative yields of active esters were obtained.

The formation of the active ester was confirmed by its reaction with benzylamine in dry DMF. For example, Cy5.205.OSu was dissolved in DMF (1 mg per 100 μL), and the aliquot (10 μL) was allowed to react with 10 μL of benzylamine in DMF (10%) for 30 min. The conjugate was precipitated with dry ethyl ether and centrifuged. A reversed-phase C18 plate was spotted with conjugate, succinimidyl ester, and hydrolyzed carboxylate product for comparison and developed with 25% methanol in water. R_f values for benzylamine conjugate and free dye Cy5.205.OH are 0.1 and 0.7, respectively. Since activation was sometimes incomplete, reversed-phase HPLC was also used to determine the percentage of the fluorochrome in the active ester form. The sample was eluted through an Alltech Econosphere 250 mm $\times$ 4.6 mm C18 RP column using a mixture of 25% acetonitrile/75% water containing 0.1% trifluoroacetic acid. The percentages of the activated and unactivated fluorophores were determined by integration of the absorbance signals from a Varian UV/vis detector. Alternatively, the percentages can be determined by TLC. For example, the diester Cy5.205.OSu (X) was dissolved in water containing 0.1% trifluoroacetic acid (1 mg per 100 μL). A small portion (25 μL) was loaded as a small strip on a C18 reversed-phase, 5 $\times$ 10 cm TLC plate. The plate was developed using a mixture of acetonitrile/water (2.5/7.5) containing 0.1% trifluoroacetic acid. The bands containing free acid, monosuccinimidyl ester and disuccinimidyl ester were scrapped from the TLC plate and extracted in equal volumes of methanol (5 mL). The absorbance of each extract was measured at 680 nm, and the fractions of the free acid and active esters were calculated from the relative absorbances of the extracts. The R_f values for Cy5.203 acid and the mono- and disuccinimidyl esters are 0.8, 0.3, and 0.15, respectively.

General Protein-Labeling Procedures. Stock solutions of the sulfobenzindocyanine succinimidyl esters were made in dry DMF (1 mg per 100 μL) and were stable for days when stored at 4 °C in a desiccator. The esters are also stable in distilled water for several hours, provided the pH of the solution is not basic. The aqueous solution of the dyes can be used for labeling antibodies if use of DMF is not suitable for certain antibody labeling. The concentration of the reactive dye in a stock solution was determined by measuring the absorbance of an aliquot of the appropriately diluted stock solution in phosphate-buffered saline (PBS) and using the extinction coefficient of the dye (Table 1). Generally, antibody-labeling reactions were carried out in 0.1 M carbonate/bicarbonate buffer (pH 9.4) for 15 min at room temperature. One milligram of sheep IgG (6.45×10^{-6} mM) was dissolved in 0.25–1 mL of buffer solution, and the desired amount of dye (e.g. 5–25 μL of the 1 mg per 100 μL of stock solution) was added during vigorous vortex mixing.

Table 1. Spectral Properties of Sulfobenzindocyanine Dyes

dye	solvent	$\lambda_{\max}$ (nm)	$E_{m\max}$ (nm)	ϵ (L mol ⁻¹ cm ⁻¹)	QY (ϕ)
Cy5.203	PBS	680	702 ^a	190 000	0.10 ^c
	MeOH	686	708 ^a	195 000	0.21 ^c
Cy5.204	PBS	682	707 ^a	197 000	0.10 ^c
	MeOH	686	708 ^a	194 000	0.23 ^c
Cy3.205	PBS	578	593 ^a	120 000	0.14 ^d
	MeOH	586	602 ^a	125 000	0.28 ^d
Cy5.205	PBS	674	694 ^b	190 000	0.23 ^c
	MeOH	680	704 ^b	195 000	0.24 ^c

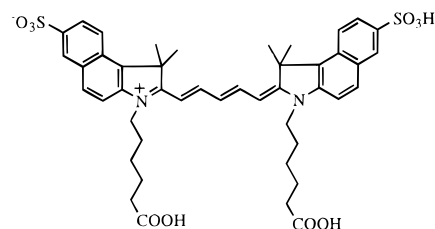
^a Excited at 632 nm. ^b Excited at 514 nm. ^c Based on Di-S-C2(5) as standard. ^d Based on rhodamine-6G as standard.

Unconjugated dye was separated from the labeled protein by gel permeation chromatography over Sephadex G-50 (0.7 × 20 cm column) using pH 7 buffer solution as eluant. A series of labeling reactions were set up as described in a procedure above for different dye/protein ratios (5–25 mol of dye per mole of protein). Absorption spectra of the labeled antibody solutions were recorded. The dye/protein ratio for each sample was determined by using the equation below with measured values of absorbance of the labeled dye (Cy3.205 at 586 nm or Cy5.205 at 676 nm) and the absorbance of protein at 280 nm.

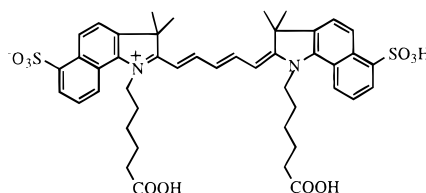
$$\frac{D}{P} = \frac{A_{\text{dye}} E_{\text{prot}}}{(A_{280} - X A_{\text{dye}}) E_{\text{dye}}}$$

The factor *X* in the denominator accounts for dye absorption at 280 nm which is a percent of the absorption of the dye at its maximum absorption (*A*_{dye}). The value of *X* is 0.13 for Cy5.205 and 0.23 for Cy3.205. This value is only 0.05 for sulfoindolenine-based cyanine dyes (*I*). The significant high absorbance at 280 nm for these dyes and blue fluorescence when excited in the UV region indicate that the properties of the benzindole moiety are retained to a greater extent. The average number of dye molecules bound per antibody molecule obtained with bis reactive Cy5.205.OSu under these labeling reaction conditions is shown in Figure 4. The efficiency of labeling under these conditions (pH 9.4) is about 35–40%.

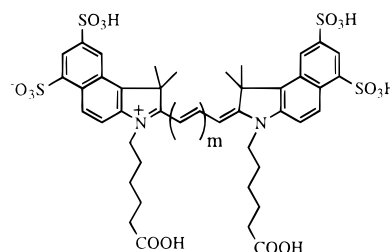
Cell Preparation and Flow Cytometry. Flow cytometry was performed on a dual laser FACS 440 instrument equipped with an Ar⁺ and a Kr⁺ laser. Lymphocyte populations were selected on the basis of forward and side scatter characteristics, and total T cell populations were identified using a biotinylated monoclonal antibody and streptavidin labeled with either Texas Red (TR) or Cy3.205.OSu. Mononuclear leukocytes were obtained from the whole blood of healthy volunteers using histopaque separation medium (Sigma). Antibody was added to 1 × 10⁶ cells per 100 mL of Hank's balanced salt solution containing 2% fetal bovine serum and 0.1% sodium azide (monoclonal wash) and incubated at 4 °C for 45 min. Optimal concentrations of streptavidin were determined by titration. After primary and secondary labeling, the cells were washed twice with monoclonal wash. Prior to analysis on the FACS, cell preparations were fixed with 1% paraformaldehyde in HBSS. The argon laser was emitting 400 mW at 488 nm, and the Krypton laser was emitting 200 mW at 568 nm. Texas Red and Cy3.205 fluorescence was collected using the delayed fluorescence signal from the Krypton laser. Data was collected and analyzed using the Consort 30 VAX and Disp4 programs. Information was collected logarithmically.



VII (Cy5.203.OH)



VIII (Cy5.204.OH)



IX m = 1 (Cy3.205.OH)

X m = 2 (Cy5.205.OH)

Figure 3. Structures of new sulfobenzindocyanines.

RESULTS

Fluorescent cyanine dye-labeling reagents developed in this work are shown in Figure 3. Following the naming scheme for reactive polymethine dyes used in the previous papers (1–4) of this series, these dyes will be referenced as Cy3.205.OSu (**IX**) and Cy5.205.OSu (**X**). In these names, the Cy portion indicates that they are cyanine dyes, while OSu indicates that the compounds have succinimidyl ester reactive groups, and OH means that the compounds are in the carboxylic acid form. The number immediately after the Cy indicates the number of bridge carbons (i.e. Cy3, *m* = 1; Cy5, *m* = 2). The number following the decimal point indicates a unique dye structure, which is determined by the heterocyclic rings and the substituents which are introduced for water solubility and reactivity.

The labels **VII** and **VIII** have two sulfonate groups, and labels **IX** and **X** have four sulfonate groups to increase their water solubility and two alkylcarboxyl groups that can be activated to form succinimidyl esters.

In the synthesis, generally, a symmetrical benzindocyanine sulfonate is formed when a quaternized benzindoleninium sulfonate (**IV–VI**), which contains an alkylcarboxyl group for later activation of the fluorophore, is linked to a second identical benzindoleninium sulfonate nucleus through a polymethine chain of three or five conjugated methine carbon atoms. The key intermediates, benzindoleninium sulfonates, required to produce new groups of labeling reagents are prepared from corresponding sulfonaphthylamines (**I–III**). Many sulfonaphthylamines are commercially available. In our initial preparations, we chose two monosulfonated naphthylamines (**I** and **II**) and converted them to respective

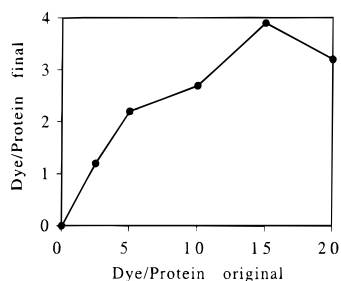


Figure 4. Plot of the ratio of moles of covalently attached Cy5.205 per mole of protein in a labeled protein vs the ratio of moles of Cy5.205.OSu per mole of protein originally present in the labeling mixture.

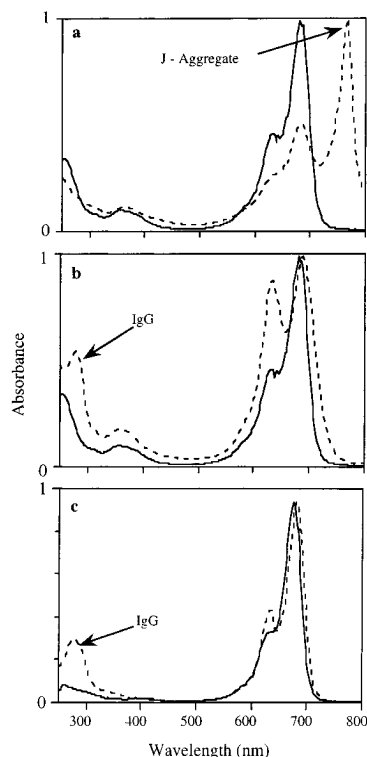


Figure 5. (a) Absorption spectra of Cy5.203 acid (—) and its disuccinimidyl ester (---) in water. The diester showed absorption maxima at 768 nm due to J-aggregate (see arrow). (b) Absorption spectra of Cy5.203 acid (—) and its antibody conjugate (---) in PBS. The conjugate showed substantial absorption at 632 nm due to dimer and no absorption for J-aggregate. (c) Absorption spectra of Cy5.203 acid (—) and its antibody conjugate (---) in PBS solution.

benzindoles (**IV** and **V**) using the Fisher indole synthesis (7). This approach is better than direct sulfonation of benzindoles. Heseltine and Lincoln (8) have synthesized several styryl dyes derived from benzindoleninium sulfonates. However, these sulfonates were obtained by direct sulfonation of the benzindole nucleus. In such a procedure, the exact orientation of the newly introduced sulfonate group(s) was not established. It is our experience that the position of the sulfonate group is very important to increase water solubility and the brightness of a fluorophore.

Following the reaction path shown in Figure 2, we have synthesized two pentamethine cyanines, Cy5.203.OH (**VII**) and Cy5.204.OH (**VIII**). These two dyes have identical spectroscopic and labeling properties, showing no effect due to change in the position of the sulfonate group. Their succinimidyl esters tend to form J-aggregates in water or PBS solution. However, their absorption spectra in methanol and alkaline buffer (pH

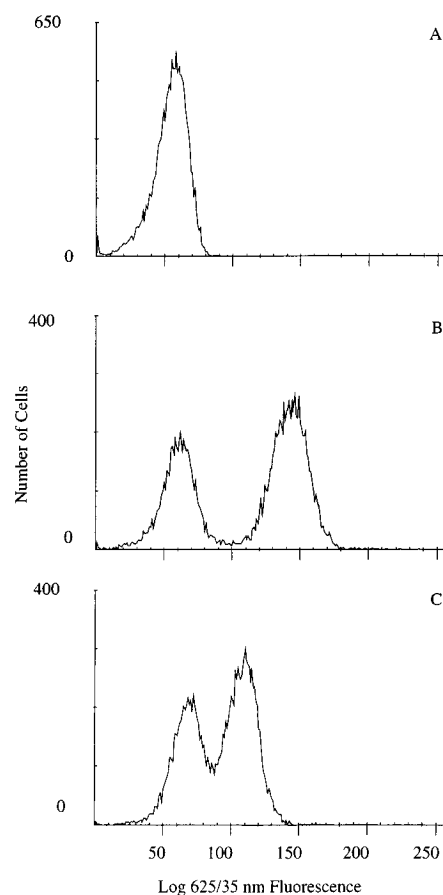


Figure 6. Normal peripheral blood lymphocytes (gated population) labeled with CD3-B followed by a streptavidin-labeled second reagent: (A) unstained control, (B) streptavidin/Cy3.205, and (C) streptavidin/Texas Red.

9.4) solution show no evidence of J-aggregate (Figure 5a). It appears that the one sulfonate group on these benzindoles is not sufficient to reduce dye-dye aggregation. The antibody conjugates of these dyes (**VII** and **VIII**) showed substantial dimer formation (Figure 5b) with associated fluorescence quenching. We, therefore, synthesized benzindocyanines with two sulfonate groups on each of the benzindolenine nuclei. The added sulfonate groups would increase the water solubility and dye-dye repulsion.

The intermediate 6-amino-1,3-naphthalenedisulfonic acid disodium salt was used for the synthesis of two tetrasulfonated fluorophores, Cy3.205.OH (**IX**) and Cy5.205.OH (**X**). After purification of the resulting fluorophores, carboxyl groups were activated to produce reactive dyes as labeling reagents. Their protein conjugates showed no evidence of dye-dye aggregation (Figure 5c) and fluorescence quenching.

Table 1 shows absorption and emission properties of the cyanine dyes. Absorption and emission wavelengths are relatively insensitive to solvent polarity. The quantum yields of the tetrasulfonated dyes are somewhat higher in both water and organic solvents. The succinimidyl ester of Cy3.205 was reacted with sheep γ -globulin in 0.1 M carbonate/bicarbonate buffer (pH 9.4). The labeling efficiency of Cy3.205.OSu (**IX**) was close to 30%. The quantum yield of the fluorophore increased by 2-fold upon conjugation to antibody and was approximately 0.25. This is consistent with our previous findings on trimethine cyanines which showed higher quantum yields in viscous media (1).

Labeling of Antibodies and Cells. Human lymphocytes were prepared and incubated with a biotinylated

Table 2. Peripheral Blood Lymphocytes Labeled with CD3-Biotin followed by Streptavidin Labeled with either Cy3.205 or Texas Red

sample cell	mean linear		positive fluorescence over negative
	negative cells	positive cells	
unstained cell (control)	9.65		
CD3-biotin + streptavidin/ Cy3.205	12.2	400	32.8
CD3-biotin + streptavidin/ Texas Red	15.98	97.84	6.12

monoclonal antibody T-cell marker (CD3). Streptavidin labeled with either Texas Red (TR) (Molecular Probes) or Cy3.205.OSu was used as a secondary reagent to mark the CD3-positive cells for analysis by flow cytometry. Samples were excited with 200 mW at the 568 nm Krypton laser line, and fluorescence was collected using a 625/35 nm band-pass filter. Cells labeled with Cy3.205.OSu gave a signal 6 times brighter than those labeled with Texas Red and showed low nonspecific binding (Table 2).

DISCUSSION

Planar and nonpolar dyes which prevail as fluorescent labels for fluorescence detection are particularly susceptible to molecular stacking. On the surface of labeled proteins, dye-dye interactions at high labeling densities generally lead to fluorescence quenching, thus limiting the brightness with labeled antibodies. Our earlier work (1-4) has shown that the addition of charged groups (such as sulfonate) on the indolenine base significantly reduces dye-dye interaction. It is also our experience that the position of the sulfonate group on the heterocyclic base affects the quantum yield and the brightness of the dye-antibody complexes. For example, oxacyanine derived from 6-sulfo-2-methylbenzoxazole is 2 times brighter than its isomeric dye obtained from 5-sulfo-2-methylbenzoxazole.¹ Bearing this observation in mind, we initially focused the present investigation on the syntheses of cyanines derived from two sulfonaphthylamines (**I** and **II**) which have only one sulfonate group. The additional benzene ring fused to sulfoindocyanine dyes shifts the dye absorption to a longer wavelength and increases the quantum yield (9). Our present results showed that the two pentamethine cyanine dyes, Cy5.203.OH (**VII**) and Cy5.204.OH (**VIII**), derived from monosulfonated benzindoles (**IV** and **V**) have the same spectral properties. The position of the sulfonate group produced no effect on the spectral properties, and both dyes showed substantial aggregation when bound to antibody. The tendency to form dye-dye aggregates on antibodies appeared to be far greater than that of previously discussed indocyanines. Even more surprisingly, disuccinimidyl esters Cy5.203.OSu (**VII**) and Cy5.204.OSu (**VIII**) form J-aggregates very freely when dissolved in water (Figure 5a). However, in pH 9.4 carbonate buffer, the same compound showed no evidence of J-aggregate. The free carboxylate ions in an alkaline solution apparently increase water solubility and reduce dye-dye aggregation. The formation of J-aggregates suggests higher-order dye-dye stacking (four dye molecules or more) (10). It is possible that the sulfonate groups of the monosulfonated naphthylamine-based dyes form tight ion pairs with the quaternary nitrogen of the adjacent dye molecule, giving rise to a ladder and stair structure which favors J-aggregates (9). The absence of J-aggregates in antibody-dye conjugate (Figure 5b) is

not a surprise. Free carboxylate ion generated after the hydrolysis of the succinimidyl ester increases water solubility. Besides, the dye-antibody solution is further diluted during Sephadex gel filtration chromatography. We have demonstrated that placement of four sulfonate groups close to the planar ring structures of the chromophore appears to provide more effective repulsion and reduces the dimer formation. The J-aggregates are not observed in the absorption spectra of Cy3.205.OSu and Cy5.205.OSu in water at neutral pH. The dye-dye aggregation is also absent in the dye-antibody conjugates as is evident from Figure 5c.

Dyes containing single reactive groups could not lead to complications from the cross-linking reactions that might seem likely to be encountered when bifunctional dyes are used. The actual experience has been that cross-linking of protein does not occur to a significant extent when disuccinimidyl ester of Cy5.205 is used for labeling at a low dye/protein ratio (10-15 dyes/protein). When proteins labeled with a high concentration of disuccinimidyl ester were subjected to sodium dodecyl sulfate (SDS) gel separation, very minimal cross-linking products were seen. This was consistent with our observations with cyanine dyes (1, 4).

The new cyanines present an opportunity to take advantage of additional laser lines that are already available or could be made available in fluorescence detection systems. A 568 nm Krypton laser excitation source and a 597 or 600/30 nm emission filter work well for flow cytometry. The longer wavelength Cy5.205 can be excited with new laser diodes emitting near 690 nm, and a 690-710 nm emission filter or Cy3.205 can give a strong fluorescence signal which is easy to detect using the standard Texas Red filter set.

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LITERATURE CITED

- (1) Mujumdar, R. B., Ernst, L. A., Mujumdar, S. R., Lewis, C. J., and Waggoner, A. S. (1993) Cyanine Dye Labeling Reagents: Sulfoindocyanine Succinimidyl Esters. *Bioconjugate Chem.* 4, 105-111.
- (2) Ernst, L. A., Gupta, R. K., Mujumdar, R. B., and Waggoner, A. S. (1989) Cyanine Dye Labeling Reagents for Sulfhydryl groups. *Cytometry* 10, 3-10.
- (3) Mujumdar, R. B., Ernst, L. A., Mujumdar, S. R., and Waggoner, A. S. (1989) Cyanine Dye Labeling Reagents containing Isothiocyanate groups. *Cytometry* 10, 11-19.
- (4) Southwick, P. L., Ernst, L. A., Tauriello, E. W., Stephen, R. P., Mujumdar, R. B., Mujumdar, S. R., Clever, H. A., and Waggoner, A. S. (1990) Cyanine Dye Labeling reagents - Carboxymethylindocyanine Succinimidyl Esters. *Cytometry* 11, 418-430.
- (5) Hamer, F. M. (1964) *The Cyanine Dyes and Related Compounds*, Wiley, New York.
- (6) Narayanan, N., and Patonay, G. (1995) A New Method for the Synthesis of Heptamine Cyanine Dyes: Synthesis of New Near-Infrared Fluorescent Labels. *J. Org. Chem.* 60, 2391-2395.
- (7) Illy, H., and Funderburk, L. (1968). Fisher Indole Synthesis-Direction of Cyclization of Isopropylmethyl Ketone Phenylhydrazones. *J. Org. Chem.* 33, 4283-4285.

¹ R. B. Mujumdar and A. S. Waggoner, unpublished data.

- (8) Heseltine, D. W., Jones, J. E., and Lincoln, L. L. (1969) Butadienyl Dyes for Photography, U.S. Patent 3,481,927.
- (9) Sturmer, D. M. (1977) Syntheses and Properties of Cyanine and Related dyes. In *Special Topics in Heterocyclic Chemistry* (W. T. Weissberger and E. C. Taylor, Eds.) pp 441–587, John Wiley & Sons, New York.
- (10) Herz, A. H. (1974) Dye-dye Interactions of Cyanines in Solution and at Silver Bromide Surfaces. *Photogr. Sci. Eng.* 18, 323–335.

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Preparation and Characterization of Poly(ethylene glycol) Vinyl Sulfone

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Synthesis of the vinyl sulfone and chloroethyl sulfone derivatives of poly(ethylene glycol) (PEG) is described. The chloroethyl sulfone (CES-PEG) is rapidly converted to the vinyl sulfone (VS-PEG) in the presence of base but is stable in water at neutral pH. Reactions with small molecules such as β -mercaptoethanol and *N*^α-acetyllysine show that the vinyl sulfone derivative is highly selective for reaction with sulfhydryl groups relative to reaction with amino groups. Also, VS-PEG is stable in water. These properties indicate that VS-PEG should be useful for selective attachment of PEG to protein cysteine groups. This hypothesis was verified by reacting VS-PEG with cysteine groups of reduced ribonuclease (RNase); the reaction is rapid and selective at pH 7–9. Reaction at lysine sites of unreduced RNase occurs slowly at pH 9.3 and is essentially complete after 100 h. Amino acid residues other than lysine and cysteine are not reactive toward VS-PEG. The covalent linkage between VS-PEG and lysine or cysteine groups is shown to be stable.

INTRODUCTION

Poly(ethylene glycol) (PEG)¹ derivatives have wide utility for a variety of biomedical and biotechnical applications (1–8), and interest continues in the synthesis of new PEG derivatives that possess novel properties (1, 9–21). Selective modification of cysteine groups is especially desirable since these groups can be introduced into specific locations in a protein, with the result that the PEG attachment site and extent of modification (i.e., “PEGylation”) are known and controlled (17).

In the present work we describe the synthesis and characterization of vinyl sulfone PEG (VS-PEG) and its precursor chloroethyl sulfone PEG (CES-PEG). These derivatives are of interest because previous work has shown that small compounds such as methyl and ethyl vinyl sulfone are selective for reaction with sulfhydryl groups relative to reaction with amino groups (22, 23), and as noted above, there is much interest in selective coupling of PEG to cysteine groups of proteins (14–21). Thus, it is of interest to determine if VS-PEG can offer advantages over the available sulfhydryl-selective PEGs such as maleimide, dithiopyridine, and iodoacetamide.

In the following we describe the synthesis of CES-PEG and VS-PEG, and we report rates for reaction of CES-PEG and VS-PEG with small thiols, small amines, the cysteine-containing tripeptide glutathione, and native and reduced RNase. The stability of the reagents and the products in aqueous medium is also determined.

EXPERIMENTAL PROCEDURES

Bovine pancreatic ribonuclease (RNase), *N*^α-acetyl-L-lysine methyl ester (ALMe), *N*-acetyl-L-cysteine, 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), fluorescamine, glutathione, methanesulfonyl chloride, and hydroxyl buffers were purchased from Sigma (St. Louis, MO). The maleimide derivative of PEG (MAL-PEG) was purchased from Shearwater Polymers (Huntsville, AL). β -Mercaptoethanol was obtained from Merck (Rahway, NJ). 4,4'-Dithiodipyridine (DTDP), triethylamine (TEA), trinitrobenzenesulfonic acid (TNBS), methoxypoly(ethylene glycol) (MW 5000) (mPEG), and *p*-nitrophenyl chloroformate were purchased from Aldrich (Milwaukee, WI).

Proton NMR spectroscopy was performed with a Bruker 200 MHz or a Varian Gemini 200 MHz instrument. Ultraviolet spectroscopy was performed with a Hewlett-Packard diode array or a Perkin-Elmer λ 2 instrument, and fluorescence was measured on a Perkin-Elmer LS-5B spectrometer. Ultrafiltration was performed with an Amicon system and a PM 10 membrane (cutoff 10 000 Da). Gel filtration chromatography was performed on a Pharmacia FPLC system using a Superose 12 TM column and a UV detector.

Synthesis of mPEG Chloroethyl Sulfone (CES-PEG). The synthesis involves four steps. First, mPEG mesylate was prepared by a modification of the method of Harris et al. (24). A toluene solution (150 mL) of mPEG 5000 (15 g) was dried by azeotropic distillation. After cooling, 50 mL of dry dichloromethane, 250 μ L (1.06 equiv) of methanesulfonyl chloride, and 440 μ L (1.06 equiv) of triethylamine were added while the solution was cooled in an ice bath. After 20 h, the solution was filtered, the volume reduced to 20 mL, and the product precipitated by addition of dry diethyl ether. The solid mesylate was dried *in vacuo*: ¹H NMR (dimethyl sulfoxide-*d*₆) 3.17 ppm (s, 3H, –SO₂CH₃), 3.23 ppm (s, 3H, CH₃O–), 3.49 ppm (s, mPEG backbone, –OCH₂–), 4.30 ppm (t, 2H, –CH₂OSO₂–).

In the second step, the mesylate was reacted with β -mercaptoethanol to form mPEG hydroxyethyl sulfide. Mesylate (14 g) was dissolved in 150 mL of distilled water, and 770 μ L (4 equiv) of β -mercaptoethanol and

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¹ Abbreviations: PEG, poly(ethylene glycol); VS-PEG, vinyl sulfone derivative of PEG; CES-PEG, chloroethyl sulfone derivative of PEG; MAL-PEG, maleimide derivative of PEG; RNase, bovine pancreatic ribonuclease; ALMe, *N*^α-acetyl-L-lysine methyl ester; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); DTDP, 4,4'-dithiodipyridine; TEA, triethylamine; TNBS, trinitrobenzenesulfonic acid; mPEG, methoxypoly(ethylene glycol) (MW 5000).

5.5 mL (4 equiv) of 2 N NaOH solution were added. The reaction was stirred at reflux for 3 h. After cooling, the product was extracted three times with dichloromethane, and the collected organic fractions were dried over anhydrous sodium sulfate. The volume was reduced to 20 mL, and the product was precipitated by addition to dry diethyl ether: ^1H NMR (dimethyl sulfoxide- d_6) 3.23 ppm (s, 3H, $\text{CH}_3\text{O}-$), 2.57 ppm (t, 2H, $-\text{SCH}_2-$), 2.65 ppm (t, 2H, $-\text{CH}_2\text{OH}$), 3.49 ppm (s, mPEG backbone, $-\text{OCH}_2-$), 4.76 ppm (t, 1H, $-\text{OH}$).

In the third step, the sulfide was oxidized to produce mPEG hydroxyethyl sulfone. Thirteen grams of the sulfide was dissolved in 20 mL of 0.123 M, pH 5.6, tungstic acid solution, and 15 mL of distilled water was added. After cooling to 0 °C, 1.04 mL of 30% hydrogen peroxide was added. The reaction was carried out overnight, and the product was extracted three times with dichloromethane. The collected organic fractions were dried over anhydrous sodium sulfate, the volume reduced to 20 mL by rotary evaporation, and the product precipitated by addition to dry diethyl ether: ^1H NMR (dimethyl sulfoxide- d_6) 3.23 ppm (s, 3H, $\text{CH}_3\text{O}-$), 3.25 ppm (t, 2H, $-\text{CH}_2\text{SO}_2-$), 3.37 ppm (t, 2H, $-\text{SO}_2\text{CH}_2-$), 3.49 ppm (s, mPEG backbone), 3.77 ppm (t, 2H, $-\text{CH}_2\text{OH}$), 5.04 ppm (t, 1H, $-\text{OH}$).

In the fourth step, the hydroxysulfone is converted to the chloride by reaction with thionyl chloride. Hydroxysulfone (13 g) was stirred overnight in 35 mL of freshly distilled (over quinoline) thionyl chloride. Excess thionyl chloride was removed by distillation. Two hundred milliliters of toluene was added and removed by distillation twice. The final product was obtained by solution in dry dichloromethane followed by precipitation upon addition to dry ethyl ether: ^1H NMR (dimethyl sulfoxide- d_6) 3.23 ppm (s, 3H, $\text{CH}_3\text{O}-$), 3.49 ppm (s, mPEG backbone), 3.64 ppm (t, 2H, $-\text{CH}_2\text{SO}_2-$), 3.79 ppm (t, 2H, $-\text{SO}_2\text{CH}_2-$), 3.94 ppm (t, 2H, $-\text{CH}_2\text{Cl}$); yield 90%; purity >95% from NMR.

Synthesis of mPEG Vinyl Sulfone (VS-PEG). The chloroethyl sulfone (CES-PEG) is readily converted to the vinyl sulfone (VS-PEG) by reaction with a variety of bases. For example, solution of 2 g of CES-PEG 5000 in 25 mL of dichloromethane containing 2 equiv of triethylamine, followed by addition to 50 mL of dry diethyl ether, and vacuum drying, produced 1.8 g of VS-PEG: ^1H NMR (dimethyl sulfoxide- d_6) 3.23 ppm (s, 3H, $\text{CH}_3\text{O}-$), 3.49 ppm (s, mPEG backbone), 3.74 ppm (t, 2H, $-\text{CH}_2\text{SO}_2-$), 6.21 ppm (m, 2H, $=\text{CH}_2$), 6.97 ppm (m, 1H, $-\text{SO}_2\text{CH}=\text{CH}_2$). The degree of end group conversion, as shown by NMR, was 100%.

The extent of end groups converted to vinyl sulfone can also be estimated by reaction of VS-PEG with excess mercaptoethanol followed by titration of remaining mercaptoethanol with DTDP. However, it should be noted that a weakness of this method is that the basic conditions used in the analytical method could convert any remaining CES-PEG to VS-PEG. VS-PEG was added under stirring to a 2 mM solution of β -mercaptoethanol in 0.1 M borate buffer (pH 9.5) containing 5 mM EDTA to minimize disulfide formation. After 15 min of stirring to complete reaction, 2 mM DTDP was added and stirred for 5 min. The absorbance was measured at 324 nm. The excess β -mercaptoethanol can be calculated from the 4-thiopyridone extinction coefficient of $19\,800\text{ M}^{-1}\text{ cm}^{-1}$ (25). This method also shows that the degree of end group conversion in synthesis of VS-PEG is 100%.

Kinetic Studies of VS-PEG and CES-PEG Reaction with Thiols. The reactivity of VS-PEG and CES-PEG toward thiols was evaluated by monitoring thiol concentration with DTDP, as described in the preceding

Table 1. Approximate Rate Constants for Reaction of CES-PEG with Model Thiols and Amines at 21 ± 1 °C

substrate	pH	k_2 ($\text{M}^{-1}\text{ min}^{-1}$)	k_1 (min^{-1})	$t_{1/2}$ (min)
thiols ^a				
β -mercaptoethanol	8.0	1.5	0.029	24
	9.0	fast ^b		≈ 1
	9.5	very fast ^c		$\ll 1$
<i>N</i> -acetylcysteine	8.0	1.0	0.020	34
	8.5	1.4	0.028	25
	9.0	8.9	0.18	3.9
glutathione	8.0	2.1	0.041	17
	8.5	3.2	0.065	11
	9.0	15	0.30	2.3
reduced RNase	8.0	3.5	0.069	10
	9.0	14	0.27	2.6
amine ^d				
<i>N</i> $^{\alpha}$ -acetyllysine methyl ester	8.0	no reaction		1 day
	9.5	0.76	0.023	30

^a Error limits $\pm 3\%$. ^b Reaction over within 15 min. ^c Reaction over within 2 min. ^d Error limits $\pm 7\%$.

paragraph (25), or with Ellman's assay (26). To obtain first-order kinetics, the mPEG derivative was used in 10 times the concentration of the thiol. The goal of these studies was to obtain qualitative kinetics, so the reactions were conducted under ambient conditions (21 ± 1 °C) rather than in a constant temperature bath. Four different thiols were studied: β -mercaptoethanol, *N*-acetyl-L-cysteine, glutathione, and reduced RNase. Rate constants for these reactions are given in Table 1.

To measure the reactivity of VS-PEG and CES-PEG with β -mercaptoethanol, the PEG derivative was added, under stirring, to a 2 mM thiol solution in 0.1 M buffer containing 5 mM EDTA. At scheduled times 50 μL aliquots of the reaction mixture were removed and quenched by addition to 950 μL of pH 7.2 buffer (0.1 M phosphate, 5 mM EDTA), followed by addition to 1.0 mL of 2 mM DTDP. After 5 min, the absorbance at 324 nm was measured. The ln of absorbance was plotted versus reaction time, and second-order rate constants were calculated by dividing the slopes of these plots by the derivative concentration (20 mM).

Kinetics of reaction with *N*-acetyl-L-cysteine and glutathione were monitored with Ellman's assay. A solution of 20 mM PEG derivative and 2 mM thiol was prepared in 0.1 M buffer (containing 5 mM EDTA). Aliquots (30 μL) were withdrawn and quenched by addition to 920 μL of pH 7.27 buffer (0.1 M phosphate, 5 mM EDTA). Fifty microliters of DTNB was added, and after 5 min, the absorbance at 412 nm was measured. Second-order rate constants were obtained by dividing the slope of the ln A versus time plot by the concentration of the derivative.

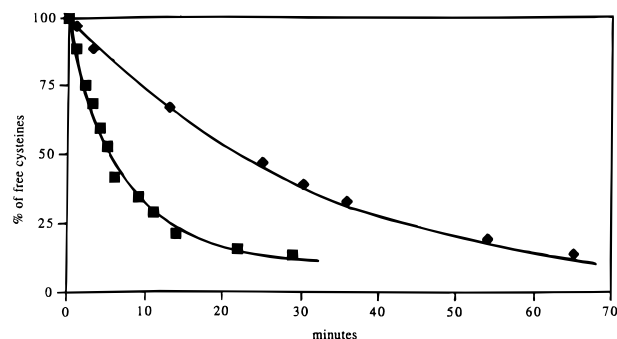
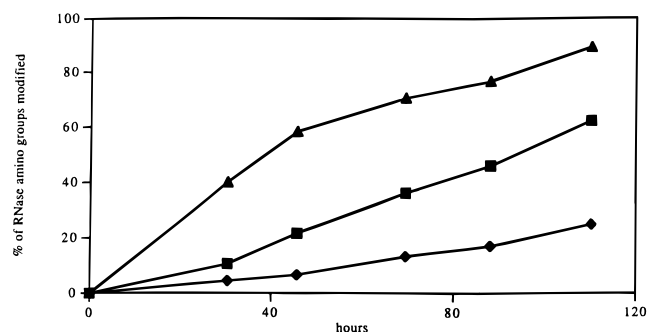
The reactivity of PEG derivatives with reduced RNase was measured as follows. Protein (MW 13 700, 14 mg) was dissolved in 300 μL of 25% β -mercaptoethanol in 4.5 M guanidinium chloride, and the solution was heated at 100 °C for 5 min. The reduced protein (four cystine disulfide bonds reduced to eight cysteine thiol groups) was isolated from excess β -mercaptoethanol by gel filtration chromatography using pH 4.3 buffer as eluent (0.05 M Tris/ethyl acetate, 0.2 M guanidinium chloride). The protein fractions were concentrated by ultrafiltration and the thiol content of the sample measured by Ellman's assay. Kinetic measurements were made, as described in the previous paragraph, using 2 mM thiol and 20 mM PEG derivative.

Kinetic Studies of VS-PEG and CES-PEG Reaction with Amines. The reactivity of VS-PEG and CES-PEG toward amines was evaluated by monitoring amine concentration with fluorescamine assay (27–29) or TNBS

Table 2. Degree of Lysine Substitution by Reaction of VS-PEG with Unreduced RNase (100 h, pH 9.3, Room Temperature) As Measured by TNBS Assay and Amino Acid Analysis

ratio of protein NH ₂ to VS-PEG ^a	degree (%) of substitution ^b by TNBS	degree (%) of substitution ^b by amino acid analysis
1:1	25	28
1:6	75	68
1:10	89	76

^a Amine concentration 0.59 M. ^b Eleven lysines are available for substitution.

**Figure 1.** Reaction between reduced RNase thiol groups and VS-PEG as evaluated from disappearance of free cysteines. The reaction was carried out at room temperature, pH 8.0 (♦) and 9.0 (■). [SH] = 0.81 mM; [VS-PEG] = 8.1 mM.**Figure 2.** Reaction between native RNase and VS-PEG evaluated from disappearance of protein lysine groups. Lysine:VS-PEG molar ratios 1:1 (♦), 1:6 (■), 1:10 (▲) were used. The reaction was carried out at room temperature and pH 9.3 ([NH₂] = 0.59 mM).

(30). To obtain first-order kinetics, the mPEG derivative was used in 10 times the concentration of the amine. Reactions were conducted under ambient conditions (21 ± 1 °C). Two different amines were studied: *N*^α-acetyl-L-lysine methyl ester (ALMe) and native RNase. Rate constants for the small molecule ALMe are given in Table 1, and reactivity data for native RNase are given in Table 2 and Figure 2.

Reactivity with ALMe was measured by following amine disappearance with the fluorescamine assay (27–29). A 10-fold excess of PEG derivative was added under stirring to 0.3 mM ALMe solution in the appropriate buffer (0.1 M phosphate, pH 8, or 0.1 M borate, pH 9.5). A 50 μL aliquot was withdrawn and quenched at scheduled times by addition to 2.95 mL of 0.1 M, pH 8.0, phosphate buffer. Fluorescamine solution (1.0 mL, at a concentration of 0.3 mg/mL in acetone) was added with vigorous mixing. Fluorescence was measured after 10 min ($\lambda_{\text{exc}} = 390$ nm, $\lambda_{\text{em}} = 475$ nm). Second-order rate constants were obtained from the slope of the plot of $\ln$ fluorescence emission intensity versus time, divided by PEG derivative concentration.

The reactivity of VS-PEG and CES-PEG with amino

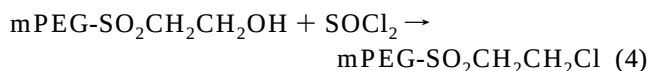
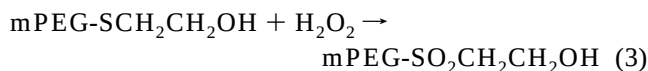
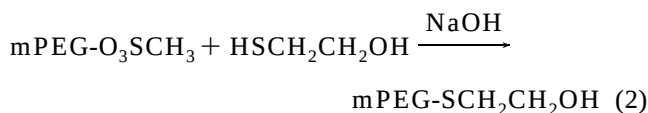
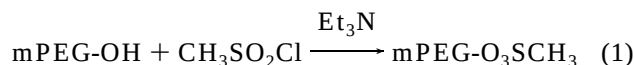
groups of unreduced RNase was determined by monitoring amine concentration with the TNBS assay (Figure 2) (30); RNase has 11 lysine groups and the N-terminal amine with which to react (31). Several tubes were prepared, each containing 600 μL of RNase solution (1.35 mg/mL in 0.2 M, pH 9.3, borate buffer). Different aliquots (50, 300, and 500 μL) of CES-PEG solution (66.6 mg/mL in the pH 9.3 borate buffer) were added to obtain molar ratios between protein amine and VS-PEG (produced by reaction of CES-PEG with pH 9.3 buffer) of 1:1, 1:6, and 1:10, respectively. The final volume of each sample was then adjusted to 1.2 mL with buffer. A control tube was also prepared without CES-PEG addition. Aliquots were withdrawn at scheduled times and analyzed by using the TNBS assay.

Degree of RNase Lysine Substitution. The extent of modification of RNase lysine groups by VS-PEG after approximately 100 h of reaction at pH 9.3 (as described in the preceding section) was determined both by TNBS assay (as described above) and by amino acid analysis (Table 2) (32). For amino acid analysis, equal volumes of 12 N HCl and phenol (final concentration, 0.5%) were added to each tube, and amino acid analysis was performed after 24 h of hydrolysis at 110 °C in closed vials. The amino acid composition of each modified sample was compared to that of the native one. Control experiments showed that VS-PEG attaches irreversibly to lysine groups, so the reduction in lysine content corresponds to modification extent.

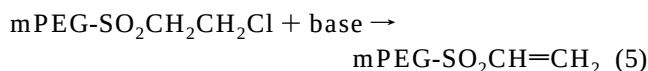
Stability of VS-PEG and CES-PEG in Aqueous Buffer. The lifetime of VS-PEG and CES-PEG at pH 7.0 and 9.0 was investigated by NMR examination using D₂O as solvent. A 5% solution of CES-PEG in D₂O was prepared, and the pH was adjusted by adding a small amount of the appropriate hydroxide buffer powder. NMR experiments were carried out at scheduled times to follow the appearance of VS-PEG and mPEG hydroxysulfone.

RESULTS AND DISCUSSION

Preparation of CES-PEG involves four steps:



The chlorosulfone is readily converted to VS-PEG by exposure to any of a variety of bases:



This final elimination step occurs rapidly at pH 9.0. A solution of CES-PEG in pH 9.0 buffer, followed within 2–3 min by NMR examination, showed only signals for VS-PEG. In contrast, a solution of CES-PEG in water at pH 7 showed only 4% VS-PEG after 48 h of incubation at room temperature; no hydroxysulfone was produced. VS-PEG was shown by these same NMR experiments to

be stable indefinitely at pH 7 and 9. This water stability of VS-PEG is highly unusual for "activated" PEGs suitable for protein modification, and such stability is of great utility for large-scale preparations and for heterofunctional reagents, one of the functional groups of which must survive during coupling of the other functional group (1, 15, 21, 33, 34). Similarly, the stability of CES-PEG at neutral pH provides a means of protecting the vinyl sulfone group.

Isolation of VS-PEG is provided by solution of CES-PEG in methylene chloride containing 2 equiv of triethylamine, followed by precipitation of the product by addition to diethyl ether.

The efficiency of each of the above synthetic steps is readily monitored by NMR characterization of the products. It was found that all of the synthesis steps were very efficient, yielding almost complete functionalization and over 90% recovery. NMR examination of the final VS-PEG showed no other functional groups. Similarly, addition of excess mercaptoethanol, followed by titration of mercaptoethanol with dithiodipyridine, showed 100% vinyl sulfone.

Experimental details are given for mPEG 5000, but we have found that the same chemistry works well for preparation of VS-PEG of molecular weights up to 20 000.

Since VS-PEG and CES-PEG are intended for selective reaction with thiol groups relative to reaction with amino groups, we have measured rates of reaction, at room temperature and in aqueous solution, of these derivatives with thiols and primary amines (Table 1). The majority of the experiments were conducted by solution of CES-PEG in basic solution, so that VS-PEG is formed in situ. As model thiols we used β -mercaptoethanol, *N*-acetylcysteine, glutathione, and reduced RNase. *N* $^{\alpha}$ -Acetyl-L-lysine methyl ester and unreduced, native RNase were used as model amines.

As can be seen from Table 1, reaction of VS-PEG with thiols at pH 8 takes place with half-lives of from 10 to 34 min. This rather large range of reactivities indicates that reaction of the large VS-PEG is sensitive to the steric environment of the thiol groups. Indeed, we have observed an even larger range of reactivity toward protein cysteine groups (35). An increase in pH to 9 gives an enhancement of VS-PEG reaction rate with thiols and reduces half-lives to 1–4 min (Table 1). At pH 9.5 reaction is too fast to measure. The extent of reaction with reduced RNase is illustrated in Figure 1. Here it is clear that cysteine modification is almost complete within 20 min at pH 9 and within 1 h at pH 8.

Reaction of VS-PEG is also effective at pH 7. Although we did not measure rate constants at pH 7, qualitative experiments were performed. Equimolar amounts (0.01 M) of VS-PEG and *N*-acetylcysteine were reacted at pH 7 and room temperature, and the reaction was monitored by the DTDP assay for free thiol. After 30 min, most thiol had reacted, and after 1.5 h, only slight amounts of thiol could be detected (data not shown—experiment repeated three times). It is important to note that protein modification would normally be done with an excess of VS-PEG, so extensive reaction would occur within 30 min. The VS-PEG conjugate with *N*-acetylcysteine was isolated by extracting into methylene chloride, drying with magnesium sulfate, and precipitating by adding this solution to diethyl ether. Solution of the pure product in deuterated water, and subsequent NMR examination, showed the conjugate to be stable indefinitely in aqueous solution (data not shown).

In contrast to thiol reactivity, reaction of VS-PEG with primary amine groups is very slow at pH 8, with no reaction detected for reaction of *N* $^{\alpha}$ -acetyllysine methyl

ester after 24 h (Table 1). Reaction with amino groups is much faster at pH 9.5 but still is quite slow compared to reaction with thiols (half-life of 30 min for amine reaction versus a thiol reaction that is too fast to measure). Thus, VS-PEG is highly selective for reaction with thiols, and this selectivity, plus the water stability of VS-PEG, shows that this derivative is suitable for selective modification of protein cysteine groups.

Despite the low reactivity of VS-PEG with amines, this derivative can be used for attachment to protein amino groups. Thus, we have found that VS-PEG gives 40% modification of lysine groups of unreduced RNase after 30 h if a 10 to 1 molar excess of VS-PEG to lysine groups is used at pH 9.3 (Figure 2). Reaction for approximately 100 h gives even more extensive modification (Table 2). Examination of the time course of this reaction, Figure 2, shows that the reaction is significantly slower than for reaction with the small molecule *N* $^{\alpha}$ -acetyl-L-lysine methyl ester. Presumably this is a reflection of the bulk of VS-PEG and steric shielding of lysine groups in the protein. Although this low reactivity of VS-PEG with protein lysine groups might appear to preclude general utilization of this reaction for protein PEGylation, the reaction could be used for introduction of a small number of PEGs into a conjugate. Presumably the more reactive amino groups will be modified first, so the slow PEGylation provided by VS-PEG could possibly be used for selective modification of lysine groups.

The extent of lysine modification was measured by two routes, the TNBS assay and amino acid analysis. The TNBS assay depends on colorimetric detection of reaction of TNBS with available lysine amino groups. Amino acid analysis is based on a reduction in the number of lysine groups that are found after protein hydrolysis. For this method to succeed, it is necessary that the lysine-ethyl sulfone linkage formed upon attachment of VS-PEG to amine be sufficiently durable to survive hydrolysis of the protein. Since the degrees of lysine modification as measured by TNBS and amino acid analysis are in reasonable agreement (Table 2), it is apparent that this linkage is very durable.

Lower values for modification extent are found from amino acid analysis than from TNBS assay at high degrees of modification, but colorimetric methods such as the TNBS assay are known to overestimate the degree of PEGylation (32). For example, overestimation by colorimetric methods was shown when colorimetric methods were compared with the exact "norleucine-PEG method" (32). This latter method involves protein modification with a mPEG bearing a norleucine group in the polymer backbone. Norleucine is an unnatural amino acid, and direct estimation of the degree of protein PEGylation is obtained by PEGylating with the norleucine PEG followed by determining the concentration of norleucine in the hydrolyzed protein. The overestimation by colorimetric assay is consistent with reduced reactivity of TNBS toward lysine residues in the mPEG-modified protein (32).

It is also noteworthy that amino acid analysis of PEGylated RNase showed complete recovery of all amino acids other than the modified lysines, especially since Masri and Friedman observed modification of histidine and tyrosine upon reaction of proteins with ethyl and methyl vinyl sulfones (22). Thus, it is apparent that VS-PEG is unreactive toward nucleophilic amino acids other than lysine and cysteine, with reactivity toward the latter group being by far the greatest.

One of the goals of our work is to compare the various thiol-selective PEG reagents. As noted above, VS-PEG is stable indefinitely in aqueous solution. It is known

from the literature that maleimides undergo slow hydrolysis (36), and we have confirmed a similar degradation pathway for PEG maleimide. In our experiment PEG maleimide is dissolved in D₂O and examined by NMR. Slow disappearance of the NMR vinyl absorptions at 7.01 ppm is observed. A half-life of 6.8 h at pH 7.5 was measured. In a continuation of the current work, which is to be published soon, we have found also that there appears to be slow hydrolysis of the amide linkages in PEG-protein conjugates prepared with MAL-PEG (35). As shown above, VS-PEG forms a stable linkage. A possible advantage of MAL-PEG is that it reacts more rapidly than VS-PEG. As described above, VS-PEG reacts completely with an equimolar solution of N-acetylcysteine at pH 7 and room temperature within 1.5 h. A companion experiment with MAL-PEG showed complete reaction in less than 1 h.

Although we have not conducted a direct comparison of VS-PEG with PEG iodoacetamide and PEG dithiopyridine, some comments can be made. The dithiopyridine derivative forms a disulfide when coupled to thiols. An advantage of this linkage is that it can be cleaved, if desired, by exposure to reducing reagents, but it is also possible that this reversibility could become a problem in certain environments (16). Iodoacetamides are also selective for reaction with thiol groups, but reaction with other groups such as histidine has been noted, and slow hydrolysis is also observed (37).

CONCLUSION

In summary, we have demonstrated that VS-PEG is readily prepared in high purity and that this PEG derivative is highly selective for reaction with thiol groups relative to amino groups. This selectivity extends to reaction with protein cysteine and lysine groups. Modification of lysines is slow but does occur at useful rates at pH 9.3. The linkages formed for reaction with both thiol and amino groups are stable. An additional useful property of VS-PEG is that the chloroethyl sulfone precursor serves as a protected form that can be deprotected by exposure to base. VS-PEG itself is stable in aqueous solution for days at pH values up to 9. The extent of protein PEGylation by VS-PEG can be monitored by colorimetric methods or by amino acid analysis. Future work concerns utilization of VS-PEG for PEGylation of therapeutically useful proteins (35).

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LITERATURE CITED

- Harris, J. M., Ed. (1992) *Poly(ethylene Glycol) Chemistry: Biotechnical and Biomedical Applications*; Plenum Press, New York.
- Mabrouk, P. A. (1994) Effect of pegylation on the structure and function of horse cytochrome c. *Bioconjugate Chem.* 5, 236–241.
- Alred, P. A., Tjerneld, F., Kozlowski, A., and Harris, J. M. (1992) Synthesis of dye conjugates of ethylene oxide-propylene oxide copolymers and application in temperature-induced phase partitioning. *Bioseparation* 2, 363–373.
- Maruyama, K., Unezaki, S., Yuda, T., Ishida, O., Takahashi, N., Suganaka, A., Huang, L., and Iwatsuru, M. (1994) Enhanced delivery and antitumor effect of doxorubicin encapsulated in long-circulating liposomes. *J. Liposome Res.* 4, 143–165.
- Shoichet, M. S., Winn, S. R., Harris, J. M., and Gentile, F. T. (1994) Poly(ethylene oxide)-grafted thermoplastic membranes for use as cellular hybrid bio-artificial organs in the central nervous system. *Biotechnol. Bioeng.* 43, 563–572.
- Jones, D. S., Hachmann, J. P., Osgood, S. A., Hayag, M. S., Barstad, P. A., Iverson, G. M., and Coutts, S. M. (1994) Conjugates of double-stranded oligonucleotides with poly(ethylene glycol) and keyhole limpet hemocyanin: a model for treating systemic lupus erythematosus. *Bioconjugate Chem.* 5, 390–399.
- Kwon, G., Suwa, S., Yokoyama, M., Okano, T., Sakurai, Y., and Kataoka, K. (1994) Enhanced tumor accumulation and prolonged circulation times of micelle-forming poly(ethylene oxide-aspartate) block copolymer-adriamycin conjugates. *J. Controlled Release* 29, 17–23.
- Sawhney, A. S., Pathak, C. P., van Rensburg, J. J., Dunn, R. C., and Hubbell, J. A. (1994) Optimization of photopolymerized bioerodible hydrogen properties for adhesion prevention. *J. Biomed. Mater. Res.* 28, 831–838.
- Nathan, A., Zalipsky, S., Ertel, S. I., Agathos, S. N., Yarmush, M. L., and Kohn, J. (1993) Copolymers of lysine and polyethylene glycol: a new family of functionalized drug carriers. *Bioconjugate Chem.* 4, 54–62.
- Chamow, S. M., Kogan, T. P., Venuti, M., Gadek, T., Harris, R. J., Peers, D. H., Mordenti, J., Shak, S., and Ashkenazi, A. (1994) Modification of CD4 immunoadhesin with monomethoxypoly(ethylene glycol) aldehyde via reductive amination. *Bioconjugate Chem.* 5, 133–140.
- Fuke, I., Hayashi, T., Tabata, Y., and Ikada, Y. (1994) Synthesis of poly(ethylene glycol) derivatives with different branchings and their use for protein modification. *J. Controlled Release* 30, 27–34.
- Zalipsky, S., Seltzer, R., and Menon-Rudolph, S. (1992) Evaluation of a new reagent for covalent attachment of polyethylene glycol to proteins. *Biotechnol. Appl. Biochem.* 15, 100–114.
- Miron, T., and Wilchek, M. (1993) A simplified method for the preparation of succinimidyl carbonate polyethylene glycol for coupling to proteins. *Bioconjugate Chem.* 4, 568–569.
- Kogan, T. P. (1992) The synthesis of substituted methoxypoly(ethylene glycol) derivatives suitable for selective protein modification. *Synth. Commun.* 22, 2417–2424.
- Akerblom, E., Dohlsten, M., Bryn , C., Mastej, M., Steringer, I., Hedlund, G., Lando, P., and Kalland, T. (1993) Preparation and characterization of conjugates of monoclonal antibodies and staphylococcal enterotoxin A using a new hydrophilic cross-linker. *Bioconjugate Chem.* 4, 455–466.
- Woghiren, C., Sharma, B., and Stein, S. (1993) Protected thiol-polyethylene glycol: a new activated polymer for reversible protein modification. *Bioconjugate Chem.* 4, 314–318.
- Kuan, C.-T., Wang, Q.-C., and Pastan, I. (1994) Pseudomonas exotoxin A mutants. *J. Biol. Chem.* 269, 7610–7616.
- Herman, S., Locuffier, J., and Schact, E. (1994) End-group modification of α -hydro- ω -methoxy-poly(oxyethylene). *Macromol. Chem. Phys.* 195, 203–209.
- Herron, J. N., Gentry, C. A., Davies, S. S., Wei, A.-p., and Lin, J.-n. (1994) Antibodies as targeting moieties: affinity measurements, conjugation chemistry and applications in immunoliposomes. *J. Controlled Release* 28, 155–166.
- Goodson, R. J., and Katre, N. V. (1990) Site directed pegylation of recombinant Interleukin-2 at its glycosylation site. *Bio/Technology* 8, 343–346.
- Zalipsky, S. (1995) Functionalized polyethylene glycols for preparation of biologically relevant conjugates. *Bioconjugate Chem.* 6, 150–165.
- Masri, M. S., and Friedman, M. (1988) Protein reactions with methyl and ethyl vinyl sulfones. *J. Protein Chem.* 7, 49–54.
- Dearfield, K. L., Harrington-Brock, D., Doerr, C. L., Rabinowitz, J. R., and Moore, M. M. (1991) Genotoxicity in mouse lymphoma cells of chemicals capable of Michael addition. *Mutagenesis* 6, 519–525.

- (24) Harris, J. M., Struck, E. C., Case, M. G., Paley, M. S., Yalpani, M., Van Alstine, J. M., and Brooks, D. E. (1984) Synthesis and characterization of poly(ethylene glycol) derivatives. *J. Polym. Sci., Polym. Chem. Ed.* 22, 341–352.
- (25) Grassetti, D. R., and Murray, J. F. (1967) Determination of sulfhydryl groups with 2,2'- or 4,4'-dithiopyridine. *J. Appl. Biochem. Biotechnol.* 119, 41–49.
- (26) Riddles, P. W., Blakeley, R. L., and Zerner, B. (1983) Reassessment of Ellman's reagent. *Methods Enzymol.* 91, 49–60.
- (27) Udenfriend, S., Stein, S., Böhlen, P., Bairman, W., Leimgruber, W., and Weigle, M. (1972) Fluorescamine: a reagent for assay of amino acid, peptides, proteins and primary amines in the picomole range. *Science* 178, 871–872.
- (28) Stocks, S. J., Jones, A. J. M., Ramey, C. W., and Brooks, D. E. (1986) A fluorometric assay of the degree of modification of proteins with polyethylene glycol. *Anal. Biochem.* 154, 232–236.
- (29) Karr, L. J., Donnelly, D. L., Kozlowski, A., and Harris, J. M. (1994) Use of poly(ethylene glycol)-modified antibody in cell extraction. *Methods Enzymol.* 228, 377–390.
- (30) Snyder, S. L., and Sobocinsky, P. Z. (1975) Improved 2,4,6-trinitrobenzenesulfonic acid method for the determination of amines. *Anal. Biochem.* 64, 284–288.
- (31) DeaKyne, C. A., and Allen, L. C. (1979) Role of active-site residues in the catalytic mechanism of ribonuclease A. *J. Am. Chem. Soc.* 101, 3951–3959.
- (32) Sartore, L., Caliceti, P., Schiavon, O., Monfardini, C., and Veronese, F. M. (1991) Accurate evaluation of the polymer content in monomethoxy-poly(ethylene glycol)-modified proteins based on amino acid analysis. *J. Appl. Biochem. Biotechnol.* 31, 213–222.
- (33) Zalipsky, S., and Barany, G. (1986) Preparation of polyethylene glycol derivatives with two different functional groups at the termini. *Polym. Prepr. (Am. Chem. Soc., Div. Polym. Chem.)* 27 (1), 1–2.
- (34) Harris, J. M., Herati, M. R. S., Sather, P. J., Brooks, D. E., and Fyles, T. M. (1992) Synthesis of new poly(ethylene glycol) derivatives. In ref 1, pp 371–381.
- (35) Kachensky, D., Kohno, T., Seely, J., Thompson, R. D., and Harris, J. M. (1996) Polyethylene glycol vinylsulfones: novel conjugation reagents for site-specific polypeptide modification. *J. Controlled Release*, in press.
- (36) Ishi, Y., and Lehrer, S. S. (1986) Effects of the state of the succinimido ring on the fluorescence and structural properties of pyrene maleimide-labeled $\alpha\alpha$ -tropomyosin. *Biophys. J.* 50, 75–80.
- (37) Means, G. E., and Feney, R. E. (1990) Chemical modification of proteins: history and applications *Bioconjugate Chem.* 1, 2–12.

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Synthesis and Binding Properties of Oligonucleotides Covalently Linked to an Acridine Derivative: New Study of the Influence of the Dye Attachment Site

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2-Methoxy-6-chloro-9-aminoacridine has been coupled via a polymethylene linker to various positions of an oligonucleotide chain: the 3'-position, using a new universal support, the 5'-position, and both 5'- and 3'-positions via a phosphate. The intercalating agent was also linked to the oligonucleotide chain via an internucleotide phosphorothiolate. The mixture of diastereoisomers was obtained as well as each pure R_p and S_p isomer. Finally, the acridine moiety was introduced to the 5-position of the deoxyuridine. The binding properties of these oligonucleotide-acridine conjugates with their DNA counterparts have been studied by absorption spectroscopy.

INTRODUCTION

The base-pairing properties of oligomeric strands of DNA which allow specific recognition of a particular base sequence in a single-stranded RNA target by a complementary base sequence are the foundation for their use as artificial regulators of gene expression via the "antisense" strategy (1). The regulation of gene expression can also be achieved by interaction of an oligonucleotide with a single-stranded DNA target in the opened transcription initiation region (2). More recently, synthetic oligonucleotides were used to regulate gene expression by hybridization with double-stranded DNA (for a review, see ref 3) via Hoogsteen base pairing. The efficiency of these synthetic oligonucleotides in regulating gene expression in living cells depends on the thermodynamic stability of the duplex or triplex structure, their resistance toward nuclease and cellular uptake. In order to increase the stability of the duplexes, intercalating agents such as acridine (4, 5), pyrene (6–9), psoralen (10–12), anthraquinone (13–16), oxazolopyridocarbazole (17), ellipticine (18), phenazinium (19, 20), fagaronine (21), coumarine (22), and ethidium (23) have been covalently coupled to different positions of oligonucleotides 5' (4, 6, 11–13, 19, 21–23), 3' (4, 17, 19, 23), and both 5' and 3' (4, 19), internucleotidic phosphate (4, 7), the 1'- (8) or 2'- (13, 14, 16) position of the sugar moiety, or nucleic bases: at the C5-position of deoxyuridine (20), at the C8-position of deoxyadenosine (10), or at the exocyclic amino function of deoxyguanosine (15) and deoxycytidine (9). Only the coupling of the intercalating agent to the 5'- or the 3'-end of the oligonucleotide led to a strong stabilization. In our previous studies carried out with 2-methoxy-6-chloro-9-aminoacridine, chosen because of its lack of specificity with respect to base or base pair interactions (24), a polymethylene linker was used to connect the acridine derivative to the oligonucleotide chain (4, 5). These results showed that a linker with five methylene groups between the 9-amino group of the acridine and the 3'-phosphate of the oligonucleotide was long enough to obtain an optimum interaction between the intercalat-

ing agent and the double-stranded structure formed by the oligonucleotide and its complementary sequence (4, 5). In order to increase the stability of the obtained duplex, one intercalating agent was added to both ends of the oligonucleotide via a phosphate. Surprisingly the presence of two intercalating agents led to the same stabilization as did the presence of one (4). The covalent attachment of the intercalating agent at an internucleotidic phosphate was also tested (4). In this case, the stabilizing effect observed was lower than that obtained by linking the intercalating agent at the end (3' or 5') of the oligomer. This is probably due to the fact that the phospho triester is a mixture of diastereoisomers having different stability. Other experiments with oligonucleotides involving "phospho triester" groups have shown that one isomer leads to the formation of a duplex having a higher stability than that of the unmodified parent while the second isomer leads to the one with lower stability (25).

In order to further explore the possibility of increasing the stability of the complexes by using more than one intercalating molecule per oligomer [to our knowledge, the stabilization of the duplex by more than an intercalating agent has never been described except in one case in which a low additional stabilization occurred (19)], we decided to covalently link the intercalating agent to the oligonucleotide chain via a polymethylene linker using either a terminal (5', 3', or both 5' and 3') or an internucleotidic phosphorothiolate or the 5-position of a deoxyuridine. In order to investigate the influence of the stereochemistry of the phosphorothiolate triester, the mixture of diastereoisomers as well as each pure R_p and S_p isomer were also synthesized. We report here the preparation of these oligonucleotides covalently linked to 2-methoxy-6-chloro-9-aminoacridine and their binding properties with a complementary single-stranded DNA sequence.

EXPERIMENTAL PROCEDURES

General Methods. All solvents used were dried, distilled, and stored as described in ref 26. All chemicals were used as obtained unless otherwise stated. 6,9-Dichloro-2-methoxyacridine, phenol, dimethylaminopyridine, acetic anhydride, succinic anhydride, *p*-nitrophenol, dicyclohexylcarbodiimide, 1,4-diaminobutane, 1,6-diaminohexane, 1,7-diaminoheptane, diisopropylethyl-

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amine, 2-(cyanoethyl)-*N,N*-diisopropylchlorophosphoramidite, dioxane, *N*-hydroxysuccinimide, and solketal were purchased from Aldrich. Triethylamine and sodium sulfate were from Merck. Pyridine and dichloromethane were from SDS. Acetonitrile was from Labo-Standa and long chain alkylamine CPG from Pierce. 6-Chloro-9-phenoxy-2-methoxyacridine synthesis was achieved as described in ref 27. $d^{55}\text{TTTCCTCTCT}^3\text{pS}(\text{CH}_2)_6\text{NHAc}$ was prepared following a previously described procedure (28, 29). Analytical thin-layer chromatography (TLC) was performed on precoated alumina plates (Merck silica gel 60F 254 ref 5554) and preparative TLC on glass-backed plates of Merck silica gel 60 F 254 ref 5717. For flash chromatography, Merck silica gel 60 (70–230 mesh) (ref 7734) and anhydrous solvents were used. All 4,4'-dimethoxytrityl-containing substances were identified as orange-colored spots on TLC plates by spraying with a 10% perchloric acid solution. Acridine-containing compounds were directly visualized on the plates. Analysis and purification by ion-exchange chromatography were carried out on a Pharmacia FPLC with a DEAE column (8 μM , 100 mm $\times$ 10 mm, Waters) with a linear gradient of NaCl in Tris/HCl (pH 7), 25 mM buffer containing 10% CH_3CN . Reversed-phase chromatography analysis was performed on a 600 E instrument (System Controller) equipped with a photodiode array detector (Waters 990) using a Lichrospher 100 RP (5 μM) column (125 mm $\times$ 4 mm) from Merck with a linear gradient of CH_3CN in 0.1 M aqueous triethylammonium acetate (pH 7) with a flow rate of 1 mL/min. For mass analysis, ion-molecular masses of the oligonucleotides were confirmed by mass spectroscopy using a Lasermat Time-of-Flight instrument (LD-TOF). The average power of the nitrogen laser (337 nm) was about 10^7 W/cm^2 . To improve the signal to noise ratio, 10 single shot spectra were accumulated and averaged. All measurements were performed using the negative detection mode. The spectrometer was calibrated with the $\text{M} - \text{H}$ and $2\text{M} - \text{H}$ mass peaks of $d^{55}(\text{Tp})_8\text{O}(\text{CH}_2)_5\text{NHAc}$ as reference. The sample preparation was performed by mixing 10 μL of a 0.5 M solution of 2,4,6-trihydroxyacetophenone in ethanol and 5 μL of a 0.1 M aqueous solution of diammonium *L*-tartrate. To this was added 1 μL of the oligonucleotide solution (10 OD/mL), and the mixture was briefly vortexed. To the probe tip was applied 1 μL of this solution, and the solvents were removed. $^1\text{H-NMR}$ spectra were recorded with a Bruker AM 300 MHz instrument. Absorption spectra were recorded with an Uvikon 860 spectrophotometer. Absorption coefficients of one acridine-containing oligomer were chosen to be $8850 \text{ M}^{-1} \text{ cm}^{-1}$ at $\lambda = 425 \text{ nm}$ and $16\,000 \text{ M}^{-1} \text{ cm}^{-1}$ at $\lambda = 425 \text{ nm}$ for oligomers containing two acridine moieties by analogy with our previously described work (30). Nucleases were purchased from Boehringer, Mannheim.

Preparation of the Modified Support 3. (3-Aminopropyl)solketal was obtained as described in ref 31.

(*N*-Acridinyl-3-aminopropyl)solketal (**1**). 2-Methoxy-6,9-dichloroacridine (3 g, 10.8 mmol) and (3-aminopropyl)solketal were stirred in phenol (8.6 g) for 90 min at 120 °C. After being cooled, the obtained mixture was poured into a 2 N NaOH aqueous solution. The precipitate was dissolved in ethyl acetate (180 mL) and washed twice with 50 mL of water. After being dried over Na_2SO_4 , the organic layer was concentrated to dryness under reduced pressure. The resultant oil was chromatographed on a silica gel column using $\text{CH}_2\text{Cl}_2/\text{CH}_3\text{OH}$ (9:1 v:v) as eluent. Compound **1** was obtained as a yellow solid with 45% yield (2 g). $R_f = 0.23$ using $\text{CH}_2\text{Cl}_2/\text{MeOH}$ (90:10 v:v) as eluent. $^1\text{H-NMR}$ (DMSO): δ 1.20 (s, 3H), 1.24 (s, 3H), 1.94 (m, 2H, $\text{CH}_2\text{CH}_2\text{CH}_2$),

3.38 (m, 2H), 3.48 (t, 2H, CH_2NH), 3.85 (m, 4H), 3.92 [s, 3H, CH_3O (Acr)], 4.03 (m, 1H), 7.24–7.83 (m, 5H, Acr), 8.26–8.31 (d, 1H, H8 Acr).

1-*O*-(4,4'-Dimethoxytrityl)-3-*O*-(*N*-acridinyl-3-aminopropyl)glycerol (**2**). (*N*-Acridinyl-3-aminopropyl)solketal (**1**) (2 g, 4.65 mmol) was dissolved in a mixture of tetrahydrofuran (45 mL) and 1 M hydrochloric acid (45 mL) and left for 30 min, and absolute ethanol (4 mL) was added. The mixture was concentrated, and the residue was redissolved in absolute ethanol (45 mL) and re-concentrated. The obtained residue was dried by coevaporation with anhydrous pyridine and dissolved in pyridine. 4,4'-Dimethoxytrityl chloride (1.56 g, 4.65 mmol) was added, and the mixture was stirred for 2 h at room temperature. Absolute ethanol (20 mL) was added, and the mixture was concentrated under reduced pressure. The residue was dissolved in CH_2Cl_2 (100 mL) and washed with water ($2 \times 30 \text{ mL}$) and with a saturated NaCl aqueous solution. The organic layer was dried over Na_2SO_4 and concentrated to dryness. The resultant oil was chromatographed on a silica gel column eluted with $\text{CH}_2\text{Cl}_2/\text{MeOH}$ (95:5 v:v) to give compound **2** (2.1 g) with 59% yield. $R_f = 0.61$ using $\text{CH}_2\text{Cl}_2/\text{MeOH}$ (90:10 v:v) as eluent. $^1\text{H-NMR}$ (DMSO): δ 1.97 (m, 2H, $\text{CH}_2\text{CH}_2\text{CH}_2$), 2.5 (m, 4H), 3.08 (m, 1H), 3.48 (t, 2H, CH_2NH), 3.65 (s, 6H, DMTr), 3.77 (t, 2H, OCH_2CH_2), 3.90 [s, 3H, CH_3O (Acr)], 5.06 (m, 1H, CHOH), 6.56–7.4 (m, 7H, DMTr), 7.40–7.90 (m, 5H, Acr), 8.26–8.88 (d, 1H, H8 Acr).

Acridine-Containing Modified Support 3. Compound **2** (1.2 g, 1.6 mmol) and dimethylaminopyridine (DMAP) (200 mg, 0.8 mmol) were dried by coevaporation with anhydrous pyridine ($3 \times 15 \text{ mL}$) and dissolved in pyridine (15 mL). Succinic anhydride (150 mg, 1.5 mmol) was added, and the solution was stirred overnight at 45 °C. Pyridine was removed by evaporation, and the obtained residue was dissolved in CH_2Cl_2 (200 mL). The organic layer was washed with an ice-cooled 10% aqueous solution of citric acid ($2 \times 100 \text{ mL}$) and then with a saturated aqueous NaCl solution. The organic layer was dried over Na_2SO_4 and concentrated under reduced pressure. The residue was purified on a silica gel column eluted with $\text{CH}_2\text{Cl}_2/\text{CH}_3\text{OH}$ (85:15 v:v) to afford 1 g of the succinate derivative. $R_f = 0.38$ and 0.35 (stereoisomers) using $\text{CH}_2\text{Cl}_2/\text{CH}_3\text{OH}$ (90:10 v:v) as eluent (yield of 77%). The succinate derivative (0.8 g, 1.1 mmol) was dried by coevaporation with anhydrous pyridine ($3 \times 10 \text{ mL}$) and redissolved in dry dioxane (3 mL). Pyridine (0.8 mL), *N*-hydroxysuccinimide (136 mg, 1.1 mmol), and DCCI (0.8 g, 2.9 mmol) were added, and the mixture was stirred overnight at room temperature. The precipitate of dicyclohexylurea was filtered, and the filtrate was concentrated under reduced pressure. The residue was chromatographed on a silica gel column eluted with acetone/hexane (6:4 v:v) to afford 0.7 g of the succinimidylated derivative in 77% yield. $R_f = 0.23$ using ethyl acetate/MeOH (85:15 v:v) as eluent.

Long chain alkylamine CPG (1 g) was added to a solution of the succinimidyl derivative (0.4 g, 0.48 mmol) in 2.5 mL of anhydrous dioxane, 0.5 mL of DMF, and 0.25 mL of NEt_3 . The suspension was gently stirred for 24 h at room temperature. The supernatant was filtered off, and the support was washed with dioxane ($2 \times 10 \text{ mL}$), CH_2Cl_2 ($2 \times 10 \text{ mL}$), methanol ($2 \times 10 \text{ mL}$) and ether ($2 \times 10 \text{ mL}$). Then a capping step was performed and is described in ref 26. The modified support **3** was obtained with a loading of 16 $\mu\text{mol/g}$.

Synthesis of the Oligonucleotides 7, 7a, and 7b Bearing the Acridine at an Internucleotidic Phosphate. Preparation of Oligonucleotide **4** Involving a Central Phosphorothioate Group and Separation of Its

Diastereoisomers. The oligodeoxyribonucleotide **4** was assembled using classical phosphoramidite chemistry except that at the site selected for the introduction of a phosphorothioate group the oxidation step by iodine was replaced by sulfuration using a 15 min treatment with a 0.5 M tetraethylthiuram disulfide solution in CH₃CN (32). The deprotection step was carried out using concentrated ammonia treatment. Purification was performed by reversed-phase chromatography using system A (5 to 42.5% CH₃CN in 50 min). Two roughly equivalent peaks with very close retention times (t_R = 20 min 51 s and 21 min 3 s) which correspond to the two diastereoisomers were obtained. These diastereoisomers were separated by using a more flat gradient (system C, see Table 2). Under these conditions, retention times were 23 min 30 s and 24 min 30 s, respectively.

Preparation of Dinucleoside $d^{5'}Tp(S)C^{3'}$. This dinucleoside used as a reference was obtained by solid-phase synthesis using tetraethylthiuram disulfide as the sulfurizing agent. After deprotection and releasing from the support, reversed-phase purification of the dinucleoside gave nearly equivalent peaks (55 and 45%, respectively) which correspond to the diastereoisomers (t_R = 21 min 48 s and 24 min 13 s using the following solvent system: 0% buffer B for 5 min, 0 to 10% in 10 min, and finally 10 to 20% in 30 min).

Synthesis of 2-Methoxy-6-chloro-9-(ω -aminooctylamino)acridine **5.** 2-Methoxy-6,9-dichloroacridine (0.3 g, 1.2 mmol), phenol (2.5 g), and 1,8-diaminooctane (0.77 g, 5.38 mmol) were reacted for 90 min at 100 °C under magnetic stirring. The mixture was diluted with methanol (5 mL) and added dropwise to a magnetically stirred 2 M sodium hydroxide solution (15 mL) for 10 min. The yellow precipitate was extracted with dichloromethane (3 $\times$ 60 mL), and the organic phase was concentrated to dryness. The obtained residue was purified on glass-backed plates of Merck silica gel 60 F 254 ref 5717 using CH₂Cl₂/MeOH (80:20 v:v) as eluent (twice) and then CH₂Cl₂/MeOH/DIEA (80:20:4 v:v). Compound **5** was obtained as a yellow powder with 70% yield. The presence of the primary amino group was verified using the ninhydrin test. R_{f5} = 0.48 using CH₂Cl₂/MeOH/DIEA (80:20:4 v:v) as eluent.

Preparation of the Iodoacetamidocridinyl Derivative **6.** Compound **5** (0.15 g, 1.03 mmol) and *p*-nitrophenyl iodoacetate (0.48 g, 1.55 mmol) were reacted in dry DMF at room temperature for 3 h under magnetic stirring. The solvent was removed by evaporation under vacuum, and the residue was purified on glass-backed plates of Merck silica gel 60 F 254 ref 5717 using CH₂Cl₂/MeOH (83:17 v:v) as eluent. Compound **6** was obtained as a yellow powder after stirring in hexane with a 75% yield. R_{f6} = 0.40 using CH₂Cl₂/MeOH (83:17 v:v) as eluent.

Synthesis of the Oligonucleotides **7, **7a**, and **7b** by Coupling of the Acridine Derivative **6** with the Oligonucleotides **4**, **4a**, and **4b**.** Oligodeoxyribonucleotide **4**, **4a**, or **4b** ($d^{5'}TpTpCpCpTp(S)CpCpTpCpT^{3'}$) (ammonium salt) (10 OD units) was dissolved in methanol (1 mL) in the presence of crown-6 ether (10 mg), and compound **6** (1 mg) was added. The mixture was kept at 45 °C for 2 h. The reaction mixture was then analyzed on a reversed-phase column using a linear gradient of 15 to 45% of buffer B in 40 min. The coupling yield was higher than 75%. t_{R4a} = 9 min 12 s, t_{R4b} = 9 min 30 s, and t_{R7} = 23 min 35 s. The two isomers of compounds **7**, **7a**, and **7b** could be better separated using a different gradient, 22 to 24% of buffer B in 25 min. t_{R7a} = 18 min 5 s, and t_{R7b} = 19 min 37 s.

5-(ω -Aminohexylamino)-5'-O-(dimethoxytrityl)-2'-deoxyuridine (8b**).** 5-Bromo-5'-O-(dimethoxytrityl)-2'-

deoxyuridine (0.3 g, 0.49 mmol) was dried by coevaporation with anhydrous CH₃CN (3 $\times$ 50 mL), kept in CH₃CN (25 mL), and 1,6-diaminohexane (0.56 g, 4.9 mmol) was added. The solution was stirred for 2 days at 65 °C. The solution was then concentrated to dryness, and the residue was dissolved in CH₂Cl₂ (100 mL) and washed with water (2 $\times$ 25 mL). The organic layer was dried over sodium sulfate and then concentrated under reduced pressure. The obtained residue was purified twice on glass-backed plates of silica gel using CH₂Cl₂/MeOH (90:10 v:v) as eluent and then CH₂Cl₂/MeOH/NEt₃ (80:15:5 v:v). Compounds **8a** and **8c** were obtained following the same protocol and using the appropriate diamine, 1,4-diaminobutane and 1,7-diaminoheptane, respectively [R_{f8a} = 0 using CH₂Cl₂/MeOH (90:10 v:v), R_{f8b} = 0 using CH₂Cl₂/MeOH (90:10 v:v), and R_{f8c} = 0 using CH₂Cl₂/MeOH (90:10 v:v)].

5-(ω -Acridinylaminohexylamino)-5'-O-(dimethoxytrityl)-2'-deoxyuridine (9b**).** **8b** (0.19 g, 0.29 mmol) was dried by coevaporation with anhydrous CH₃CN and pyridine and redissolved in CH₂Cl₂ and CH₃CN, and 6-chloro-9-phenoxy-2-methoxyacridine (0.2 g, 0.58 mmol) was added. The mixture was allowed to react at 65 °C with magnetic stirring. After 1 night, the solvent was removed by evaporation and the yellow residue was purified twice on glass-backed plates of silica gel 60 using CH₂Cl₂/MeOH (90:10 v:v) as eluent. R_{f9b} = 0.26 using CH₂Cl₂/MeOH (87:13) (twice) as eluent. Yield = 65%. Compounds **9a** and **9c** were obtained starting from compounds **8a** and **8c**, respectively, and by using the same protocol. R_{f9a} = 0.18 using CH₂Cl₂/MeOH (87:13 v:v) (twice) as eluent. Yield = 68%. R_{f9c} = 0.30 using CH₂Cl₂/MeOH (87:13 v:v) (twice) as eluent. Yield = 70%.

9a. MS (DIC): m/z = 857 (M^+)Cl/NH₃.

9a. ¹H-NMR (DMSO): δ 1.60–1.90 (m, 4H, NCH₂CH₂CH₂CH₂NHAc), 2.00–2.10 (m, 2H, H-2'', H-2'); 2.85 (m, 2H, NCH₂), 3.55 (m, 2H, H-5', H-5''), 3.90 [br s, 5H, CH₃O (Acr) + CH₂N], 4.25 (m, 1H, H-4'), 4.48 (m, 1H, H-3'), 5.0 (m, 1H, 3'-OH), 5.18 (d, 1H, 5'-OH), 6.24 (t, 1H, H-1'), 6.82 (s, 1H, H-6), 7.29–7.58 (m, 5H, Acr), 8.39–8.42 (d, 1H, H8 Acr), 11.28 (s, 1H, NH).

Preparation of the Modified Support **10.** This was achieved as described in ref 26 using compounds **9a** and **9b** instead of 5'-O-(dimethoxytrityl)thymidine.

Preparation of 5-(ω -Acridinylaminobutylamino)-5'-O-(dimethoxytrityl)-3'-O-[(2-cyanoethyl)-*N,N*-diisopropylamidophosphite]-2'-deoxyuridine (11a**).** To a dichloromethane solution (4 mL) of 5-*N*-[2-methoxy-6-chloro-9-(ω -butylamino)acridinyl]-5'-O-(dimethoxytrityl)-2'-deoxyuridine (**9a**) (0.14 g, 0.14 mmol) previously dried by coevaporation with anhydrous pyridine and kept under vacuum overnight was added excess diisopropylethylamine (0.135 mL, 0.093 g, 0.72 mmol) under a nitrogen atmosphere. (2-Cyanoethyl)-*N,N*-diisopropylchlorophosphite (0.040 g, 0.17 mmol) was added dropwise at room temperature with magnetic stirring. The reaction was monitored by TLC on silica gel with ethyl acetate/triethylamine (90:10 v:v) as eluent. After 30 min, the starting material (R = 0.40) was fully transformed into a compound having a higher R_f (0.50). After the usual workup, the residue was purified on a silica gel column using ethyl acetate/triethylamine (95:5 v:v) as eluent. The fraction containing the pure product was pooled and the solvent removed under reduced pressure. The obtained residue was precipitated in hexane. R_{f11a} = 0.38 using ethyl acetate/NEt₃ (90:10 v:v) as eluent. Yield = 80%. R_{f11c} = 0.46 using ethyl acetate/NEt₃ (90:10 v:v) as eluent. Yield = 76%.

Preparation of the Acridine-Containing Oligodeoxynucleotides **12a, **12b**, **13**–**17**, and **22**.** Chain elongation

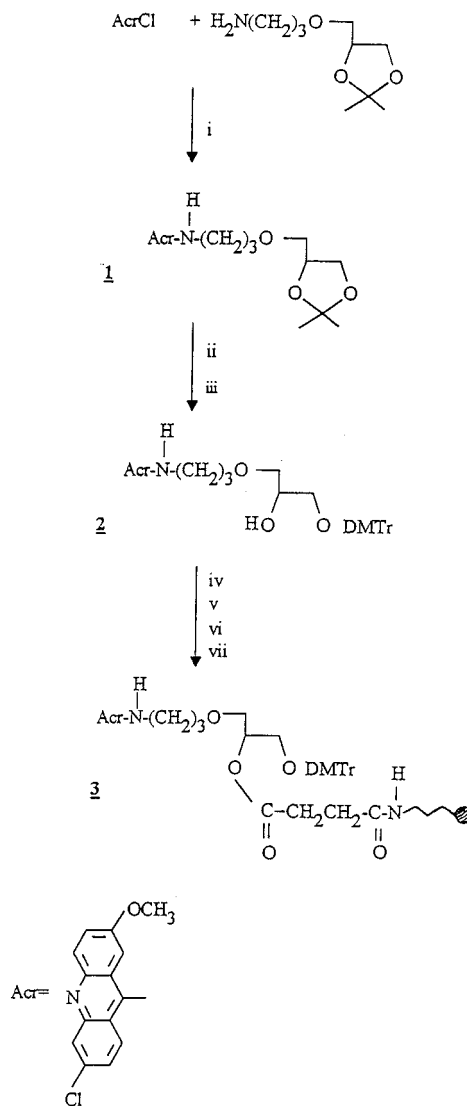
was performed by the classical phosphoramidite procedure on a modified support **3**, **10a**, or **10b** (at the 0.5 or 1 μ mol scale, depending on the loading of the modified support) and/or the acridine-containing phosphoramidites **11a** or **11c** with increased coupling time (5 min) for the latter. The oligonucleotides, bound to the support, were submitted to an additional detritylation step to deprotect the 5'-hydroxyl function and then treated with a 0.4 M sodium hydroxide solution in H₂O/MeOH (50:50 v:v) mixture for 3 h. The pHs of the solutions were then adjusted to 7, and the methanol was removed by evaporation. After extraction with ethyl acetate and filtration using a 0.45 μ m disposable filter, the aqueous oligodeoxyribonucleotide solutions were purified by ion-exchange or reversed-phase chromatography using the systems described in General Methods.

RESULTS AND DISCUSSION

Synthesis of the Oligonucleotide-Acridine Conjugates. *Synthesis of the Universal Support 3.* 3'-Acridine-linked oligonucleotides were obtained using a new universal support, while in our previous work, one modified support was needed for each nucleotide at the 3'-position of the sequence (29). The synthesis of the universal support **3** was achieved following a five-step procedure (Scheme 1). Reaction of 3-aminosolketal (obtained as described in ref 31) with 6,9-dichloro-2-methoxyacridine in a phenol solution gave the (*N*-acridinyl-3-aminopropyl)solketal (**1**) in 45% yield. The acridine derivative **1** was treated with a mixture of 1 M hydrochloric acid and tetrahydrofuran (1:1) to remove the isopropylidene group. Without purification, the product was reacted with 4,4'-dimethoxytrityl chloride in anhydrous pyridine to give 1-*O*-(4,4'-dimethoxytrityl)-3-*O*-(ω -acridinylaminopropyl)glycerol (**2**) which was purified by silica column chromatography in a 59% yield. Starting from compound **2**, the acridine-containing modified CPG support **3** was obtained following a three-step procedure with a loading of 16 μ mol of acridine per gram of support. The oligonucleotides containing the acridine moiety at the 3'-position via a phosphate group were assembled at the micromolar scale using classical phosphoramidite chemistry (26) (Table 1).

Attachment of the Acridine Moiety via an Internucleotide Phosphorothiolate. For the introduction of the acridine moiety via an internucleotide phosphorothiolate, we chose a previously described method (33) involving a phosphorothioate group as the site for alkylation by an acridine derivative (Scheme 2). Using this strategy, a phosphoramidite coupling at a preselected position is followed by a sulfuration step instead of oxidation to generate the phosphorothionotriester which is stable in the subsequent oxidation steps necessary to generate the internucleotidic phosphodiester in the remainder of the oligodeoxyribonucleotide. Our previous results (28, 29) showed that the synthesis of a 20-mer oligonucleotide involving a 3'-phosphorothioate group was carried out on a modified support involving a phosphorothiono triester group without significant loss of sulfur. The 11-mer d^{5'}-TTTCCTp(S)CCTCT^{3'} **4** was obtained as a mixture of stereoisomers whose structures are given in Scheme 3. Reversed-phase analysis of compound **4** exhibited two roughly equivalent peaks (56 and 44%, respectively) with very close retention times (Table 1). The two diastereoisomers **4a** (fast eluted) and **4b** (slowly eluted) were separated by reversed-phase chromatography (see Experimental Procedures for the conditions used) and assigned to be the *R_p* isomer (fast eluted) and *S_p* (slowly eluted) after digestion by the nuclease P1 followed by alkaline phosphatase treatment. This led to the full

Scheme 1. Synthesis of the Universal Acridinyl-Modified Support **3**^a



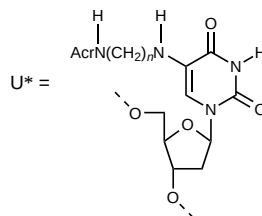
^a (i) phenol, 120 °C; (ii) aqueous HCl, THF; (iii) DMTrCl, Py; (iv) succinic anhydride, DMAP, Py; (v) *N*-hydroxysuccinimide, Py, DCC, dioxane; (vi) NH₂ with cross-hatched circle = LCAA-CPG, NEt₃, DMF; (vii) acetic anhydride.

degradation of the *S_p* isomer in dC and dT, while the *R_p* isomer, under the same conditions, led to dC and dT and to the *R_p* dinucleoside phosphorothioate d^{5'}Tp(S)C^{3'} identified by comparison with an authentic sample of the *R_p* dinucleoside d^{5'}Tp(S)C^{3'} (**34**). It must be noted that the ratio obtained for the *R_p* and *S_p* dinucleosides d^{5'}Tp(S)-C^{3'} was in accordance with recently reported data (35). Reaction of compound **4** (Scheme 2a) as a mixture of diastereoisomers or as a pure *R_p* or *S_p* isomer with the iodoacetamidoacridine derivative AcrN(H)(CH₂)₈N(H)C(O)CH₂I (**6**) whose preparation is described in Scheme 2b yielded the corresponding mixture of diastereoisomers **7** (Figure 1) of the acridine-labeled 11-mer [d^{5'}TTTCCTp-[SCH₂C(O)N(H)(CH₂)₈NHAc]CCTCT^{3'}] or the pure *R_p* **7a** and *S_p* **7b** isomers whose structures are given in Scheme 3. The acridine derivative **6** was chosen with an 11-atom linker since the CPK model has shown that this arm is better suited than the previously used one involving five methylene groups (4), although this size is long enough when the acridine derivative is attached to the 3'- or 5'-end of an oligonucleotide. In these cases, the structures involving a phosphodiester linkage between the linker and the oligonucleotide are less restricted than when the

Table 1. Structures of the Modified Oligodeoxyribonucleotides Synthesized and Reversed-Phase Analysis of the Oligodeoxyribonucleosides Used in This Study Performed on a Lichrospher 100 RP (5 μ m) Column (125 mm $\times$ 4 mm) from Merck

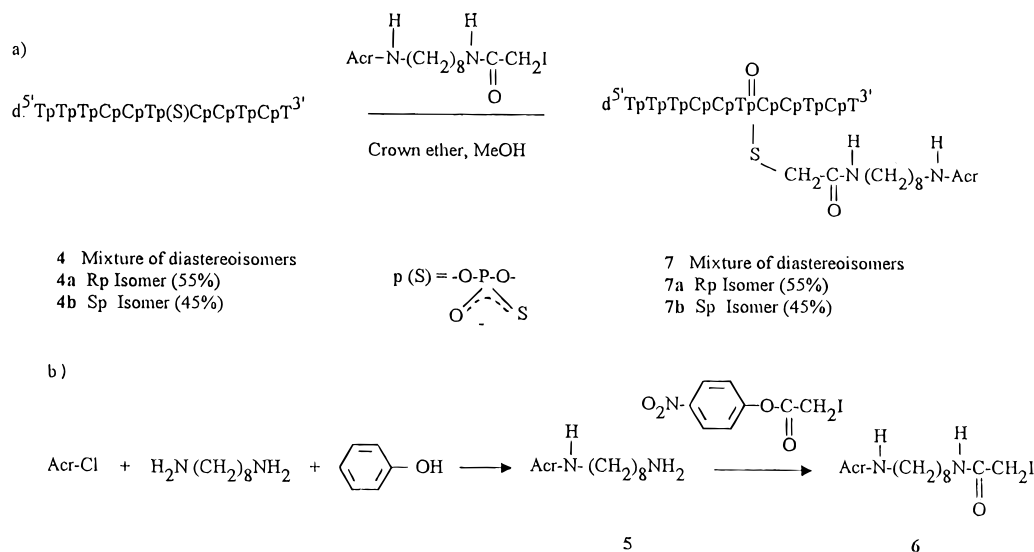
compounds		reversed-phase analysis
d ^{5'} TTTCCTp(S)CCTCT ^{3'}	4 (mixture of diastereoisomers)	20 min 52 s ^b 21 min 2 s ^b 23 min 30 s ^c
d ^{5'} TTTCCTp(S X)CCTCT ^{3'}	4a (<i>R</i> _p isomer)	24 min 30 s ^c
	4b (<i>S</i> _p isomer)	29 min 36 s ^b
	7 (mixture of diastereoisomers)	18 min 5 s ^d
	7a (<i>R</i> _p isomer)	19 min 37 s ^d
d ^{5'} TTTCCTCCTCU* ^{3'}	7b (<i>S</i> _p isomer)	19 min 6 s ^a
	12a (<i>n</i> = 4)	21 min 14 s ^a
d ^{5'} TTTCCTCCTCT ^{3'} p-Y	12b (<i>n</i> = 6)	19 min 26 s ^a
	13	19 min ^a
d ^{5'} TTTCCU*CCTCT ^{3'}	14 (<i>n</i> = 4)	37 min 52 s ^a
d ^{5'} TTTCCU*CCTCU* ^{3'}	15 (<i>n</i> = 4)	23 min 9 s ^a
Z-O-p-d ^{5'} TTTCCTCCTCT ^{3'} p-Y	16	33 min ^b
Z-O-p-d ^{5'} TTTCCU*CCTCT ^{3'}	17 (<i>n</i> = 4)	21 min 52 s ^a
Z-O-p-d ^{5'} TTTCCTCCTCT ^{3'}	18	21 min 30 s ^a
d ^{5'} TTTCCTCCTCT ^{3'} p-S-Z	19	17 min 18 s ^b
5'-dAGTAGAGGAGGAAATAG ^{3'}	20	16 min 18 s ^b
d ^{5'} TTTCCTCCTCT ^{3'}	21	34 min 10 s ^c
5'-dTTTTCTTU*TC ^{3'}	22	16 min 42 s ^b
5'-TTTAAAGAAAAGGG ^{3'}	23	17 min 18 s ^b
5'-dTTTTCTTTTC ^{3'}	24	

X = CH₂C(O)NH(CH₂)₈NHAc
Y = OCH₂CH(OH)CH₂O(CH₂)₃NHAc
Z = AcrNH(CH₂)₆



^a System A: 5 to 42.5% CH₃CN in 50 min. ^b System B: 5% CH₃CN for 5 min and then 5 to 42.5% CH₃CN in 50 min. ^c System C: 0 to 40% CH₃CN in 50 min. ^d System D: 21.5 to 23% CH₃CN in 40 min.

Scheme 2. Synthesis of the Acridine Linker 6 and of the Oligodeoxyribonucleotides 7, 7a, and 7b Involving an Acridine Attached at an Internucleotidic Phosphorothioate



acridine is linked to an internucleotidic phosphate via a phosphothiotriester group. The iodoacetamido derivative **6** was used since coupling with the ω -bromoundecyl derivative Br(CH₂)₁₁NHAc was not possible, probably due to the lower reactivity of an internucleosidic phosphorothioate compared to that of a terminal phosphorothioate. This was achieved in a two-step procedure (Scheme 2b), in which 2-methoxy-6,9-dichloroacridine was reacted with an excess (5 equiv) of 1,8-diaminooctane to yield the ω -amino derivative **5** whose reaction with *p*-nitrophenyl iodoacetate gave the expected acridine derivative **6**.

Attachment of the Acridine Moiety to the 5-Position of the Deoxyuridine. The attachment of the intercalator to the 5-position of deoxyuridine was developed following Scheme 4. First, 5'-O-(4,4'-dimethoxytrityl)-5-bromodeoxyuridine was reacted with 1,4-diaminobutane, 1,6-diaminohexane, or 1,7-diaminoheptane to give 5'-O-(dimethoxytrityl)deoxyuridine substituted at the 5-position with (4-aminobutyl)amino, (6-aminohexyl)amino, or (7-aminohexyl)amino linker **8a**, **8b**, or **8c**, respectively. Then, the latter were reacted with 6-chloro-9-phenoxy-2-methoxyacridine (obtained as described in ref 27) in acetonitrile to give compounds **9a**, **9b**, and **9c**, respec-

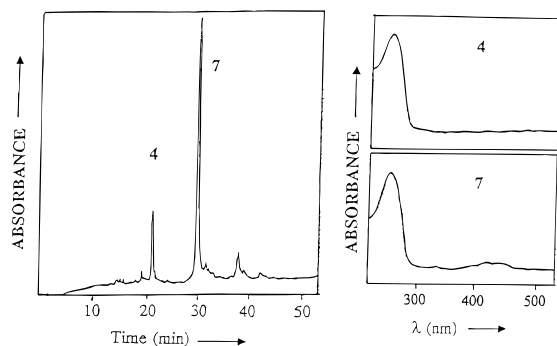
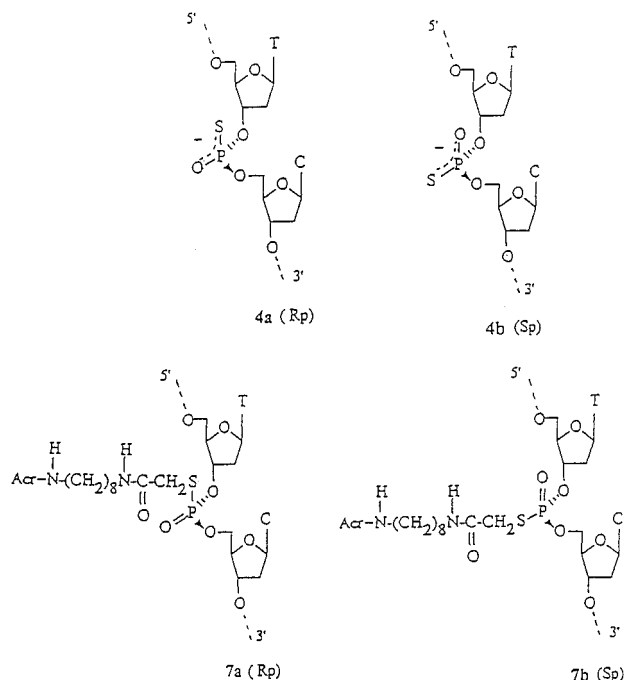


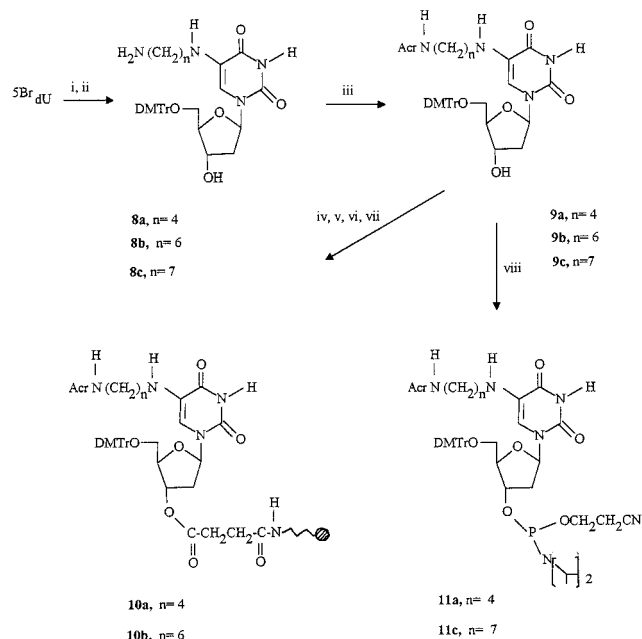
Figure 1. Reversed-phase analysis of the coupling reaction of the oligonucleotide phosphorothioate $d^5\text{TpTpTpCpCpTp(S)-CpCpTpCpT}^{3'}$ ($t_R = 21$ min) with the acridinyl linker derivative **6**. The oligonucleotide–acridine conjugate **7** ($t_R = 29$ min 26 s) has been obtained with a yield of 75% (left). The insets show the absorption spectra, recorded between $\lambda = 220$ nm and $\lambda = 550$ nm, of the starting oligonucleotide **4** (upper right) and of the oligonucleotide–acridine conjugate $d^5\text{TpTpTpCpCpTp-[SCH}_2\text{C(O)NH(CH}_2)_8\text{NHAcrlCpCpTpCpT}^{3'}$ **7** (lower right). Analysis has been performed on a Lichrospher 100 RP-18 ($5\ \mu\text{m}$) column (125×4 mm) using a linear gradient of CH_3CN in 0.1 M aqueous triethylammonium acetate buffer (pH 7) with a flow rate of 1 mL/min (0 to 50% buffer B in 50 min).

Scheme 3. Structures of the R_p Isomers **4a** and **7a** and S_p Isomers **4b** and **7b** of the Oligonucleotides **4** $d^5\text{TTTCCTp(S)CCTCT}^{3'}$ and $d^5\text{TpTpTpCpCpTp-[SCH}_2\text{C(O)NH(CH}_2)_8\text{NHAcrlCpCpTpCpT}^{3'}$ **7**



tively, with a yield ranging from 65 to 70%. In order to incorporate acridine-containing modified deoxyuridine into any position of the oligonucleotide chain, both the modified support derivatized with the acridine-modified deoxyuridine and the corresponding 3'-phosphoramidite derivative must be obtained. Starting from the modified nucleosides **9a** and **9b**, the modified derivatized supports **10a** and **10b** were obtained following the classical procedure (26) with a loading of 8 and 12 $\mu\text{mol/g}$, respectively. Phosphitylation of compound **9** was carried out using 1.1 equiv of (2-cyanoethyl)(*N,N*-diisopropylamido)chlorophosphite in dichloromethane in the presence of *N,N*-diisopropylethylamine. Under these conditions, the desired products **11a** and **11c** were obtained after silica gel chromatography and precipitation in hexane with 75 and 80% yields, respectively.

Scheme 4. Synthesis of the Modified Supports **10a,b** and of the Phosphoramidites **11a,b**^a



^a DMTr = dimethoxytrityl; cross-hatched circle attachment = LCAA-CPG. (i) DMTrCl, Py; (ii) $\text{H}_2\text{N(CH}_2)_n\text{NH}_2$ ($n = 4, 6$, or 7), CH_3CN , 65°C ; (iii) 6-chloro-9-phenoxy-2-methoxyacridine, CH_3CN ; (iv) succinic anhydride, DMAP, Py; (v) *p*-nitrophenol, Py, DCC, dioxane; (vi) LCA-CPG, NEt_3 , DMF; (vii) acetic anhydride, Py; (viii) ClPN(*i*Pr)₂OCH₂CH₂CN, DIEA, CH_2Cl_2 .

Synthesis of the Oligonucleotides. Using the above-mentioned supports **3**, **10a**, and **10b** and the phosphoramidites **11a** and **11c**, oligonucleotides bearing one or two acridine moieties whose structures are given in Table 1 were assembled. In order to compare the stabilization due to the acridine using the new described linkages with that obtained with previously described acridine–oligonucleotide conjugates, the compounds $\text{AcrNH(CH}_2)_6\text{-p-}d^5\text{TTTCCTCCTCT}^{3'}$ (**18**) and $d^5\text{TTTCCTCCTCT}^{3'p}\text{S(CH}_2)_6\text{-NHAcrl}$ (**19**) have been prepared by using our previously described procedures (28). After the chain assembly, the removal of the protective group and the releasing from the support of the acridine-containing oligonucleotides were achieved by 0.4 M sodium hydroxide treatment. Using NH_4OH with acridine-containing oligonucleotides must be avoided in order to prevent the cleavage of the bond between the acridine ring and the nitrogen atom of the linker (28). After full deblocking of the oligonucleotides, the purification step was achieved either by ion-exchange or by reversed-phase chromatography. Attempts to purify an undecamer involving two U acridine-modified bases at internal positions (3 and 9) ($d^5\text{TTU}^*\text{CCTCCU}^*\text{CT}^{3'}$) ($n = 4$) by either chromatography or polyacrylamide gel electrophoresis under denaturing conditions failed. However, no major problem occurred during the purification of compound **16** with one acridine at each end and compound **15** with one acridine at one end and the second on U at an internal location. After purification, analyses of the oligonucleotide–acridine conjugates were performed on both ion-exchange and reversed-phase columns (Table 1). The structures of oligonucleotide derivatives **7**, **7a**, **7b**, **12a**, **12b**, and **13–19** were confirmed by different methods. Full deprotection and base composition analysis have been ascertained by reversed-phase analysis after nuclease degradation (Figures 2 and 3). The obtained modified oligomers were also analyzed by Maldi mass spectroscopy (36, 37). The results are in accordance with the calculations (see

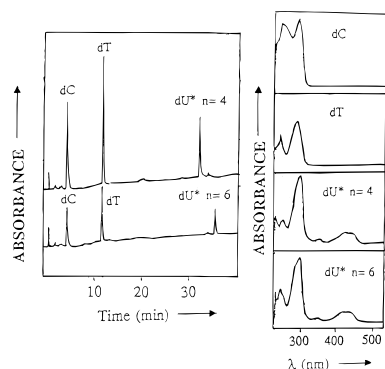


Figure 2. Reversed-phase HPLC analysis of the hydrolysate of the compounds **12a** (d⁵TTTCCTCCTCU*) ($n = 4$) (upper) and **12b** (d⁵TTTCCTCCTCU*) ($n = 6$) (lower) with nucleases (P1 from *Penicillium citrinum* and alkaline phosphatase) on a Lichrospher 100 RP-18 (5 μ m) column (125 $\times$ 4 mm) using a linear gradient of CH₃CN in 0.1 M aqueous triethylammonium acetate buffer (pH 7) with a flow rate of 1 mL/min (0% CH₃CN for 5 min, then 0 to 20% in 20 min, and then 20 to 50% CH₃CN in 15 min). The right parts show the absorption spectra, recorded between $\lambda = 220$ nm and $\lambda = 550$ nm, of the obtained compounds: dC, dT, and dU* ($n = 4$) and dU* ($n = 6$) from top to bottom, respectively.

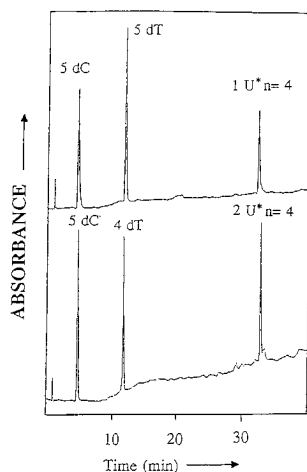


Figure 3. Reversed-phase analysis of the hydrolysate of the compounds **12a** (upper) and **15** (lower) with nucleases (P1 from *P. citrinum* and alkaline phosphatase). The conditions are the same as those described in the legend of Figure 2. The ratio of the peaks corresponding to the monomers dC, dT, and U* indicates that the compound **15** (d⁵TTTCCU*CCTCU*) ($n = 4$) contains two acridinyl-modified U* compared to the compound **12a** (d⁵TTTCCTCCTCU*) ($n = 4$) which contains only one acridine residue.

Experimental Procedures for conditions used). The UV/visible spectra of the acridine derivatives showed absorption maxima corresponding to the oligonucleotide domain at 260 nm and to the 2-methoxy-6-chloro-9-aminoacridine at 425 nm. Comparison of the spectra of the oligonucleotide and dye with those of the oligonucleotide-acridine conjugates and nuclease degradation analysis confirm both the existence and the number of intercalating residues in the molecular structure (Figures 2 and 3). As expected, the mobility of the oligonucleotide derivatives using a Lichrosorb RP 18 (5 μ M) HPLC column (125 $\times$ 4 mm) decreases with the introduction of the hydrophobic dye residue. As shown in Table 1, the length of the polymethylene linker also affects the mobility of the acridine derivatives. Ion-exchange analysis performed on a 8 μ M DEAE (100 mm $\times$ 10 mm) column with a linear gradient of NaCl (0 to 0.9 M in 30 min) in a Tris/HCl (pH 7) 25 mM buffer containing 10% CH₃CN shows very close retention times for all the compounds analyzed.

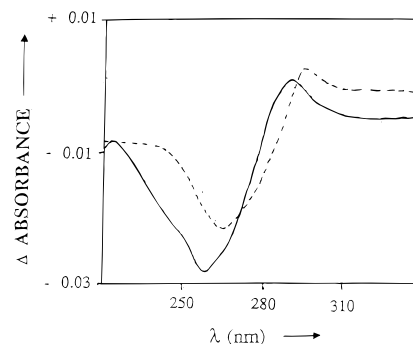


Figure 4. Difference between the absorption spectrum of a 1:1 mixture (1 μ M each strand) of the oligonucleotides **22** (d⁵-TTTCTTU*TC³) and **23** (d⁵TTTAAAAGAAAAGGG³) (—) or **24** (d⁵TTTCTTTTC³) and **23** (d⁵TTTAAAAGAAAAGGG³) (---) and the sum of the absorption spectra of the separated components **22** + **23** and **24** + **23**, respectively. The spectra were recorded between $\lambda = 220$ nm and $\lambda = 340$ nm at 2 $^{\circ}$ C in tandem cells. Measurements were carried out in a pH 7 buffer containing 10 mM sodium cacodylate and 0.1 M NaCl.

It must be noted that the acridine is protonated at pH 7 and the retention times are proportional to the total number of negative charges on the molecule. Therefore, compounds **21** (unmodified, 10 phosphate groups) and **13** (11 phosphate groups + 1 acridine) showed the same retention time (11 min 6 s), while compounds **12a**, **12b**, and **14** (10 phosphate groups + 1 acridine) eluted earlier (10 min 48 s).

Interaction with the Complementary Sequence.

To determine the stabilizing effect of the acridine residue on the hybridization of the acridine-oligonucleotide conjugates with the complementary sequences, depending on its attachment site, the duplexes formed by the undecanucleotides **7** and **12–19** with an heptadecadeoxynucleotide (5'-d-AGTAGAGGAGGAAATAG³) **20** involving the complementary sequence were studied. The undecadeoxynucleotide (5'-dTTTCCTCCTCT³) **21** was used as a reference.

The interaction of the duplexes formed between oligomers **7**, **12–19**, and **21** and the complementary target DNA sequence **20** was followed by absorption spectroscopy in the visible range where only the acridine ring absorbed light and in the UV range in the absence of acridine. Addition of increasing concentrations of oligonucleotide **20** to acridine-oligonucleotide conjugate solutions at 4 $^{\circ}$ C led to changes in the absorbance spectra of compounds **12a**, **12b**, **13**, **16**, **18**, and **19**. When absorbance at 425 or 260 nm was plotted versus the target concentration, a sharp break occurred with a nearly 1:1 ratio, indicating a specific interaction between the acridine-oligonucleotide conjugates and their targets. In the case of oligonucleotide-acridine conjugates **12a**, **12b**, **13**, **16**, and **19**, a red shift of the visible acridine spectrum was observed together with a strong hypochromism, while in the case of the binding of oligomers **14** (d⁵-TTTCCU*CCTCT³) and **22** (d⁵-TTTTCTTU*TC³) with the internal acridine-modified U, except for a slight hypochromism, no significant change of the visible spectrum of the acridine was observed during the addition of the target sequences **20** (5'-dAGTAGAGGAGGAAATAG³) and **23** (d⁵TTTAAAAGAAAAGGG³), respectively. This probably indicates that different interactions occur between the acridine moiety and the double-stranded duplex when the acridine is linked to uridine located inside the sequence. Nevertheless, a variation of the UV spectrum confirms the formation of a complex between the oligonucleotides **14** and **20** or **22** and **23** (Figure 4).

When the temperature of the duplex solutions formed at 4 $^{\circ}$ C is increased, the changes of the absorbance

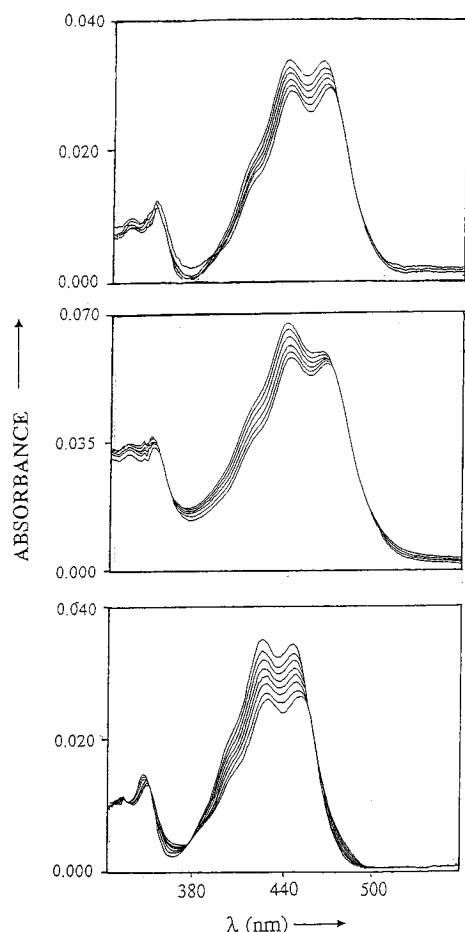


Figure 5. Change in absorbance spectrum with the temperature of 1:1 mixtures of oligomer **20** with oligonucleotide-acridine conjugates. Compounds **18** [AcrNH(CH₂)₆-d⁵TTT-CCTCCTCT³] (upper), **16** [AcrNH(CH₂)₆-p-d⁵TTTCCTCCTCT³-pOCH₂CH(OH)CH₂(CH₂)₃NHAc] (middle), and **12a** (d⁵TTT-CCTCCTCU*³) (*n* = 4) (lower). Measurements were carried out in a pH 7 buffer containing 0.01 mM sodium cacodylate and 0.1 M NaCl. The oligonucleotide concentration was 4 μM (each strand).

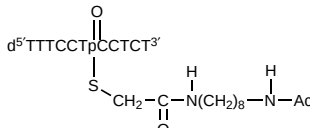
spectra observed during the addition of the target sequence **20** (5'-dAGTAGAGGAGGAAATAG³) are reversed (Figure 5). Plotting the difference in absorbance at $\lambda = 425$ nm or $\lambda = 260$ nm of a 1:1 mixture versus temperature gave the melting curves. When the acridine is covalently linked to either the 3'- or the 5'-position of the oligonucleotides, the T_m values (at which half of the duplex dissociation occurred) are not very different [**12a**, d⁵TTTCCTCCTCU*³ (*n* = 4), $T_m = 46.2 \pm 0.5$ °C; **12b**, d⁵TTTCCTCCTCU*³ (*n* = 6), $T_m = 46 \pm 0.5$ °C; **13**, d⁵TTTCCTCCTCT³-pOCH₂CH(OH)CH₂(CH₂)₃NHAc, $T_m = 45 \pm 0.5$ °C; **19**, d⁵TTTCCTCCTCT³-pS(CH₂)₆NHAc, $T_m = 48.4 \pm 0.5$ °C; and **18**, AcrNH(CH₂)₆-p-d⁵TTTCCTCCTCT³, $T_m = 47 \pm 0.5$ °C]. In any case, a strong stabilization was observed when compared to the T_m value (34.4 ± 0.5 °C) of the duplex formed by oligonucleotide **21** without acridine and the complementary sequence **20** (Table 2).

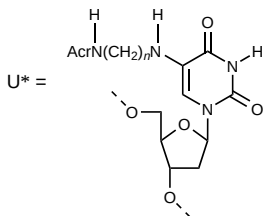
When the acridine is linked to an internal position of the oligonucleotide chain either to the 5-position of U or to an internucleotidic phosphate, different results are observed. The introduction of a single acridine-modified deoxyuridine to the middle of the sequence (compound **14**, d⁵TTTCCU*CCTCT³) leads to slight destabilization of the duplex (**14** + **20**) ($T_m = 32.8 \pm 0.5$ °C) compared to that of the unmodified parent duplex (**20** + **21**) ($T_m = 34.4 \pm 0.5$ °C). Despite the results obtained with oligo-

nucleotides **12a** (*n* = 4) and **12b** (*n* = 6) showing that the introduction of the acridine residue at the 5-position of deoxyuridine gives good stability of the duplex, we have chosen to use a longer linker between the acridine and the deoxyuridine. Thus, a 7-methylene linker instead of a 4-methylene arm [compound **14** (d⁵TTTCCU*CCTCT³), *n* = 4] was chosen. This longer linker arm with a higher degree of freedom should allow the acridine label to intercalate more efficiently. Along with this modification, we also decided to change the neighbor base pair at the possible intercalating site. Although in the sequence **14** the modified labeled deoxyuridine is located between two cytosines, we opted for the preparation of a new sequence in which an internally modified deoxyuridine is surrounded by two thymines (**22**, d⁵TTTCTTU*TC³, *n* = 7). Once again, the covalent linking of the acridine moiety to the 5-position of an internal deoxyuridine via a heptamethylene linker had a destabilizing effect on the duplex ($T_m = 16 \pm 0.5$ °C) formed with the complementary sequence **23** (5'-dTTTAAAAGAAAAGGG³) compared to the complex obtained with the unmodified parent **24** (5'-dTTTCTTTTC³) ($T_m = 19.8 \pm 0.5$ °C). Reported data (9) have shown that the incorporation of a sole linker with a terminal aminoalkyl group at the 5-position of an internal U in an oligonucleotide had a destabilizing effect. However, at pH 7, the primary amine linker arm was protonated and could thus help counteract the electrostatic barrier to duplex formation by the negatively charged oligomers. A similar effect has been described more recently (38). The incorporation of 5-hexyl-2'-deoxyuridine at an internal position of a dodecamer pyrimidine sequence had a strong destabilizing effect on the duplex formation with the complementary sequence ($\Delta T_m = -4.5$ °C in 50 mM NaCl).

Consistent with these literature data and with the fact that the linking of the acridine ring to the 5-position of a uracil had a strong stabilizing effect when the modified U is located at a terminal position of an oligonucleotide (compounds **12a** and **12b**) and a slight destabilizing one when the modified U is located at an internal position (compound **14**), one explanation could be that the covalent linking of an acridine to internal positions changes the hydration spin inside the groove, thus disturbing the duplex stability. Another explanation could be that the best position for the intercalation of the acridine ring is the 9-position in the minor groove while the linker attached to the 5-position of U* protrudes in the major groove. Along these lines, NMR studies (39) have proven that, when linked to an oligonucleotide via a 3'-phosphate, the 2-methoxy-6-chloro-9-aminoacridine intercalates with the 9-amino group in the minor groove. Thus, when the acridine-modified deoxyuridine is located at the 3'-end of the oligonucleotide (compound **12a** or **12b**), the base can rotate around the glycosidic bond to bring the acridine moiety into the minor groove. In addition, it must be noted that compounds **12a** and **12b** involving a 4- and 6-methylene linker length, respectively, form duplexes with very similar stability. This probably means that the linkers used have the optimum length to induce maximum stabilization of the duplex when the acridine-modified U* is located at the end of the oligomer. On the contrary, when the acridine-modified U* is located inside the oligonucleotide sequence (compound **14**), rotation of the acridine-modified base around the glycosidic bond is more unlikely due to the steric hindrance preventing a favorable position for the acridine moiety to intercalate. Nevertheless, the rotation around the glycosidic bond to allow intercalation of the acridine ring would induce mismatched a base pair and so a strong destabilization for the duplex. The increase of the linker

Table 2. Duplex Melting Temperatures^a

	duplexes		T_m (°C) (+0.5 °C)
20	d ^{3'} GATAAAGGAGGAGATGA ^{5'}	(target)	
21	d ^{5'} TTTCCTCCTCT ^{3'}		34.4
19	d ^{5'} TTTCCTCCTCT ^{3'} p-S-(CH ₂) ₆ NHAc		48.4
13	d ^{5'} TTTCCTCCTCT ^{3'} p-OCH ₂ CH(OH)CH ₂ O(CH ₂) ₃ NHAc		45.2
12	d ^{5'} TTTCCTCCTCU* ^{3'}	12a (n = 4)	46.2
		12b (n = 6)	46
18	AcrNH(CH ₂) ₆ O-p-d ^{5'} TTTCCTCCTCT ^{3'}		47
16	AcrNH(CH ₂) ₆ O-p-d ^{5'} TTTCCTCCTCT ^{3'} p-OCH ₂ CH(OH)CH ₂ O(CH ₂) ₃ NHAc		52.4
4	d ^{5'} TTTCCTp(S)CCTCT ^{3'}	4a (<i>R_p</i> isomer)	33
		4b (<i>S_p</i> isomer)	33.3
7		7a (<i>R_p</i> isomer)	45.2
		7b (<i>S_p</i> isomer)	51.4
14	d ^{5'} TTTCCU*CCTCT ^{3'}	(n = 4)	32.8
15	d ^{5'} TTTCCU*CCTCU* ^{3'}	(n = 4)	no T_m
17	AcrNH(CH ₂) ₆ Op-d ^{5'} TTTCCU*CCTCT ^{3'}	(n = 4)	26
23	d ^{3'} GGGAAAAGAAAATT ^{5'}	(target)	
24	d ^{5'} TTTTCTTTTC ^{3'}		19.8
22	d ^{5'} TTTTCTTU*TC ^{3'}	(n = 7)	16



^a All duplexes were in T_m buffer: 0.01 M sodium cacodylate and 0.1 M NaCl (pH 7). The concentration (each strand) were 4 μ M except for duplexes **4a** or **4b** + **20** (3.6 μ M) and for duplexes **14** + **20** and **22** or **24** + **23** (1 μ M). Unless stated otherwise in the text absorbance measurements were carried out at 425 nm for the acridine-containing compounds and at 260 nm for the compounds without acridine.

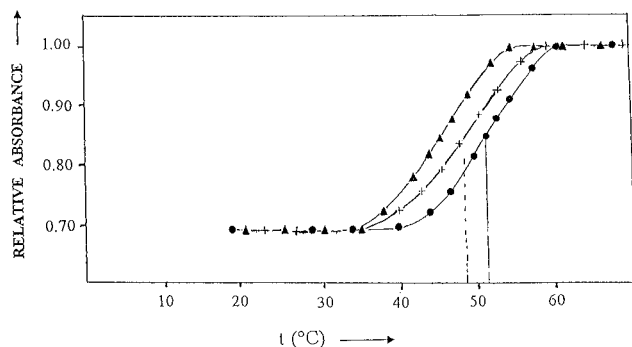


Figure 6. Melting curves at 425 nm of duplex **7** [d^{5'}-TpTpTpCpCpTp[SCH₂C(O)NH(CH₂)₈NHAc]CpCpTpCpT^{3'}] + **20** (+), duplex **7a** (*R_p* isomer) + **20** (Δ) and duplex **7b** (*S_p* isomer) + **20** (●) at a 4 μ M concentration. Measurements were carried out in a pH 7 buffer containing 10 mM sodium cacodylate and 0.1 M Na Cl.

length between the acridine residue and base did not lead to any stabilizing effect (compound **22**).

On the contrary, when the acridine moiety is attached to an internucleotidic phosphorothiolate **7** (d^{5'}TTTCCTp-[SCH₂C(O)N(H)(CH₂)₈NHAc]CCTCT^{3'}), a strong stabilization is observed when using either the mixture of diastereoisomers (T_m for **7** + **20** = 48.6 $\pm$ 0.5 °C) or each pure *R_p* isomer **7a** (T_m = 45 $\pm$ 0.5 °C) or *S_p* isomer **7b** (T_m = 51.4 $\pm$ 0.5 °C) (Figure 6). These results confirm our hypothesis that in our previous studies the linker used to connect the acridine ring to the internucleotidic phosphate of the oligonucleotide was too short (**4**). It is important to note that, under very close conditions (3.6 instead of 4 μ M duplex concentrations), the *R_p* and the *S_p* isomers of the 11-mer phosphorothioate **4** form duplexes with similar T_m values very close to that of the

duplex used as reference (T_m = 34.4 $\pm$ 0.5 °C). Concerning the varying stability obtained with *R_p* and *S_p* isomers **7a** and **7b**, one explanation could be that as observed on the CPK model and in accordance with a literature report (40) when the oligomer **7** is hybridized with its complementary sequence the sulfur of the *R_p* isomer would direct the covalently attached acridine moiety rather toward the major groove of the double helix in a position unfavorable for intercalation whereas the sulfur of the *S_p* isomer would direct the intercalating agent in a position allowing its interaction in either the minor or the major groove. When interaction takes place in the major groove, the linker is able to fold back, allowing only partial intercalation of the acridine which can explain the different stability ΔT_m = 6.2 °C observed for the two isomers.

When two acridine moieties are present on the same oligonucleotide, different behaviors are observed. When a second acridine moiety is added to the 5'-end of the conjugate **12a** to give the compound **16** (AcrNH(CH₂)₆p-d^{5'}TTTCCTCCTCT^{3'}pOCH₂CH(OH)CH₂(CH₂)₃NHAc), additional stability can be observed (T_m = 52.4 $\pm$ 0.5 °C). On the other hand, the addition of one acridine molecule to the 5'-end of the undecanucleotide **12a** to give the bis-acridine conjugate **17** (AcrNH(CH₂)₆p-d^{5'}TTTCCU*-CCTCT^{3'}) induces a duplex destabilization (T_m = 26 $\pm$ 0.5 °C). This large destabilization is probably due to the presence of the acridine on a modified U in the middle of the sequence as observed with compound **14** and also to intramolecular interactions. In the case of compound **15** (d^{5'}TTTCCU*CCTCU*^{3'}) involving two acridine residues linked to the 5-position of a modified deoxyuridine located at the middle and at the 3'-terminal positions of the sequence, respectively, changes in absorbance did not allow us to determine a T_m value. In addition, during

the synthesis, we did not succeed in the purification of an oligonucleotide involving two U modified with acridine at internal positions of the sequence.

We have reported the synthesis and binding properties of oligodeoxyribonucleotides covalently linked to an acridine derivative via various positions of the oligonucleotide chain. First, the preparation of a universal modified support **3** which allows the synthesis of oligonucleotides bearing the acridine moiety via a 3'-phosphodiester linkage has been described. The obtained oligonucleotide-acridine conjugate **13** ($T_m = 45 \pm 0.5$ °C) has binding properties very close to those of compound **19** ($T_m = 48.4 \pm 0.5$ °C) described previously. The addition of a second acridine moiety via the 5'-phosphate group (compound **16**) leads to better stability ($T_m = 52.4 \pm 0.5$ °C), similar to that obtained by Zarytova *et al.*, who have used the phenazinium intercalating agent. Then, oligodeoxyribonucleotides bearing the acridine moiety at the 5-position of the deoxyuridine not involved in the Watson-Crick base pair formation have been synthesized. When the acridine-modified deoxyuridine is located at the 3'-end of the oligonucleotide (oligomers **12a** and **12b**), strong stabilization of the complexes can be observed ($T_m = 46.2 \pm 0.5$ and 46 ± 0.5 °C, respectively), very similar to that obtained with the oligomers **13** and **19** bearing the acridine moiety via a 3'-phosphodiester or phosphothiolodiester, respectively. Surprisingly, when the acridine-modified deoxyuridine is located inside the oligonucleotide sequence (compounds **14** or **22**), a slight destabilization of the complex can be observed ($T_m = 32.8 \pm 0.5$ and 16 ± 0.5 °C, respectively) compared to the stability of the complexes obtained with the unmodified parent (compounds **21** or **24**, $T_m = 34.4 \pm 0.5$ and 19.8 ± 0.5 °C). The acridine moiety has also been incorporated at the middle position of the sequence via a phosphorothioate group. The mixture of diastereoisomers (compound **7**) as well as each pure R_p and S_p isomer (compounds **7a** and **7b**, respectively) were obtained also. The mixture of diastereoisomers led to a slightly more stable complex ($T_m = 48.6 \pm 0.5$ °C) than did the 3'-acridine-linked oligonucleotide prepared on the universal support **13** ($T_m = 45 \pm 0.5$ °C) and similar to that obtained with the oligomer **19** linked to the acridine via a 3'-phosphothiolodiester ($T_m = 48.4 \pm 0.5$ °C). The R_p isomer **7a** ($T_m = 45.2 \pm 0.5$ °C) led to lower stability than the racemic mixture did while the S_p isomer **7b** ($T_m = 51.4 \pm 0.5$ °C) to the most stable duplex of all those studied.

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LITERATURE CITED

- (1) J. S. Cohen Ed. (1989) Oligodeoxynucleotides. Antisense Inhibitors of Gene Expression. In *Topics in Molecular and Structural Biology*; Mac Millan Press, London.
- (2) Hélène, C., Montenay-Garestier, T., Saison, T., Takasugi, M., Toulmé, J. J., Asseline, U., Lancelot, G., Maurizot, J. C., Toulmé, F., and Thuong, N. T. (1985) Oligodeoxynucleotides covalently linked to intercalating agents: a new class of gene regulatory substances. *Biochimie* 67, 773–783.
- (3) Nguyen, T., Thuong, N. T., and Hélène, C. (1993) Sequence-specific recognition and modification of double-helical DNA by oligonucleotides. *Angew. Chem., Int. Ed. Engl.* 32, 666–690.
- (4) Asseline, U., Toulmé, F., Thuong, N. T., Delarue, M., Montenay-Garestier, T., and Hélène, C. (1984) Oligodeoxynucleotides covalently linked to intercalating dyes as base sequence-specific ligands. Influence of dye attachment site. *EMBO J.* 3, 795–800.
- (5) Asseline, U., Delarue, M., Lancelot, G., Toulmé, F., Thuong, N. T., Montenay-Garestier, T., and Hélène, C. (1984) Nucleic acid-binding molecules with high affinity and sequences specificity: intercalating agents covalently linked to oligodeoxynucleotides. *Proc. Natl. Acad. Sci. U.S.A.* 81, 3297–3301.
- (6) Mann, J. S., Shibata, Y., and Meehan, T. (1992) Synthesis and properties of an oligonucleotide modified with a pyrene derivative at the 5'-phosphate. *Bioconjugate Chem.* 3, 554–558.
- (7) Yamana, K., and Letsinger, R. L. (1985) Synthesis and properties of oligonucleotides bearing a pendant pyrene group. *Nucleic Acids Symp. Ser.* 16, 169–172.
- (8) Ono, A., Dan, A., and Matsuda, A. (1993) Nucleosides and nucleotides. 121. Synthesis of oligonucleotides carrying linker groups at the 1'-position of sugar residues. *Bioconjugate Chem.* 4, 499–508.
- (9) Telser, J., Cruickshank, K. A., Morrison, L. E., and Netzel, T. L. (1989) Synthesis and characterization of DNA oligomers and duplexes containing covalently attached molecular labels: comparison of biotin, fluorescein and pyrene labels by thermodynamic and optical spectroscopic measurements. *J. Am. Chem. Soc.* 111, 6966–6976.
- (10) Pielles, U., Sproat, B. S., Neuner, P., and Cramer, F. (1989) Preparation of a novel psoralen containing deoxyadenosine building block for the facile solid-phase synthesis of psoralen-modified oligonucleotides for a sequence specific crosslink, to a given target sequence. *Nucleic Acids Res.* 17, 8967–8978.
- (11) Lee, B. L., Murakami, A., Blake, K. R., Lin, S. B., and Miller, P. S. (1988) Interaction of psoralen-derivatized oligodeoxyribonucleotide methyl phosphonates with single-stranded DNA. *Biochemistry* 27, 6736–6739.
- (12) Thuong, N. T., Hélène, C., and Asseline, U. (1984) European Patent 84-400143-8; (1989) U.S. Patent 4,835,263.
- (13) Lin, K.-Y., and Matteucci, M. (1991) 5',3' hybridization properties of deoxyligoligonucleotides containing anthraquinone pseudonucleosides. *Nucleic Acids Res.* 19, 3111–3114.
- (14) Yamana, K., Nishijima, Y., Ikeda, T., Gokota, T., Ozaki, H., Nakano, H., Sangen, O., and Shimidzu, T. (1990) Synthesis and interactive properties of an oligonucleotide with anthraquinone at the sugar fragment. *Bioconjugate Chem.* 1, 319–324.
- (15) Casale, R., and McLaughlin, L. W. (1990) Synthesis and properties of an oligodeoxynucleotide containing a polycyclic aromatic hydrocarbon site specifically bound to the N² amino group of a 2'-deoxyguanosine residue. *J. Am. Chem. Soc.* 112, 5264–5271.
- (16) Deshmukh, H. M., Joglekar, S. P., and Broom, A. D. (1995) Self-complementary oligodeoxyribonucleotides containing 2'-O-(anthraquinone-2-methyl)adenosine. *Bioconjugate Chem.* 6, 578–586.
- (17) Gautier, C., Morvan, F., Rayner, B., Huynh-Dinh, T., Igolen, J., Imbach, J. L., Paoletti, C., and Paoletti, J. (1987) α -Anomeric and β -anomeric tetrathymidylates covalently linked to intercalating oxazopyridocarbazole. Synthesis, physicochemical properties and poly(rA) binding. *Nucleic Acids Res.* 15, 6625–6641.
- (18) Perrouault, L., Asseline, U., Rivalle, C., Thuong, N. T., Bisagni, E., Giovannangeli, C., Le Doan, T., and Hélène, C. (1990) Sequence-specific artificial photo-induced endonucleases based on triple-helix forming oligonucleotides. *Nature* 344, 358–360.
- (19) Lokhov, S. G., Podyminogin, M. A., Sergeev, D. S., Silnikov, V. N., Kutavyn, I. V., Shishkin, G. V., and Zarytova, V. P. (1992) Synthesis and high stability of complementary complexes of N-(2-hydroxyethyl)phenazinium derivatives of oligonucleotides. *Bioconjugate Chem.* 3, 414–419.
- (20) Levina, A. S., Tabatadse, D. R., Khalinskaya, L. M., Prichodko, T. A., Shishkin, G. V., Alexandrova, G. V., and Zarytova, V. P. (1993) Oligonucleotide derivatives bearing reactive and stabilizing groups attached to C5 of deoxyuridine. *Bioconjugate Chem.* 4, 319–325.
- (21) Chen, J. K., Carlson, D. V., Weith, H. L., O'Brien, J. A., Goldman, M. E., and Cushman, M. (1992) Synthesis of an oligonucleotide-intercalator conjugate in which the linker

- chain is attached via the phenolic hydroxyl group of fagarone. *Tetrahedron Lett.* 33, 2275–2278.
- (22) Balbi, A., Sottofattori, E., Grandi, T., Mazzei, M., Abramova, T. V., Lokhov, S., and Lebedev, A. V. (1994) Synthesis and complementary complex formation properties of oligonucleotides covalently linked to new stabilizing agents. *Tetrahedron* 50, 4009–4018.
- (23) Mergny, J.-L., Boutorine, A. S., Garestier, T., Belloc, F., Rougée, M., Bulychov, N. V., Koshkin, A. A., Bourson, J., Lebedev, A. V., Valeur, B., Thuong, N. T., and Hélène, C. (1994) Fluorescence energy transfer as a probe for nucleic acid structures and sequences. *Nucleic Acids Res.* 22, 920–928.
- (24) Weisblum, B., and De Hasth, P. L. (1972) Quinacrine, a chromosome stain specific for deoxyadenylate-deoxythymidylate rich regions in DNA. *Proc. Natl. Acad. Sci. U.S.A.* 69, 629–633.
- (25) Asseline, U., Barbier, C., and Thuong, N. T. (1986) Oligothymidylates comportant la structure alternée alkylphosphotriester-phosphodiester et liés de façon covalente à un agent intercalant (Oligothymidylates with alternating alkyl phosphotriester and phosphodiester structure covalently bonded to an intercalating agent). *Phosphorus Sulfur Relat. Elem.* 26, 63–73.
- (26) Atkinson, T., and Smith, M. (1984) Solid-phase synthesis of oligodeoxyribonucleotides by the phosphite triester approach. In *Oligonucleotides Synthesis: A Practical Approach*. (M. J. Gait, Ed.) IRL Press, Oxford.
- (27) Jäger, A., Levy, M. J., and Hecht, S. M. (1988) Oligonucleotide *N*-Alkylphosphoramidates: synthesis and binding to polynucleotides. *Biochemistry* 27, 7237–7246.
- (28) Thuong, N. T., and Asseline, U. (1991) Oligonucleotides attached to intercalators, photoreactive and cleavage agents. In *Oligonucleotides and Analogues: A Practical Approach* (F. Eckstein, Ed.) pp 283–308, IRL Press, Oxford.
- (29) Asseline, U., Bonfils, E., Kurfürst, R., Chassignol, M., Roig, V., and Thuong, N. T. (1992) Solid-phase preparation of 5'-3' heterobifunctional oligodeoxyribonucleotides using modified solid supports. *Tetrahedron* 48, 1233–1254.
- (30) Asseline, U., and Thuong, N. T. (1988) Oligothymidylates substitués par un dérivé de l'acridine en position 5', à la fois en position 5' et 3' ou sur un phosphate internucléotidique (Oligothymidylates substituted by an acridine derivative in the 5'-position; at both the 5'- and 3'-positions, or on the internucleotidic phosphate). *Nucleosides Nucleotides*, 431–455.
- (31) Misiura, K., Durrant, I., Evans, M. R., and Gait, M. J. (1990) Biotinyl and phosphotyrosinyl phosphoramidite derivatives useful in the incorporation of multiple reporter groups on synthetic oligonucleotides. *Nucleic Acids Res.* 18, 4345–4354.
- (32) Vu, H., and Hirschbein, B. L. (1991) Internucleotide phosphite sulfurization with tetraethylthiuram disulfide: Phosphorothioate oligonucleotide synthesis via phosphoramidite chemistry. *Tetrahedron Lett.* 32, 3005–3008.
- (33) Conway, N. E., Fidanza, J. A., O'Donnell, M. J., Narekian, N. D., Ozaki, H., and Mc Laughlin, L. W. (1991) Site-specific attachment of labels to the DNA backbone. In *Oligonucleotides and Analogues: A Practical Approach* (F. Eckstein, Ed.) pp 211–239, IRL Press, Oxford.
- (34) Connolly, B. A., Potter, B. V. L., Eckstein, F., Pingould, A., and Grotjahn, L. (1984) Synthesis and characterization of an octanucleotide containing the *Eco*R1 recognition sequence with a phosphorothioate group at the cleavage site. *Biochemistry* 23, 3443–3453.
- (35) Wilk, A., and Stec, W. J. (1995) Analysis of oligo(deoxynucleoside phosphorothioate)s and their diastereoisomeric composition. *Nucleic Acids Res.* 23, 530–534.
- (36) Piles, U., Zürcher, W., Schär, M. K. L., and Moser, H. E. (1993) Matrix-assisted laser desorption ionization time-of-flight mass-spectrometry: a powerful tool for the mass and sequence analysis of natural and modified oligonucleotides. *Nucleic Acids Res.* 21, 3191–3196.
- (37) Nordhoff, E., Cramer, R., Karas, M., Hillenkamp, F., Kirpekar, F., Kristiansen, K., and Roepstroff, P. (1993) Ion stability of nucleic acids in infrared matrix-assisted laser desorption ionization mass spectrometry. *Nucleic Acids Res.* 21, 3347–3357.
- (38) Hashimoto, H., Nelson, M. G., and Switzer, C. (1993) Zwitterionic DNA. *J. Am. Chem. Soc.* 115, 7128–7134.
- (39) Lancelot, G., and Thuong, N. T. (1986) Nuclear magnetic resonance studies of complex formation between the oligonucleotide d(TATC) covalently linked to an acridine derivative and its complementary sequence d(GATA). *Biochemistry* 25, 5357–5363.
- (40) LaPlanche, L., James, T., Powell, C., Wilson, W., Uznanski, B., Stec, W., Summers, M., and Zon, G. (1986) Phosphorothioate-modified oligodeoxyribonucleotides. III. NMR and UV spectroscopic studies of the R_p - R_p , S_p - S_p , and R_p - S_p duplexes, [d(GG₅AATTCC)]₂, derived from diastereomeric *O*-ethylphosphorothioates. *Nucleic Acids Res.* 14, 9081–9093.

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TECHNICAL NOTES

S-[2-(4-Azidosalicylamido)ethylthio]-2-thiopyridine: Radioiodinatable, Cleavable, Photoactivatable Cross-Linking Agent

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S-[2-(4-Azidosalicylamido)ethylthio]-2-thiopyridine (AET) contains a 2-thiopyridyl moiety, which permits cysteine-specific incorporation into protein through a cleavable disulfide bond, and a 4-azidosalicylamido moiety, which permits radioiodination and photoactivatable cross-linking. In contrast to the related compound S-[2-[N-[4-(4-azidosalicylamido)butyl]carbamoyl]ethylthio]-2-thiopyridine [APDP; Zecherle, G., Oleinikov, A., and Traut, R. (1992) *Biochemistry* 31, 9526], AET contains a relatively short linker arm between the 2-thiopyridyl moiety and the 4-azidosalicylamido moiety. In a previous paper, it was shown that AET could be used in site-specific protein–protein photo-cross-linking to identify nearest-neighbor protein domains within a multiprotein complex [Chen, Y., Ebright, Y., and Ebright, R. (1994) *Science* 265, 90]. In this paper, the synthesis, radioiodination, and incorporation into protein of AET are described.

INTRODUCTION

Site-specific protein–protein photo-cross-linking followed by cross-link cleavage and radiolabel transfer is a powerful procedure for defining interactions within multiprotein complexes (1–4). The procedure permits identification of proteins—and amino acids within those proteins—that are close to a site of interest *within an intact, fully-assembled, functional multiprotein complex* (3, 4). The procedure involves four steps: (i) incorporation of a radiolabeled, cleavable, photoactivatable cross-linking agent at a site of interest within one protein of a multiprotein complex; (ii) formation and photoirradiation of the derivatized multiprotein complex; (iii) cleavage of the resulting cross-link, with concomitant radiolabel transfer to the protein(s) and amino acid(s) at which cross-linking occurs; and (iv) identification of the protein(s) and amino acid(s) at which cross-linking occurs.

¹²⁵I is a radioisotope of choice in site-specific protein–protein photo-cross-linking followed by cross-link cleavage and radiolabel transfer, due to its ease of incorporation under mild conditions into phenol-containing agents and its availability at very high specific activities (up to 90 Bq/fmol) (3–5). In published work, two radioiodinatable agents have been used in site-specific protein–protein photo-cross-linking followed by cross-link cleavage and radiolabel transfer: S-[2-[N-[4-(4-azidosalicylamido)butyl]carbamoyl]ethylthio]-2-thiopyridine (APDP; 3) and S-[2-(4-azidosalicylamido)ethylthio]-2-thiopyridine (AET or ACT; 4). The azidosalicylamido moiety in each agent permits radioiodination and photoactivatable cross-linking. The 2-thiopyridyl moiety present in each agent permits cysteine-specific incorporation into a protein through a disulfide linkage—a linkage cleavable under mild conditions. Each agent can be incorporated at a

single, defined site of interest within a protein by a two-step procedure consisting of (i) site-directed mutagenesis to introduce a unique surface cysteine at the site of interest, followed by (ii) cysteine-specific chemical modification.

APDP results in a long linker arm between Cα of the amino acid of interest and the photoreactive atom (21 Å if fully extended). The length of this linker arm is comparable to the diameter of an average protein domain (25 Å; 6) and is greater than the diameters of the smallest protein domains (19–20 Å; see 7). As such, the length of this linker arm precludes reliable identification of nearest-neighbor protein domains—much less nearest-neighbor amino acids—in multiprotein complexes.

AET results in a shorter linker arm between Cα of the amino acid of interest and the photoreactive atom (14 Å if fully extended) and, as such, permits identification of nearest-neighbor protein domains—and, possibly, nearest-neighbor amino acids—within multiprotein complexes.

In this report, we describe the synthesis, radioiodination, and conjugation to protein of AET.

MATERIALS AND METHODS

S-(2-Aminoethylthio)-2-thiopyridine Hydrochloride. S-(2-Aminoethylthio)-2-thiopyridine hydrochloride was synthesized according to a modification of the procedure in ref 8. Thiopyridyl disulfide [Aldrich (Aldrichiol-2); 4.41 g, 20.0 mmol] was dissolved in 20 mL of methanol and 0.8 mL of acetic acid. Into this solution was added dropwise over a period of 0.5 h 2-aminoethylthiol hydrochloride (Aldrich; 1.14 g, 10.0 mmol) in 10 mL of methanol. The reaction mixture was stirred for an additional 48 h and then evaporated under high vacuum to a yellow oil. The product was washed with 50 mL of diethyl ether and was dissolved in 10 mL of methanol. The product was precipitated by addition of 200 mL of diethyl ether, chilled for 12 h at –20 °C, and collected by vacuum filtration (twice). Yield: 1.7 g, 77%; TLC (silica gel; ammonium hydroxide/95% ethanol, 1:99

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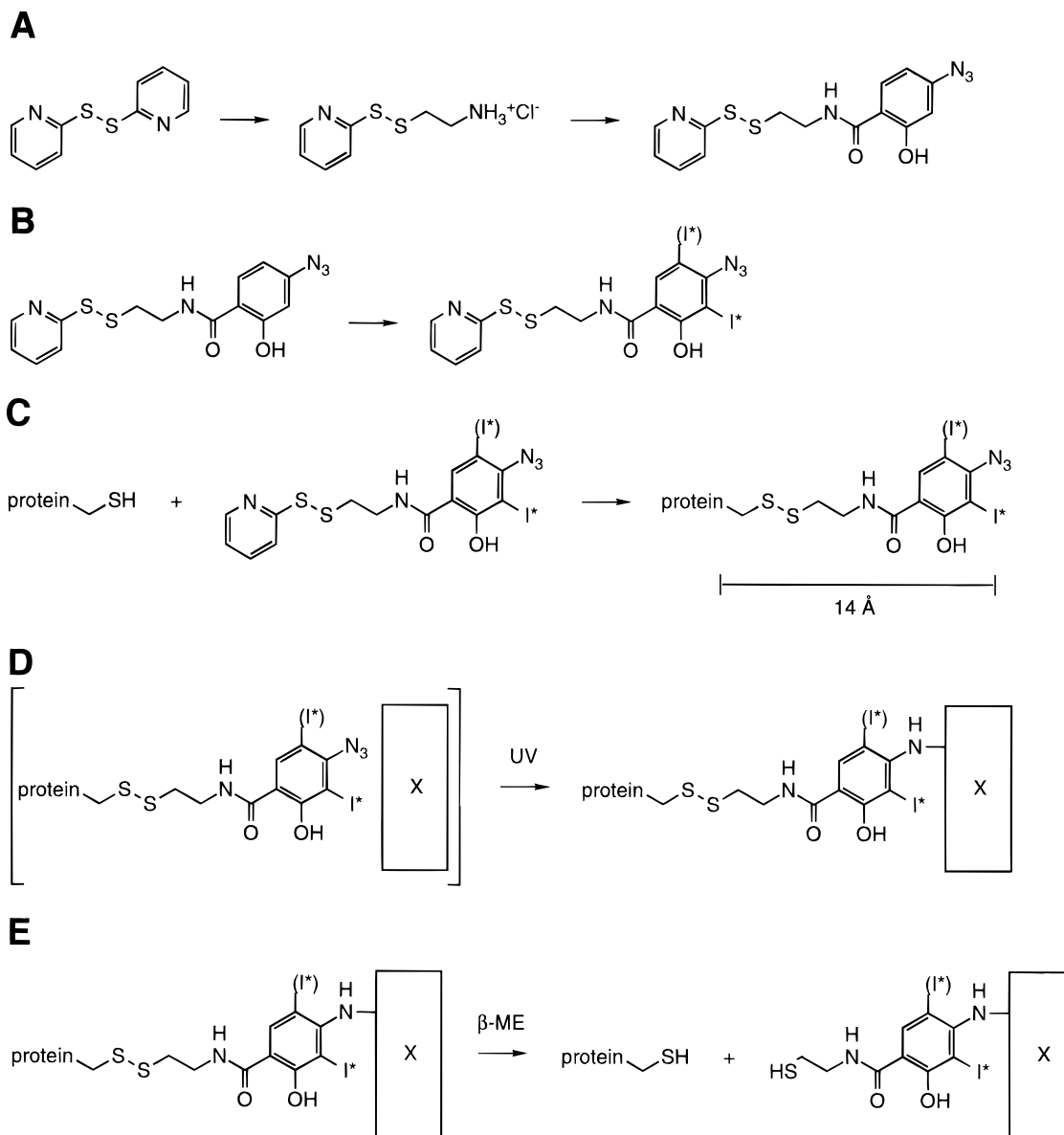


Figure 1. (A) Synthesis of AET. (B) Radioiodination of AET, yielding [^{125}I]AET. (C) Reaction of [^{125}I]AET with a protein having a unique surface cysteine residue, yielding a conjugate of the form [S-[2-([^{125}I]iodo-4-azidosalicylamido)ethylthio]-Cys]protein. In the conjugate, the length of the linker arm between the α carbon of the cysteine residue and the photoreactive atom is 14 Å (1.9 times the length of an arginine side chain). The length of the linker arm is suitable for analysis of interactions occurring at, or just beyond, side-chain contact distance. (D) UV irradiation of a multiprotein complex containing the conjugate and nearest-neighbor protein X. (E) Cleavage of the cross-link, with concomitant radiolabel transfer to nearest-neighbor protein X.

v/v) $R_f = 0.8$; NMR (CD_3OD ; tetramethylsilane as reference) δ 3.15 (2H, t, $J = 5.8$ Hz), 3.30 (2H, t, $J = 6.0$ Hz), 7.32 (1H, t, $J = 5.0$ Hz), 7.64 (1H, d, $J = 8.3$ Hz), 7.80 (1H, t, $J = 7.4$ Hz), 8.54 (1H, d, $J = 4.4$ Hz).

S-[2-(4-Azidosalicylamido)ethylthio]-2-thiopyridine (AET). S-(2-Aminoethylthio)-2-thiopyridine hydrochloride (90 mg, 0.40 mmol) and sodium bicarbonate (70 mg, 0.80 mmol) were dissolved in 1.5 mL of water. Into this solution was added in three aliquots over a period of 1 h *N*-hydroxysuccinimidyl-4-azidosalicylic acid (9; Pierce; 100 mg, 0.36 mmol) in 4.5 mL of tetrahydrofuran. The reaction mixture was stirred for an additional 0.5 h and then extracted three times with 5 mL of diethyl ether. The ether extracts were pooled, washed twice with 10 mL of 1% HCl and once with 10 mL of brine, dried over anhydrous sodium sulfate, and evaporated. The resulting clear oil was dissolved in 1 mL of methanol. White crystals formed upon standing for 3 h at 4 °C and were collected by vacuum filtration. Yield: 39 mg, 31%; TLC (silica gel; ethyl acetate/chloroform, 1:3 v/v) $R_f = 0.8$;

NMR (CD_3OD ; tetramethylsilane as reference) δ 3.18 (2H, t, $J = 5.3$ Hz), 3.75 (2H, t, $J = 5.3$ Hz), 6.53–6.62 (2H, m, $J = 2.0$ Hz), 7.31 (1H, t, $J = 6.0$ Hz), 7.58 (1H, d, $J = 8.3$ Hz), 7.77–7.87 (2H, m, $J = 4.0$ Hz), 8.65 (1H, d, $J = 4.9$ Hz), 8.95 (1H, br s); FAB-MS m/e 348 ($\text{M} + \text{H}$) $^+$.

All operations in this and subsequent steps were performed in darkness or under safelamp illumination.

S-[2-(Iodo-4-azidosalicylamido)ethylthio]-2-thiopyridine (IAET). In standard-scale reactions, 1,3,4,6-tetrachloro-3 α ,6 α -diphenylglycoluril [10, 11; Pierce (IODOGEN); 52 μg , 120 nmol] was plated on the walls of a 1.5 mL flat-bottom vial by evaporation under nitrogen of a 100 μL chloroform solution. AET (5.2 μg , 15 nmol) in 100 μL of 100 mM sodium borate (pH 8.4) and 0.5% dimethyl sulfoxide was mixed with KI (Aldrich; 2.5 μg , 15 nmol) in 20 μL of 30 mM sodium borate (pH 8.4), and the mixture immediately was added to the vial containing 1,3,4,6-tetrachloro-3 α ,6 α -diphenylglycoluril. After 30 s at 22 °C with vigorous stirring, reactions were terminated by transfer to a vial containing 15 μL of 8 mM methionine

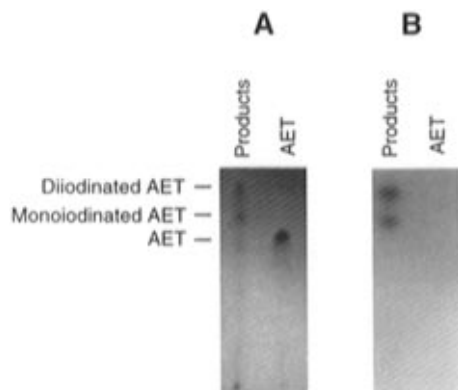


Figure 2. Radioiodination of AET. AET was allowed to react with $^{125}\text{I}^-$ and the solid-phase oxidant 1,3,4,6-tetrachloro-3 α ,6 α -diphenylglycoluril (10, 11). Reaction products were analyzed by TLC, with detection by UV shadowing (A) or autoradiography (B).

and 1 mM tyrosine. TLC (silica gel; chloroform/ethyl acetate, 95:5 v/v) $R_f = 0.75$ (monoiodinated AET) and 0.88 (diiodinated AET).

In preparative-scale reactions, 1,3,4,6-tetrachloro-3 α ,6 α -diphenylglycoluril [10, 11; Pierce (ODO-GEN); 200 mg, 460 μmol] was plated on the walls of a 500 mL Erlenmeyer flask by evaporation under house vacuum of a 25 mL chloroform solution. AET (20 mg, 60 μmol) in 2 mL of dimethyl sulfoxide and KI (10 mg, 60 μmol) in 26 mL of 100 mM sodium borate (pH 8.4) were mixed and then added to the prepared flask containing 1,3,4,6-tetrachloro-3 α ,6 α -diphenylglycoluril. After 15 min at 22 $^\circ\text{C}$ with vigorous stirring, reaction mixtures were filtered and extracted three times with 20 mL of chloroform. The chloroform extracts were pooled, washed with 20 mL of brine, dried over anhydrous sodium sulfate, and evaporated to an oil. Products were purified by preparative TLC (silica gel, 1000 μm ; ethyl acetate/chloroform, 95:5 v/v) $R_f = 0.7$ (monoiodinated AET) and 0.8 (diiodinated AET); FAB-MS m/e 474 [monoiodinated AET, $(M + H)^+$] and 599 [diiodinated AET, $(M + H)^+$].

S-[2-([^{125}I]Iodo-4-azidosalicylamido)ethylthio]-2-thiopyridine ([^{125}I]IAET). Standard-scale reactions

were performed as described for IAET using KI (Aldrich; 2.2 μg , 13 nmol) and Na^{125}I (New England Nuclear; 0.30 μg , 2.0 nmol, 190 MBq). Vials were equipped with rubber septa vented through 10 g activated-charcoal filters, and transfers were made using syringes (see 5).

Photolysis of AET and IAET. Samples contained 6 μM AET in 100 mM sodium borate (pH 8.4), 0.5% dimethyl sulfoxide or 4 μM IAET in 80 mM sodium borate (pH 8.4), 0.4% dimethyl sulfoxide, 0.9 mM methionine, and 0.1 mM tyrosine. Photolyses were carried out for 0–640 s at 22 $^\circ\text{C}$ (350 nm; $1 \times 10^5 \text{ erg mm}^{-2} \text{ s}^{-1}$) in a Rayonet RPR100 photochemical reactor (Southern New England Ultraviolet). Reaction vessels were 1 mL polystyrene microcentrifuge tubes (Fisher) held inside 13 $\times$ 100 mm borosilicate glass tubes; these reaction vessels exclude wavelengths less than 290 nm. Absorbance spectra were recorded using a Lambda 3A UV–vis spectrophotometer (Perkin-Elmer).

[S-[2-([^{125}I]Iodo-4-azidosalicylamido)ethylthio]-Cys152;Ser178]CAP ([^{125}I]IAE)CAP. Reaction mixtures contained (in 100 μL) the following: 70 μM [^{125}I]IAET (12 Bq/fmol; radioiodinated immediately before use); 4 μM [Cys152;Ser178]CAP or [Ser178] (prepared as described for [Ser178] in ref 12); 50 mM sodium borate (pH 8.3); 8 mM Tris-HCl (pH 8.3); 80 mM KCl; 0.4 mM EDTA; 5 mM methionine; 0.6 mM tyrosine; 2% glycerol; and 0.2% dimethyl sulfoxide. Reactions were carried out for 20 min at 22 $^\circ\text{C}$. [^{125}I]IAE)CAP was purified by chromatography on Bio-Gel P6DG (Bio-Rad) and was stored at -70°C in 40 mM Tris-HCl (pH 8.0), 100 mM KCl, 10 mM MgCl_2 , and 5% glycerol. ^{125}I was quantified using a Gamma 5500 gamma counter (Beckmann), 2-thiopyridone was quantified using a Lambda 3A UV–vis spectrophotometer (Perkin-Elmer) [$\epsilon_{343} = 8080 \text{ M}^{-1} \text{ cm}^{-1}$ (1)], and protein was quantified using Protein Gold reagent (Integrated Separation Systems).

RESULTS AND DISCUSSION

Synthesis of AET. AET was prepared in two steps: (i) reaction of 2-aminoethylthiol hydrochloride with thiopyridyl disulfide, yielding S-(2-aminoethylthio)-2-thiopyridine hydrochloride; and (ii) reaction of S-(2-amino-

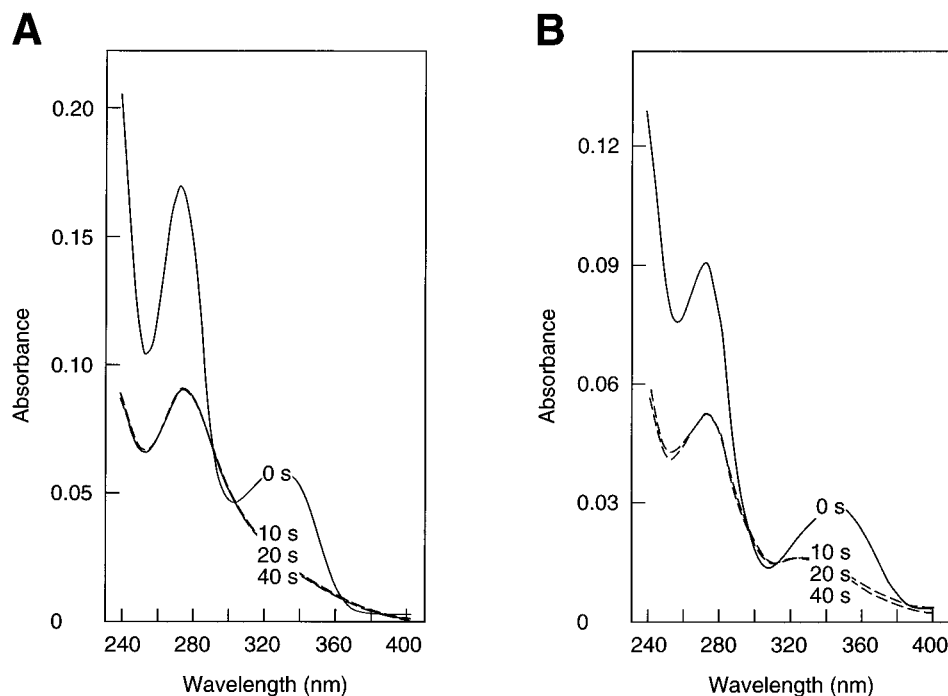


Figure 3. Photolysis of AET (A) and IAET (B). Photolyses were carried out for 0–40 s at 22 $^\circ\text{C}$ (350 nm; $1 \times 10^5 \text{ erg mm}^{-2} \text{ s}^{-1}$).

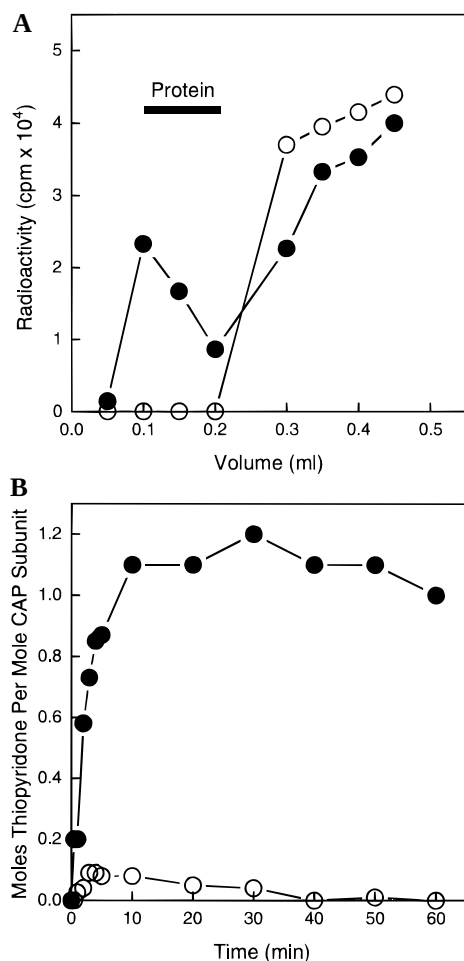


Figure 4. Incorporation into protein. In parallel experiments, [¹²⁵I]IAET in large molar excess was allowed to react with [Cys152;Ser178]CAP (a CAP derivative having a single surface cysteine residue; solid circles) and [Ser178]CAP [a CAP derivative having no surface cysteine residues (12); open circles]. Reaction products were isolated by gel filtration chromatography, and efficiencies of reaction were determined by quantitation of ¹²⁵I incorporated (A) and 2-thiopyridone released (B).

ethylthio)-2-thiopyridine hydrochloride with *N*-hydroxy-succinimidyl-4-azidosalicylic acid (Figure 1A). The overall yield was 24%.

Radioiodination of AET. AET was radioiodinated using ¹²⁵I⁻ and the solid-phase oxidant 1,3,4,6-tetrachloro-3 α ,6 α -diphenylglycoluril (10, 11) (Figure 1B). TLC indicated that >90% of the AET was consumed and that two main products were generated, in the ratio 1.5:1 (Figure 2A) (cf. 2, 11). TLC followed by autoradiography indicated that the two products contained iodine (Figure 2B). After a parallel reaction using nonradioactive I⁻, the two products were isolated by preparative TLC, analyzed by FAB-MS, and found to have masses corresponding to monoiodinated AET (3-iodo-AET) and diiodinated AET (3,5-diiodo-AET) (see Materials and Methods).

All further analyses—photolysis, attachment to protein, and site-specific protein–protein photo-cross-linking (see below and ref 3)—were carried out using unfractionated reaction products (i.e., monoiodinated AET and diiodinated AET). The specific activity of the unfractionated reaction products was 70% of the specific activity of the input ¹²⁵I⁻.

Photolysis of AET and IAET. AET has absorbance maxima at 273 ($\epsilon = 26\,000\text{ M}^{-1}$) and 330 nm ($\epsilon = 9500\text{ M}^{-1}\text{ cm}^{-1}$) (Figure 3A). UV irradiation at 350 nm and $1 \times 10^5\text{ erg mm}^{-2}\text{ s}^{-1}$ for 10 s results in loss of absorbance

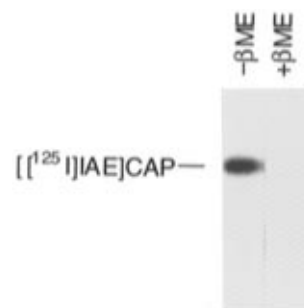


Figure 5. Cleavage of linkage to protein. [S-[2-([¹²⁵I]iodo-4-azidosalicylamido)ethylthio]-Cys152;Ser178]CAP was allowed to react with 0.25 mM β -mercaptoethanol for 5 min at 22 °C. Reaction products were analyzed by SDS–PAGE followed by autoradiography.

at 273 and 330 nm, indicative of destruction of the azido group (Figure 3A). Further photolysis results in no further loss of absorbance.

IAET has absorbance maxima at 273 ($\epsilon = 23\,000\text{ M}^{-1}$) and 343 nm ($\epsilon = 8000\text{ M}^{-1}\text{ cm}^{-1}$) (Figure 3B). UV irradiation at 350 nm and $1 \times 10^5\text{ erg mm}^{-2}\text{ s}^{-1}$ for 2–4 s results in loss of absorbance at 273 and 343 nm, and further photolysis results in no further loss of absorbance (Figure 3B and data not shown).

The ability to carry out photolysis with relatively long wavelengths (350 nm) and relatively short times (<10 s)—conditions that minimize damage to biological macromolecules—is an important advantage for site-specific protein–protein photo-cross-linking.

Incorporation into Protein. *Escherichia coli* catabolite activator protein (CAP) is a sequence-specific DNA binding protein and transcription activator protein (reviewed in ref 13). [Cys152;Ser178]CAP is a CAP derivative engineered to have a unique surface cysteine residue at position 152—a position adjacent to the transcription “activating region” of CAP (positions 156–164; see refs 4 and 14–17). [¹²⁵I]IAET in large molar excess was allowed to react with [Cys152;Ser178]CAP, and the resulting conjugate, [S-[2-([¹²⁵I]iodo-4-azidosalicylamido)ethylthio]-Cys152;Ser178]CAP ([¹²⁵I]IAE]CAP), was isolated by gel filtration chromatography (Figures 1C and 4A).

Quantitation of ¹²⁵I by gamma counting indicated that reaction was 100% complete: i.e., 1 mol of [2-([¹²⁵I]iodo-4-azidosalicylamido)ethylthio]– group was incorporated per mole of [Cys152;Ser178]CAP subunit (Figure 4A). Control experiments with [Ser178]CAP, which has no surface cysteine residues (12), indicated that reaction was 100% site-specific: i.e., 0 mol of [2-([¹²⁵I]iodo-4-azidosalicyl)ethylthio]– was incorporated per mole [Ser178]CAP (Figure 4A).

Efficiencies of incorporation also were determined by quantitation of released 2-thiopyridone by measurement of UV absorbance at 343 nm (see ref 1). Efficiencies of incorporation as determined by gamma counting agreed with efficiencies of incorporation as determined by quantitation of released 2-thiopyridone within 5% (Figure 4B).

Treatment of [¹²⁵I]IAE]CAP with 0.25 mM β -mercaptoethanol for 5 min at 22 °C resulted in complete loss of the [2-([¹²⁵I]iodo-4-azidosalicylamido)ethylthio]– group from the protein, indicating that the disulfide linkage between [2-([¹²⁵I]iodo-4-azidosalicylamido)ethylthio]– and the protein can be cleaved quantitatively under mild conditions (Figure 5).

Prospect. We anticipate that the reagent and procedures of this paper will be broadly useful for analysis of protein–protein and protein–nucleic acid interactions (see ref 4). We successfully have used the reagent of

this in site-specific protein–protein photo-cross-linking with CAP derivatives site-specifically derivatized at each of four positions (4; Y.C., Y.K., and R.H.E., unpublished results) and with TATA-binding protein derivatives site specifically derivatized at each of two positions (P. Padmanabhan, Y.K., and R.H.E., unpublished results).

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LITERATURE CITED

- (1) Chong, P., and Hodges, R. (1981) A new heterobifunctional cross-linking reagent for the study of biological interactions between proteins. I. Design, synthesis, and characterization. *J. Biol. Chem.* 256, 5064–5070.
- (2) Chong, P., and Hodges, R. (1981) A new heterobifunctional cross-linking reagent for the study of biological interactions between proteins. II. Application to the troponin C-troponin I interaction. *J. Biol. Chem.* 256, 5071–5076.
- (3) Zecherle, G., Oleinikov, A., and Traut, R. (1992) The C-terminal domain of *Escherichia coli* ribosomal protein L7/L12 can occupy a location near the factor-binding domain of the 50S subunit as shown by cross-linking with *N*-[4-(*p*-azidosalicylamido)butyl]-3-(2'-pyridylthio)propionamide. *Biochemistry* 31, 9526–9532.
- (4) Chen, Y., Ebright, Y., and Ebright, R. (1994) Identification of the target of a transcription activator protein by protein-protein photocrosslinking. *Science* 265, 90–92.
- (5) Wilbur, D. S. (1992) Radiohalogenation of proteins: an overview of radionuclides, labeling methods, and reagents for conjugate labeling. *Bioconjugate Chem.* 3, 433–470.
- (6) Schulz, G., and Schirmer, R. (1979) *Principles of Protein Structure*, Springer-Verlag, New York.
- (7) Lee, M., Gippert, G., Soman, K., Case, D., and Wright, P. (1989) Three-dimensional solution structure of a single zinc finger DNA-binding domain. *Science* 245, 635–637.
- (8) Ebright, Y., Chen, Y., Pendergrast, P. S., and Ebright, R. (1992) Incorporation of an EDTA-metal complex at a rationally selected site within a protein: application to EDTA-iron DNA affinity cleaving with catabolite gene activator protein (CAP) and Cro. *Biochemistry* 31, 10664–10670.
- (9) Ji, T., and Ji, I. (1982) Macromolecular photoaffinity labeling with radioactive photoactivable heterobifunctional reagents. *Anal. Biochem.* 121, 286–289.
- (10) Fraker, P., and Speck, J. (1978) Protein and cell membrane iodinations with a sparingly soluble chloramide, 1,3,4,6-tetrachloro-3a,6a-diphenylglycoluril. *Biochem. Biophys. Res. Commun.* 80, 849–857.
- (11) Shepard, E., de Beer, F., von Holt, C., and Hapgood, J. (1988) The use of sulfosuccinimidyl-2-(*p*-azidosalicylamido)-1,3'-dithiopropionate as a crosslinking reagent to identify cell surface receptors. *Anal. Biochem.* 168, 306–313.
- (12) Zhang, X., Gunasekera, A., Ebright, Y., and Ebright, R. (1991) Derivatives of CAP having no solvent-accessible cysteine residues, or having a unique solvent-accessible cysteine residue at amino acid 2 of the helix-turn-helix motif. *J. Biomol. Struct. Dyn.* 9, 463–473.
- (13) Kolb, A., Busby, S., Buc, H., Garges, S., and Adhya, S. (1993) Transcriptional regulation by cAMP and its receptor protein. *Annu. Rev. Biochem.* 62, 749–795.
- (14) Ebright, R. (1993) Transcription activation at class I CAP-dependent promoters. *Mol. Microbiol.* 8, 797–802.
- (15) Zhou, Y., Zhang, X., and Ebright, R. (1993) Identification of the activating region of CAP: isolation and characterization of mutants of CAP specifically defective in transcription activation. *Proc. Natl. Acad. Sci. U.S.A.* 90, 6081–6085.
- (16) Niu, W., Zhou, Y., Dong, Q., Ebright, Y., and Ebright, R. (1994) Characterization of the activating region of *Escherichia coli* catabolite gene activator protein (CAP): I. Saturation and alanine-scanning mutagenesis. *J. Mol. Biol.* 243, 595–602.
- (17) Zhou, Y., Merkel, T., and Ebright, R. (1994) Characterization of the activating region of *Escherichia coli* catabolite gene activator protein (CAP): II. Role at class I and class II CAP-dependent promoters. *J. Mol. Biol.* 243, 603–610.

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Improved Method for Converting an Unmodified Peptide to an Energy-Transfer Substrate for a Proteinase

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This paper describes refinements to a method for generating energy-transfer-based fluorogenic substrates for proteinases [Geoghegan, K. F., et al. (1993) *Bioconjugate Chem.* 4, 537–544]. An unprotected peptide is taken as starting material and coupled by three steps of chemistry to two dyes that form an energy donor–energy acceptor pair. The donor group is Lucifer Yellow CH (λ_{ex} 420–430 nm, λ_{em} 530 nm), and the acceptor is 5-carboxytetramethylrhodamine, a strong chromophore that effectively quenches Lucifer Yellow fluorescence in intact substrates. Periodate oxidation of N-terminal Ser gives the peptide an α -N-glyoxylyl moiety, OHC–CO–peptide, which is allowed to react with Lucifer Yellow CH (a carbonylhydrazide derivative) to attach the dye through an adequately stable hydrazone bond. Derivatization is completed by allowing the succinimidyl ester of 5-carboxytetramethylrhodamine to react with the ϵ -amino group of a single Lys placed near the C terminus. In the revised method, the first two steps of chemistry are completed sequentially in a single vessel to eliminate the labor and losses associated with isolating the α -N-glyoxylyl intermediate. In addition, peptides taken as starting material for this method are now designed according to the scheme (Ser)-(cleavable sequence)-(Lys-Arg). The addition of Arg at the C terminus promotes aqueous solubility of the final substrate without complicating the chemistry; multiple Arg might also be used, though this has not been tried here. It has also been found that relative reversed-phase HPLC retention of Lucifer Yellow/5-carboxytetramethylrhodamine substrates is predictable on the basis of the hydrophobicity of the original peptide. Substrates prepared from peptides with Bull and Breese indices of >200 cal/mol are readily separated from residual dye in the second and final chromatographic step of the synthesis, simplifying the only moderately difficult step in the preparation. As with all fluorogenic substrates, assays using these substrates are subject to internal filtering effects that can lead to serious error. This problem becomes significant at a range of substrate concentration that is predictable when the size and geometry of the fluorescence cell are taken into account. The reasoning applied in determining this range is applicable to substrates constructed with any donor–acceptor pair.

INTRODUCTION

Fluorogenic “energy-transfer” substrates (1–3) are increasingly chosen as the basis of assays for highly specific proteinases (4–15). Their function depends on their molecular design, in which a peptide cleaved by the enzyme is flanked by (or partly embodies) two groups that form an energy donor–energy acceptor pair (16). Qualities desired in these substrates include ease of synthesis, adequate aqueous solubility, and convenient optical properties.

A recently introduced route to energy-transfer substrates begins from an unprotected peptide and requires only three steps of chemistry (17). This paper describes an improved format for the method that saves time and reduces potential complications in the final step.

In the method as first described (17), a suitable peptide sequence of about 10 residues was placed between an N-terminal Ser and a C-terminal Lys, i.e., (Ser)-(cleavable sequence)-(Lys). Each of these terminal residues had an amino group, and no other amino group could appear in the peptide apart from these two. Solution-phase chemistry was used to derivatize the peptide with a pair of chromophores comprising an energy donor–energy acceptor pair. First, periodate oxidation of the N-terminal Ser at pH 7.0 gave an α -N-glyoxylyl peptide, OHC–CO–peptide, which was recovered by HPLC and dried. This

product was reacted with Lucifer Yellow CH at pH 4.5 to form a stable hydrazone adduct that was purified by HPLC and dried. For the third and final step, this product was tagged with 5-carboxytetramethylrhodamine at its remaining amino group by reaction with the succinimidyl ester of the dye. In the resulting doubly tagged peptide, fluorescent emission of Lucifer Yellow at 520–530 nm was effectively quenched by 5-carboxytetramethylrhodamine. Enzyme-catalyzed peptide cleavage relieved this quenching, allowing activity to be followed through the development of Lucifer Yellow fluorescence.

Refinements described here include (i) performing the first two steps sequentially in one vessel to avoid the labor and losses associated with isolating the α -N-glyoxylyl-peptide; (ii) introducing an Arg residue placed to the C-terminal side of the single Lys, to improve solubility in aqueous buffers; and (iii) rationalizing HPLC isolation of the final product, which has been the only technically demanding element of the method. The process is illustrated by synthesis of a putative fluorogenic substrate for TNF convertase, an incompletely characterized enzyme responsible for the maturation of tumor necrosis factor α (18, 19).

EXPERIMENTAL PROCEDURES

Peptide. The peptide SLAQAVRSSKR was obtained through custom synthesis performed by AnaSpec Inc., San Jose, CA. HPLC and amino acid analysis by the vendor indicated that the peptide was 97% pure and that the sample contained 71% peptide by weight. Lucifer Yellow CH and 5-carboxytetramethylrhodamine succin-

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imidyl ester were obtained from Molecular Probes Inc., Eugene, OR. The molar absorptivity for Lucifer Yellow CH was taken as $\epsilon_{428} = 12\,000\text{ M}^{-1}\text{ cm}^{-1}$ following information from the vendor's catalog. For 5-carboxytetramethylrhodamine conjugated to a peptide, a provisional value of $\epsilon_{560} = 60\,000\text{ M}^{-1}\text{ cm}^{-1}$ was used on the basis of amino acid analysis (with an internal standard) of a solution of the final product with measured A_{560} . The free carboxylate form of the dye has about 30% higher absorptivity, based on the absorbance of solutions prepared from weighed samples.

Chromatography. Preparative HPLC in this laboratory was performed using a Hewlett-Packard 1050 pump, a Rheodyne Model 7125 injector with sample loops varying from 50 μL to 5 mL, and two variable-wavelength detectors connected in series. These were an LDC/Milton Roy spectroMonitor IIID with conventional fixed-scale function and a Rainin UV-1 detector equipped with expanded range function based on an L-shaped flow cell. The UV-1 detector allows relative absorbance of the sample to be measured up to a value of about 10, which is convenient for collecting peaks of peptides conjugated to strong chromophores. Pump control and data acquisition were accomplished using Hewlett-Packard 2D HPLC Chemstation software running under Microsoft Windows version 3.1 on a 33 MHz 80486-based microcomputer. The HPLC columns used are specified below. Analytical HPLC was performed on a Hewlett-Packard 1090 Series II system equipped with a diode array detector.

Periodate Oxidation and Reaction with Lucifer Yellow. Purified SLAQAVRSSKR (13.1 mg, with a calculated peptide content of 9.3 mg, i.e., 7.7 μmol) was dissolved in 0.25 mL of 0.025 M sodium phosphate, pH 7.0. The resulting acidic solution was titrated with 1 μL drops of 1 M NaOH until the pH, as estimated by spotting 1 μL drops onto pH paper, had risen from its initial value near pH 2.0 to a value near pH 7. A 1 μL sample was then withdrawn for HPLC analysis, after which the solution was treated with 0.25 mL of 0.04 M NaIO_4 dissolved in 0.025 M sodium phosphate, pH 7.0 (final pH of the periodate solution, pH 6.7, not further adjusted), to create a reaction solution at 22 °C containing 0.02 M NaIO_4 and 0.015 M peptide. After 3 min, a 2 μL portion of the reaction solution was withdrawn and analyzed by HPLC to verify the expectation that 100% of the peptide had been converted to α -*N*-glyoxylyl-LAQAVRSSKR (see below). When this had been confirmed (14 min after the reaction was started), the solution was treated with 1 mL of 0.03 M Lucifer Yellow CH in 0.1 M sodium acetate, pH 4.5, and the resulting 1.5 mL of reaction mixture was incubated at 22 °C for 1 h. After this time, HPLC analysis of 6 μL of the sample indicated that about 80% of the oxidized peptide had been converted to the Lucifer Yellow hydrazone. Incubation was continued for a further 1 h, after which time the product was recovered by HPLC.

For this HPLC step, the column was a Poros R1/M 10/100 (Perseptive Biosystems) running at 10 mL/min with the detectors set to 220 and 420 nm (to detect the Lucifer Yellow chromophore). This column is a reversed-phase column that functions like a C_{18} . Solvents were (A) 0.1% TFA and (B) 0.085% TFA in 80% CH_3CN . The gradient was formed from 0 to 40% B in the period from 1 to 7 min after the start of the method, and the product peak was hand-collected on the basis of visual monitoring of the detector signal at 420 nm. The product fractions from a series of three injections of 400–500 μL each were pooled, and the pool was stripped of CH_3CN by rotary evaporation before being dried on a lyophilizer.

Reaction with 5-Carboxytetramethylrhodamine Succinimidyl Ester. The dried, bright yellow product (about 11 mg; see Results for yield information) was redissolved in 2.25 mL of 0.1 M NaHCO_3 and treated with 0.75 mL of 5-carboxytetramethylrhodamine succinimidyl ester prepared as a 20 mM suspension in CH_3CN . After 1 h of incubation at 22 °C, progress of the reaction was checked by HPLC and a further 0.15 mL of the suspension of 5-carboxytetramethylrhodamine succinimidyl ester was added to drive the reaction toward completion.

The HPLC fractionation at this stage used a Vydac C_{18} column (type 218TP510) with the same solvents as above, a flow rate of 4 mL/min, and detectors set to 540 and 420 nm for analytical runs or to the off-peak-maxima values of 510 and 400 nm for preparative runs. The desired product uniquely gave a strong peak at both of the wavelengths monitored due to the fact that it contained both the Lucifer Yellow and 5-carboxytetramethylrhodamine chromophores.

Because the reaction mixture contained CH_3CN , it was diluted with aqueous buffer before being injected into the HPLC (e.g., 1 mL of reaction mixture was mixed with 1 mL of 0.025 M sodium phosphate buffer, pH 7.0).

The desired product from two major preparative runs and other minor fractionations was pooled, divided into aliquots that would give a useful stock concentration when redissolved in a chosen volume, and dried (Speed-Vac concentrator, Savant).

Mass Spectrometry. Plasma desorption mass spectrometry was performed on a Bio-Ion 20 system.

RESULTS

Tumor necrosis factor (TNF) is a macrophage-derived 17 kDa cytokine that originates as a 26 kDa precursor (20–22). Proteolysis of the bond $\text{Ala}^{76}\text{--Val}^{77}$ releases the mature cytokine from the pro-form, and the responsible proteinase is an attractive potential target for new antiinflammatory medicines (18, 19). We recently required a peptide substrate for use in characterizing the convertase and based it on the local amino acid sequence around the cleavage site. To this end, the peptide SLAQAVRSSKR was designed with a core sequence (LAQAVRSS), matching residues 73–80 of human pro-TNF. This was flanked at the N-terminal end by Ser to form the target for periodate oxidation and attachment of Lucifer Yellow CH. The C terminus had Lys to accommodate 5-carboxytetramethylrhodamine at an ϵ -amino group, and Arg was added to promote solubility. The overall design of the starting peptide was (Ser)-(cleavable sequence)-(Lys-Arg).

Our original procedure (17) called for periodate oxidation of the peptide at pH 7.0 to be followed by HPLC purification of the α -*N*-glyoxylyl product. The dried product was then redissolved in pH 4.5 buffer for coupling to Lucifer Yellow CH. This was a methodical approach, but the HPLC operation inevitably reduced overall yield as well as consuming time and labor. As an alternative, the first two steps of chemistry can usually be performed sequentially in a single vessel.

When SLAQAVRSSKR was dissolved to a concentration of 0.03 M in 0.025 M sodium phosphate buffer, pH 7.0, the solution had a pH near 2 due to the acidity of the peptide sample. The pH was adjusted to about pH 7 by adding 1 μL aliquots of 1 M NaOH while monitoring the pH by applying a droplet (<1 μL) to pH paper after each addition. As the peptide concentration remained essentially unaltered at 0.03 M, the peptide could then be treated with a small excess (4:3) of periodate by adding an equal volume of 0.04 M sodium

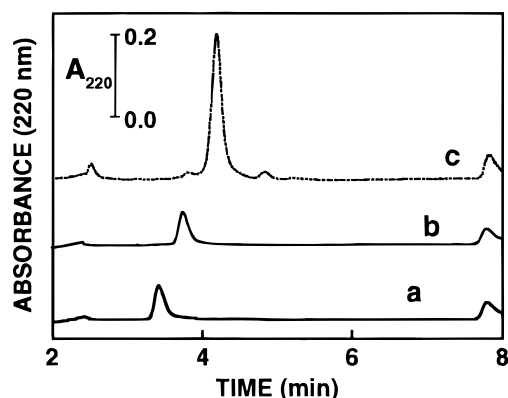


Figure 1. Use of HPLC to follow the first two steps of chemistry on an analytical scale. A small shift to later retention time accompanies both periodate oxidation of the N-terminal Ser and the subsequent condensation of the resulting α -N-glyoxylyl-peptide with Lucifer Yellow CH. (a) Peptide starting solution; (b) peptide after 3 min of reaction with sodium periodate at pH 7.0; (c) analysis of a sample from the reaction of Lucifer Yellow CH with the α -N-glyoxylyl-peptide, in which excess dye was eluted with the injection peak (i.e., prior to 2 min). The HPLC column was a Poros R2/H 2.1/30 running at 1 mL/min with a gradient of 0–56% acetonitrile in 0.1% TFA from 1 to 7 min after injection.

Table 1. Predicted and Observed Masses for Intermediates and Product^a

	MH ⁺ theor	MH ⁺ obsd
SLAQAVRSSKR	1203.4	1202.7
α -N-glyoxylyl-LAQAVRSSKR	1172.4 (unhydrated)	1172.7
	1190.4 (hydrated)	1190.2
LY-LAQAVRSSKR	1599.8	1598.8
LY-LAQAVRSSK(-CTMR)R	2011.8	2011.5

^a Mass analyses were performed by PDMS (see Experimental Procedures). Abbreviations: LY, Lucifer Yellow CH in hydrazone linkage with residue of α -N-glyoxylyl moiety at peptide N terminus; CTMR, 5-carboxytetramethylrhodamine in amide linkage with ϵ -amino group of C-terminal Lys.

periodate made up in the phosphate buffer (final pH of the periodate solution was pH 6.7). Successful oxidation of the N-terminal Ser, a rapid reaction (23), was confirmed by comparing the HPLC elution of the unreacted peptide (Figure 1a) with that for the periodate-treated peptide (Figure 1b). This analysis indicated that the peptide was quantitatively oxidized within 3 min of the addition of periodate. Periodate oxidation of the N-terminal Ser invariably causes a slight increase in retention in reversed-phase HPLC due to replacement of the cationic N-terminal Ser with the neutral α -N-glyoxylyl moiety (24). The identity of the oxidized peptide as α -N-glyoxylyl-LAQAVRSSKR was confirmed by PDMS, where α -N-glyoxylyl-peptides typically are detected in both unhydrated and hydrated forms with masses differing by 18 Da (Table 1) (17, 24).

At this point, the benefit of using such a dilute phosphate buffer for the first step was obtained. To the pH 7 solution of oxidized peptide was added 2 volumes of 0.1 M sodium acetate buffer, pH 4.5, containing 0.03 M Lucifer Yellow CH. The mixed solution had a pH of 4.5 (by pH paper) and initially contained 0.02 M Lucifer Yellow CH and 0.005 M α -N-glyoxylyl-LAQAVRSSKR. HPLC analysis according to the same protocol as before was used to follow dye-to-peptide coupling (Figure 1c). When this was largely complete after 2 h, the Lucifer Yellow conjugate was purified by HPLC. An overall yield of 75% for the combined opening two steps of the procedure was determined from the absorption spectrum of the product pool before it was lyophilized (Table 2).

Table 2. Yield Report for Synthesis of LY-LAQAVRSSK(-CTMR)R

step	quantity (mg)	quantity (μ mol)	yield (%)	how estimated
SLAQAVRSSKR (starting material)	9.3	7.7	100	AAA
LY-LAQAVRSSKR	9.3	5.8	75	A ₄₂₈
LY-LAQAVRSSK(-CTMR)R	9.2	4.6	60	A ₅₆₀

^a For Lucifer Yellow, $\epsilon_{428} = 12\,000\text{ M}^{-1}\text{ cm}^{-1}$. For CTMR, $\epsilon_{560} = 60\,000\text{ M}^{-1}\text{ cm}^{-1}$ (see Experimental Procedures).

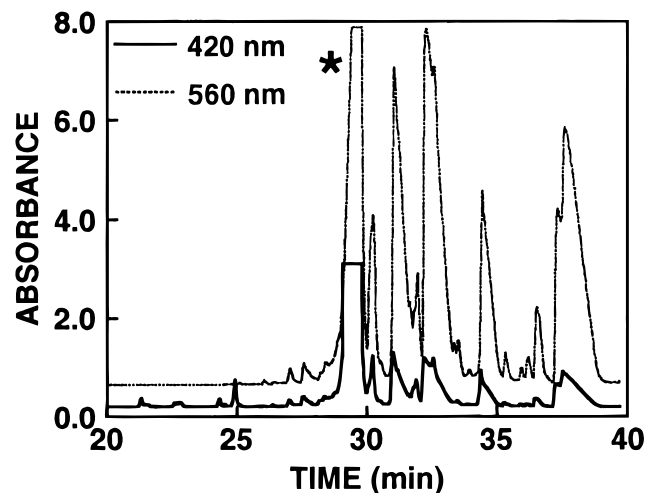


Figure 2. HPLC fractionation of the reaction mixture for the final step. The peak marked with an asterisk is the one with strong absorbance at both 420 (solid trace) and 560 nm (dotted trace), indicating the presence of both Lucifer Yellow and 5-carboxytetramethylrhodamine chromophores in the same product. Absorbance values in excess of 2.0 are the output of a detector with extended range function (see Experimental Procedures).

For the final coupling, dried LY-LAQAVRSSKR¹ was redissolved in 0.1 M NaHCO₃ and treated with 5-carboxytetramethylrhodamine succinimidyl ester suspended (largely dissolved) in CH₃CN. This reaction, though selective and relatively rapid, leads to the potentially complicated step of HPLC recovery of the doubly tagged peptide product. The source of concern is that free 5-carboxytetramethylrhodamine (in the various forms present) is retained by reversed-phase HPLC columns and gives a series of peaks that may be close to or even overlap the peak of the desired peptide product. In this case, however, there was no problem; LY-LAQAVRSSK(-CTMR)R was eluted cleanly ahead of the dye peaks (Figure 2).

The purity and mass of the product were characterized by HPLC (Figure 3) and PDMS (Figure 4), respectively. The major peak accounted for 87% of the total peak area detected at 540 nm, and the mass agreed closely with the theoretical mass for LY-LAQAVRSSK(-CTMR)R (Table 1).

In the preparation of these substrates, it is very desirable that the product be easily separated from dye in the final step. This problem can be approached rationally, because accumulated experience (see data below) has made somewhat predictable the HPLC be-

¹Abbreviations: LY-, Lucifer Yellow CH in hydrazone linkage with the α -N-glyoxylyl N terminus of a periodate-oxidized peptide; -CTMR, 5-carboxytetramethylrhodamine; EDANS, 5-(2'-aminoethyl)aminonaphthalenesulfonic acid; AAA, amino acid analysis; DABCYL, 4-[4'-(dimethylamino)phenylazo]benzoic acid; 0.025 M sodium phosphate buffer, pH 7.0, a 0.025 M solution of NaH₂PO₄ that is brought to pH 7.0 by addition of NaOH solution before final adjustment to volume.

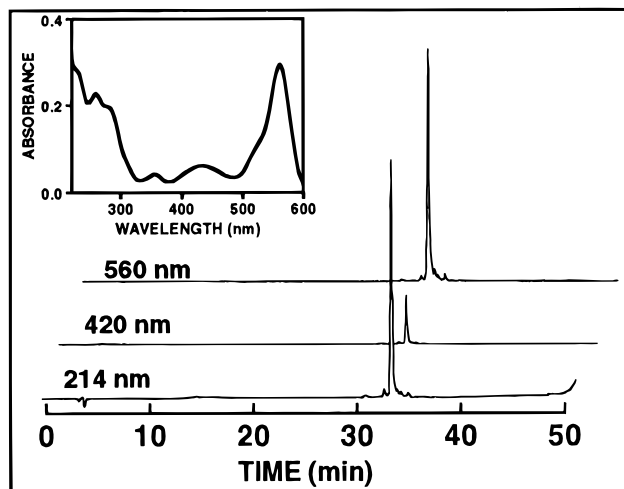


Figure 3. Analytical HPLC to gauge purity of the final product. Chromatograms collected at three different wavelengths for a single analysis are shown. (Inset) Absorption spectrum of the product peak.

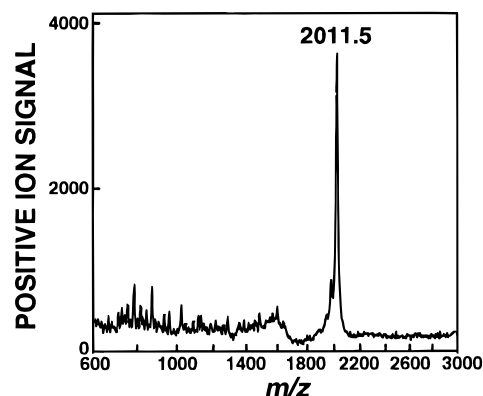


Figure 4. Plasma desorption mass spectrometry of the final product, LY-LAQAVRSSK(-CTMR)R. The predicted value of MH^+ for the product was 2011.8. The spectrum was calibrated using external standards.

havior of the LY-CTMR substrate prepared from any peptide in the relevant size range. If the peptide sequence is sufficiently hydrophilic, the doubly dye-conjugated product is eluted *before* the peaks of 5-carboxytetramethylrhodamine succinimidyl ester, its hydrolysis product, and related minor species (as in Figure 2). A considerably more hydrophobic substrate could be eluted *after* the dye peaks, as occurred for a renin substrate (see Figure 3 of ref 17). Thus, it may pay to design the substrate for favorable HPLC retention in the final step.

Hydrophilic substrates are usually preferred, not least because their concentrated stock solutions (e.g., 400 μM for a number of the substrates noted in Figure 5) can be prepared in aqueous buffers. Hydrophilicity can be increased by adding an Arg (Table 3) (or multiple arginyl residues, although this has not been tried) to the peptide sequence after the Lys that accepts 5-carboxytetramethylrhodamine, where it is remote from the sequence recognized by the enzyme. Our laboratory has not yet tried intentionally increasing the hydrophobicity of a peptide to cause the doubly tagged product to be eluted after dye peaks, but there seems to be no reason this could not be done. It could best be achieved by opting for a suitably hydrophobic core sequence, but one might try adding a C-terminal Leu (for example) if the core sequence could not be varied or extended.

Experience with six substrates provides the basis for predicting the relative HPLC retention of future sub-

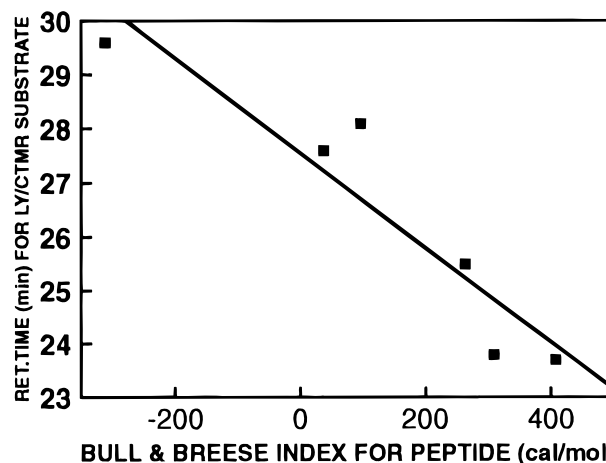
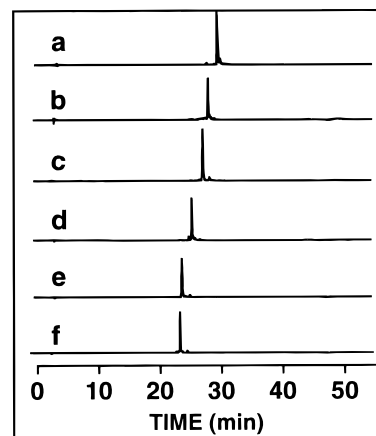


Figure 5. Effect of sequence on HPLC retention of substrates. (Upper panel) Reversed-phase HPLC analysis of six substrates containing Lucifer Yellow CH and 5-carboxytetramethylrhodamine and based, respectively, on the following peptides: (a) SIHPFHLVIHTK; (b) SA-Nle-RGGAFSWKR; (c) SGPLGLRAK; (d) SLAQAVRSSKR; (e) SITEGEARGSVKR; (f) SALAGASHKR. These are analytical chromatograms; see Figure 2 for an example of a preparative separation. (Lower panel) Plot of the peak retention times shown in the upper panel against Bull and Breese indices for the respective peptides (Table 3).

Table 3. HPLC Retention Time of Doubly Tagged Substrate as a Function of Starting Peptide Sequence

peptide	Bull and Breese value of peptide (cal/mol)	retention time for LY-CTMR substrate (min)	retention relative to dye peaks
SALAGGASHKR	407	23.7	before
SITEGEARGSVKR	309	23.8	before
SLAQAVRSSKR	263	25.5	just before
SA-Nle-RGGAFSWKR	96	28.1	not separated
SGPLGLRAK	37	27.6	not separated
SIHPFHLVIHTK	-312	29.6	after

^a Analytical chromatography, for which retention times are given, was performed on a Vydac C_{18} narrowbore column (2.1 mm $\times$ 250 mm) running at 0.2 mL/min and using an gradient of 0–58% acetonitrile in 0.1% trifluoroacetic acid from 2 to 32 min after injection; these are the chromatograms shown in Figure 5 (upper panel). The “retention relative to dye peaks” results are from preparative chromatography performed as described under Experimental Procedures and exemplified in Figure 2.

strates. A comparison of chromatograms for these six substrates (Figure 5, upper panel) and a numerical evaluation of their hydrophobicity (Table 3) show that substrates made from more hydrophilic peptides are eluted earlier from a C_{18} column (Figure 5, lower panel). The Bull and Breese index (calculated in PCGENE software) is a computational tool that expresses the hydrophobicity of peptides in a single numerical value.

The substrates made from three peptides with Bull and Breese values greater than 200 cal/mol were eluted significantly earlier than three substrates made from peptides with Bull and Breese values less than 100 cal/mol (Table 3). Preparative chromatography for LY-SLAQAVRSSK(-CTMR)R is shown in Figure 2, where it may be seen that this substrate was eluted just ahead of excess dye reagent. The collected data suggest that, whenever possible, peptides should be designed to have a Bull and Breese value of >200 cal/mol. Alternatively, it is likely but unproven that peptides with hydrophobicity comparable to that of SIHPFHLVIHTK, or rather less, will yield other substrates that are eluted after the dye.

When LY-LAQAVRSSK(-CTMR)R was cleaved by an enzyme (trypsin), fluorescence at 530 nm increased about 26-fold, indicating that it was >95% quenched in the intact molecule. The excitation wavelength was 420 nm. The result was very similar to the one shown in Figure 5 of ref 17. Tracking the fluorescence at 530 nm allowed the reaction progress curve to be monitored, offering the capability to analyze relative reaction rates in the presence of inhibitors. Application of the substrate to studies of the putative TNF convertase will not be reported here.

DISCUSSION

As knowledge of the scope and specificity of proteolytic action in biology is rapidly extended, there is a frequent need for convenient assays for newly discovered enzymes. Energy-transfer substrates are exceptionally useful for this purpose, but their application can sometimes be delayed by difficulty in their synthesis. The method updated in this paper is the one favored in our laboratory, where it is considered rapid, convenient, and simple. It separates introduction of the energy donor-acceptor pair from assembly of the peptide, requires the effort of a single chemist for less than two full workdays, is directly applicable to cleavable sequences containing any amino acid except Lys or Cys (17, 24), and produces substrates with functional properties that appear to be as satisfactory as those of substrates designed otherwise.

While simplicity and convenience are subjective concepts, experience with six different substrates indicates that the method is predictable and reliable as well as exceptionally brief. Major points related to the design were discussed in an earlier paper (17).

The newly abbreviated synthesis does create one minor new uncertainty. This is the effect of leaving unreacted periodate and periodate reaction products (presumed mainly to be iodate) in the reaction mixture when the pH is shifted to pH 4.5 to support condensation of the α -N-glyoxylyl-peptide with Lucifer Yellow CH. If the peptide undergoing modification contains amino acid residues capable of being oxidized by these agents, there is an increased risk that such oxidation will occur in the new procedure. This needs to be watched on a case-by-case basis. As a precaution against the possibility of difficulty, we used oxidation-proof norleucine in place of methionine in one substrate prepared recently. A substrate that contained Trp did not undergo any oxidative side reaction at that residue.

A practical measure against the risk of unwanted oxidation is to add a scavenger compound such as methionine in large (e.g., 50-fold) excess over the peptide (25). In this case, all reactions on the peptide will be slowed, but the desired N-terminal oxidation is still relatively rapid, while the secondary reactions are intended to be slowed to a point of vanishing significance. It is also possible to add a quenching agent such as ethylene glycol when the wanted reaction is complete (26), but one must then consider the possible action of

formaldehyde generated by allowing all of the remaining periodate to react with this agent.

Internally quenched fluorogenic substrates provide excellent activity assays for many proteinases (1, 5-12, 14, 15, 27, 28), but their apparent simplicity conceals a pitfall that has caught many in error, including ourselves. This is a general factor in fluorescence spectroscopy known as the inner filter or internal filtering effect (29), which holds special dangers for enzymologists measuring the effects of substrate concentration on reaction initial velocity. Thorough analysis of the inner filter is a complex matter (29), and only a single case is dealt with here.

Many fluorimeters detect emitted fluorescence on a path lying at a right angle to the beam of exciting light. The inner filter effect arises when the solution has significant absorbance at the exciting wavelength, the emitted wavelength, or both. When this occurs, some of the sample's potential fluorescence is suppressed. Inner filter effects are also concentration-sensitive (because absorbance rises with substrate concentration) and are more severe as substrate concentration increases. To understand this problem, it is essential to recall that absorbance $A = \log(I_0/I)$, where I_0 is the intensity of incident light and I is the light transmitted through the sample. An absorbance of 1 corresponds to 90% of incident light being trapped, and an absorbance of 2 denotes 99% of incident light being trapped. From the definition of absorbance, $I/I_0 = 10^{-A}$, and this quantity is the fraction of incident light transmitted completely through the sample.

Here we will take it that the cuvette has a 1 cm $\times$ 1 cm square cross section and that emission is read from a window that straddles the midline of the cuvette when viewed from the side. The average molecule of substrate or fluorescent product is located halfway across the cuvette in the cross-sectional dimensions, and light traveling to it (420 nm excitation) or from it (530 nm emission) runs a risk of being absorbed that is equal to one-half of the absorbance per centimeter at the respective wavelength. For our average molecule, the fraction of incident light at λ_{ex} that reaches it is $10^{-0.5A_{ex}}$, where A_{ex} is the absorbance per centimeter at λ_{ex} . Likewise, the fraction of emitted fluorescence that escapes from the cuvette is $10^{-0.5A_{em}}$, where A_{em} is the absorbance per centimeter at λ_{em} . Overall, detected fluorescence F_{obs} at any substrate concentration will be a fraction of the fluorescence F_0 that would be observed if no inner filter effect were present, and this fraction is described by the expression

$$F_{obs} = F_0/10^{0.5(A_{ex} + A_{em})} \quad (1)$$

(This equation is a restatement for a specific case of one derived and given in general terms in ref 29. Note that it includes a factor of 0.5 related to the specific value of the cuvette path length, and this must be altered for cuvettes of different dimensions or geometries.)

When absorbance is zero or negligible at both wavelengths, $F_{obs} = F_0$. Should the sum of absorbances per centimeter at λ_{ex} and λ_{em} be equal to 0.2, the inner filter effect reduces the detected fluorescence to near 80% of the unfiltered value. Greater absorbances effect further reductions in the percentage of potential fluorescence that can be detected. The range in which such effects occur can be predicted using eq 1 or a suitable variant. Using calculated absorbance values for a range of substrate concentrations, a spreadsheet program can furnish a plot of predicted F_{obs}/F_0 the shape of which reveals the

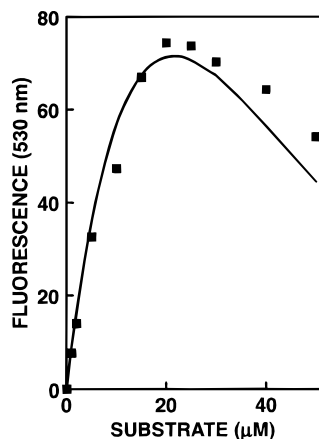


Figure 6. Fluorescence from simulated hydrolysis reactions of LY-LAQAVRSSK(-CTMR)R. An experiment in which Lucifer Yellow CH was added to solutions of LY-LAQAVRSSK(-CTMR)R to simulate the first 10% of hydrolysis (i.e., a 1:10 ratio of Lucifer Yellow to substrate existed at each point). Data points show experimentally measured fluorescence at 530 nm. The line is theoretical, corresponding to the calculated values of fluorescence at each substrate concentration as predicted by eq 1; an experimentally determined value of the fluorescence per unit concentration of Lucifer Yellow CH was used to allow comparison of the y-range of the theoretical curve with the experimental data.

range of substrate concentration at which *optical effects alone* will cause a plot of velocity against substrate concentration to be nonlinear. To make this plot, some value must be assigned to the yield of fluorescence per unit concentration of the energy donor in its free form. This can be any arbitrary number, in which case the exercise still yields valid information about the substrate concentration at which inner filtering is important, but values on the y-axis are strictly relative. It can also be experimentally obtained.

Figure 6 shows a plot of this kind for the TNF convertase substrate and a comparison with experiment. Even when the substrate is intact, equimolar quantities are present of Lucifer Yellow (the energy donor) and 5-carboxytetramethylrhodamine (the energy acceptor). The fluorescence of Lucifer Yellow is suppressed, but the chromophores contribute to absorbance at λ_{ex} and λ_{em} as determined by the absorption spectrum (inset to Figure 3). Enzymatic cleavage abolishes the quenching (which is inversely proportional to the sixth power of the distance separating the dyes) (16), and the fluorescence of Lucifer Yellow becomes detectable. This unmasking can be simulated by adding free Lucifer Yellow CH to substrate. With substrate in 10-fold excess over Lucifer Yellow CH, the solution approximates one in which 10% of the substrate has been cleaved, i.e., the end-point of a typical initial rate assay. Fluorescence readings for a series of such samples approximate measurements of the effect of substrate concentration on initial rate. Figure 6 shows that eq 1 predicts quite well the change in fluorescence values as a function of concentration. Imperfections in the agreement may be due to experimental error, some uncertainty in the molar absorptivity value for the absorption spectrum of the substrate, and (at higher substrate concentrations) the fact that secondary fluorescence has not been considered (29).

There has been considerable discussion of means to correct for inner filter effects (11, 29), but the first priority is at least to be aware of them. Holskin et al. (15) recently provided an illustration of the effect as seen in velocity data obtained with a cytomegalovirus proteinase substrate based on energy transfer between EDANS and DABCYL groups.

The present approach to substrate design has been successful in our laboratory and has the virtue of being available today using easily accessible materials and methods. By no means, however, will it represent the last word, as new chemical methods for bioconjugate formation continue to emerge. Zhang and Tam (30), for example, describe condensation of a 1,2-aminothiol with an aldehyde (such as an α -N-glyoxylyl-peptide) to form thiazolidine adducts that have good potential for this type of work. Their study further extends the list of ways to form useful conjugates at aldehydes created in peptides and proteins, including hydrazones (24, 26, 31), reduced hydrazones (26, 32), oximes (33, 34), and thiazolidines (30). With the recent reintroduction of metal-catalyzed transamination as a route to creating α -oxoacyl sites at protein N termini (25, 35), there is now considerable versatility available in this chemical toolbox.

LITERATURE CITED

- (1) Latt, S. A., Auld, D. S., and Vallee, B. L. (1972) Fluorescence determination of carboxypeptidase A activity based on electronic energy transfer. *Anal. Biochem.* 50, 56–62.
- (2) Carmel, A., Zur, M., Yaron, A., and Katchalski, E. (1973) Use of substrates with fluorescent donor and acceptor chromophores for the kinetic assay of hydrolases. *FEBS Lett.* 30, 11–14.
- (3) Knight, C. G. (1995) Fluorimetric assays of proteolytic enzymes. *Methods Enzymol.* 248, 18–34.
- (4) Geoghegan, K. F., Spencer, R. W., Danley, D. E., Contillo, L. G., and Andrews, G. C. (1990) Fluorescence-based continuous assay for the aspartyl protease of human immunodeficiency virus-1. *FEBS Lett.* 262, 119–122.
- (5) Matayoshi, E. D., Wang, G. T., Krafft, G. A., and Erickson, J. (1990) Novel fluorogenic substrates for assaying retroviral proteases by resonance energy transfer. *Science* 247, 954–958.
- (6) Ng, M., and Auld, D. S. (1989) A fluorescent oligopeptide energy transfer assay with broad applications for neutral proteases. *Anal. Biochem.* 183, 50–66.
- (7) Stack, M. S., and Gray, R. D. (1989) Comparison of vertebrate collagenase and gelatinase using a new fluorogenic substrate peptide. *J. Biol. Chem.* 264, 4277–4281.
- (8) Garcia-Echeverria, C., and Rich, D. H. (1992) New intramolecularly quenched fluorogenic peptide substrates for the study of the kinetic specificity of papain. *FEBS Lett.* 297, 100–102.
- (9) Niedzwiecki, L., Teahan, J., Harrison, R. K., and Stein, R. L. (1992) Substrate specificity of the human matrix metalloproteinase stromelysin and the development of continuous fluorometric assays. *Biochemistry* 31, 12618–12623.
- (10) Bickett, D. M., Green, M. D., Berman, J., Dezube, M., Howe, A. S., Brown, P. J., Roth, J. T., and McGeehan, G. M. (1993) A high throughput fluorogenic substrate for interstitial collagenase (MMP-1) and gelatinase (MMP-9). *Anal. Biochem.* 212, 58–64.
- (11) Wang, G. T., Chung, C. C., Holzman, T. F., and Krafft, G. A. (1993) A continuous fluorescence assay of renin activity. *Anal. Biochem.* 210, 351–359.
- (12) Goudreau, N., Guis, C., Soleilhac, J. M., and Roques, B. P. (1994) Dns-Gly-(p-NO₂)Phe-beta Ala, a specific fluorogenic substrate for neutral endopeptidase 24.11. *Anal. Biochem.* 219, 87–95.
- (13) Nagase, H., Fields, C. G., and Fields, G. B. (1994) Design and characterization of a fluorogenic substrate selectively hydrolyzed by stromelysin 1 (matrix metalloproteinase-3). *J. Biol. Chem.* 269, 20952–20957.
- (14) Bickett, D. M., Green, M. D., Wagner, C., Roth, J. T., Berman, J., and McGeehan, G. M. (1994) A high throughput fluorogenic substrate for stromelysin (MMP-3). *Ann. N.Y. Acad. Sci.* 732, 351–355.
- (15) Holskin, B. P., Bukhtiyarova, M., Dunn, B. M., Baur, P., de Chastonay, J., and Pennington, M. W. (1995) A continuous fluorescence-based assay of human cytomegalovirus protease using a peptide substrate. *Anal. Biochem.* 227, 148–155.

- (16) Stryer, L. (1978) Fluorescence energy transfer as a spectroscopic ruler. *Annu. Rev. Biochem.* 47, 819–846.
- (17) Geoghegan, K. F., Emery, M. J., Martin, W. H., McColl, A. S., and Daumy, G. O. (1993) Site-directed double fluorescent tagging of human renin and collagenase (MMP-1) substrate peptides using the periodate oxidation of N-terminal serine. An apparently general strategy for provision of energy-transfer substrates for proteases. *Bioconjugate Chem.* 4, 537–544.
- (18) McGeehan, G. M., Becherer, J. D., Bast, R. C., Jr., Boyer, C. M., Champion, B., Connolly, K. M., Conway, J. G., Furdon, P., Karp, S., Kidao, S., McElroy, A. B., Nichols, J., Pryzwansky, K. M., Schoenen, F., Sekut, L., Truesdale, A., Verghese, M., Warner, J., and Ways, J. P. (1994) Regulation of tumour necrosis factor- α processing by a metalloproteinase inhibitor. *Nature* 370, 558–561.
- (19) Mohler, K. M., Sleath, P. R., Fitzner, J. N., Cerretti, D. P., Alderson, M., Kerwar, S. S., Torrance, D. S., Otten-Evans, C., Greenstreet, T., Weerawarna, K., Kronheim, S. R., Petersen, M., Gerhart, M., Kozlosky, C. J., March, C. J., and Black, R. A. (1994) Protection against a lethal dose of endotoxin by an inhibitor of tumour necrosis factor processing. *Nature* 370, 218–220.
- (20) Pennica, D., Nedwin, G. E., Hayflick, J. S., Seeburg, P. H., Derynck, R., Palladino, M. A., Kohr, W. J., Aggarwal, B. B., and Goeddel, D. V. (1984) Human tumour necrosis factor: precursor structure, expression and homology to lymphotoxin. *Nature* 312, 724–729.
- (21) Shirai, T., Yamaguchi, H., Ito, H., Todd, C. W., and Wallace, R. B. (1985) Cloning and expression in *Escherichia coli* of the gene for human tumour necrosis factor. *Nature* 313, 803–806.
- (22) Wang, A. M., Creasey, A. A., Ladner, M. B., Lin, L. S., Strickler, J., Van Arsdel, J. N., Yamamoto, R., and Mark, D. F. (1985) Molecular cloning of the complementary DNA for human tumor necrosis factor. *Science* 228, 149–154.
- (23) Fields, R., and Dixon, H. B. F. (1968) Spectrophotometric determination of periodate. *Biochem. J.* 108, 883–887.
- (24) Geoghegan, K. F., and Stroh, J. G. (1992) Site-directed conjugation of nonpeptide groups to peptides and proteins via periodate oxidation of a 2-amino alcohol. Application to modification at N-terminal serine. *Bioconjugate Chem.* 3, 138–46.
- (25) Gaertner, H. F., and Offord, R. E. (1996) Site-specific attachment of functionalized poly(ethylene glycol) to the amino terminus of proteins. *Bioconjugate Chem.* 7, 38–44 (and references cited therein).
- (26) Gaertner, H. F., Rose, K., Cotton, R., Timms, D., Camble, R., and Offord, R. E. (1992) Construction of protein analogues by site-specific condensation of unprotected fragments. *Bioconjugate Chem.* 3, 262–268.
- (27) Pennington, M. W., and Thornberry, N. A. (1994) Synthesis of a fluorogenic interleukin-1 β converting enzyme substrate based on resonance energy transfer. *Pept. Res.* 7, 72–76.
- (28) Contillo, L. G., Singleton, D. H., Andrews, G. C., Spencer, R. W., Faraci, W. S., Martin, W. H., and Stock, I. A. (1994) A general strategy for the synthesis of eosin fluorescein energy transfer substrates for high sensitivity screening of protease inhibitors. *Techniques in Protein Chemistry V* (J. W. Crabb, Ed.) pp 493–500, Academic Press, New York.
- (29) Lloyd, J. B. F. (1981) Inner filter effects, sample cells and their geometry in fluorescence spectrometry. *Standards in Fluorescence Spectrometry* (J. N. Miller, Ed.) pp 27–43, Chapman and Hall, New York.
- (30) Zhang, L., and Tam, J. P. (1996) Thiazolidine formation as a general and site-specific conjugation method for synthetic peptides and proteins. *Anal. Biochem.* 233, 87–93 (and references cited therein).
- (31) Maassen, J. A., Thielen, T. P., and Moller, W. (1983) Synthesis and application of two reagents for the introduction of sulfhydryl groups into proteins. *Eur. J. Biochem.* 134, 327–330.
- (32) Gaertner, H. F., Offord, R. E., Cotton, R., Timms, D., Camble, R., and Rose, K. (1994) Chemo-enzymic backbone engineering of proteins. Site-specific incorporation of synthetic peptides that mimic the 64–74 disulfide loop of granulocyte colony-stimulating factor. *J. Biol. Chem.* 269, 7224–7230.
- (33) Alouani, S., Gaertner, H. F., Mermod, J. J., Power, C. A., Bacon, K. B., Wells, T. N., and Proudfoot, A. E. (1995) A fluorescent interleukin-8 receptor probe produced by targeted labelling at the amino terminus. *Eur. J. Biochem.* 227, 328–334.
- (34) Shao, J., and Tam, J. P. (1995) Unprotected peptides as building blocks for the synthesis of peptide dendrimers with oxime, hydrazone, and thiazolidine linkages. *J. Am. Chem. Soc.* 117, 3893–3899.
- (35) Dixon, H. B. F., and Fields, R. (1972) Specific modifications of NH₂-terminal residues by transamination. *Methods Enzymol.* 25, 409–419.

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COMMUNICATIONS

Synthesis and Anticoagulant Activity of Sulfated Glucoside-Bearing Polymer

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Poly(glucosyloxyethyl methacrylate) sulfate [poly(GEMA) sulfate], which contains sulfated glucoside residues, was prepared by the reaction with *N,N*-dimethylformamide (DMF)/sulfur trioxide (SO₃) complex. The degree of sulfation was easily controlled by changing the amount of DMF/SO₃ complex added and reaction time. The total human blood clotting time in the presence of poly(GEMA) sulfate was prolonged by increasing the dose or the degree of sulfation of the polymer. The anticoagulant activity of poly(GEMA) sulfate was also compared with that of dextran sulfate, poly(styrenesulfonic acid), poly(vinylsulfuric acid), and heparin. The result suggests that sulfated saccharide residues are essential for endowing anticoagulant activity to synthetic polymer.

It is well-known that heparin has many biological activities such as an anticoagulant activity, a cell growth stimulation activity, an antiviral activity, and a plasma clearing activity. Among them, the anticoagulant activity is the essential activity for heparin. The physiological activity of heparin, however, has not been completely clarified, and it is not so easy to immobilize heparin onto solid polymer surfaces. To solve these problems, various heparinoids, which are synthetic polymers or analogues of naturally occurring heparin, were synthesized and their biological activities studied. From the viewpoint of the synthesis of heparinoid polymers, naturally occurring polysaccharides such as dextran and chitin were sulfated and their anticoagulant activities evaluated (1–4). Even for synthetic polymers, it has been believed that sulfation is effective for acquisition of anticoagulant

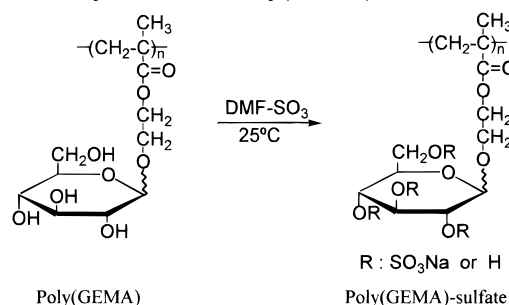
activity (5–7). It has been considered that not only the anionic charge but also the linearity of the polymer backbone, such as a polysaccharide chain, are important in revealing the anticoagulant activity (8). The anticoagulation mechanism of the heparinoid polymers, however, has not been clarified with regard to the importance of monosaccharide units yet.

In our previous study, free radical polymerization and copolymerization of glucosyloxyethyl methacrylate (GEMA) were found to give highly water soluble polymers and hydrogels, which have glucoside residue in their side chains, and their properties were reported (9–11). They can be considered as one of the polysaccharide model compounds. In this paper, we report the synthesis of sulfated poly(GEMA) and its anticoagulant activity to determine whether sulfated saccharide residues are important for acquisition of the anticoagulant activity.

Scheme 1 illustrates the synthesis of sulfated poly(GEMA). Poly(GEMA) was obtained by free radical polymerization of GEMA in distilled water using ammonium peroxodisulfate (APS) as an initiator (10–12).

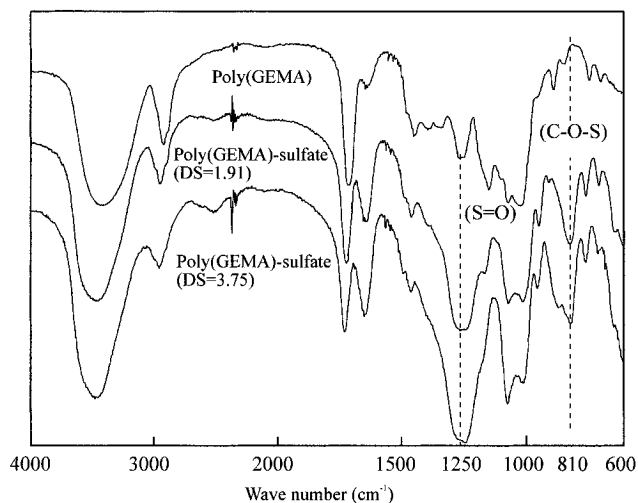
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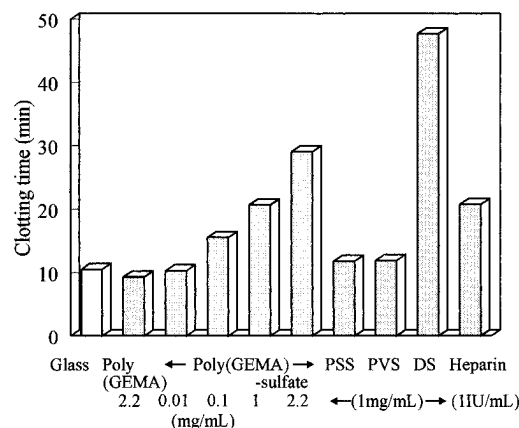
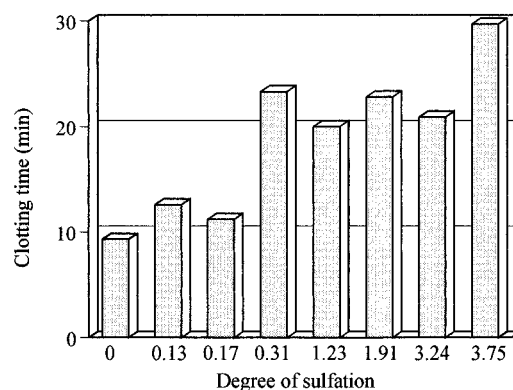
Scheme 1. Synthesis of Poly(GEMA) Sulfate**Table 1. Sulfation of Poly(GEMA)^a**

run no.	reaction time (h)	degree of sulfation ^b
1	0.3	1.91
2	1.8	3.24
3	5.0	3.33
4	12.0	3.42
5	24.0	3.75

^a Reaction temperature was room temperature. ^b Degree of sulfation was estimated by elemental analysis.

**Figure 1.** IR spectra of poly(GEMA) and sulfated poly(GEMA).

The reaction of poly(GEMA) and excess *N,N*-dimethylformamide (DMF)/sulfur trioxide (SO₃) in DMF at room temperature gives poly(GEMA) sulfate in analogy with the sulfation of chitin (3). As shown in Table 1, the degree of sulfation is able to change according to reaction conditions such as reaction time. [The synthesis of poly-(2-glucosyloxyethyl methacrylate) sulfate was as follows: Poly(GEMA) (2 g) and SO₃ (3 g) were mixed in *N,N*-dimethylformamide (DMF) (50 g) in molar ratio of 1:3. The mixture was stirred under nitrogen atmosphere, and the temperature of the mixture was not allowed to rise over 25 °C. At the end of the reaction time, the reaction product was neutralized with NaOH (3 g) in a 1:10 water (27 g)–methanol (270 g) mixture solution and purified by dialysis against distilled water for 3 days to remove sodium sulfate. Then, polymer solution was freeze-dried for 4 days. The degree of sulfation was estimated by elemental analysis.] After 24 h, most of the hydroxy groups on the glucoside residue were converted to the sulfate group. The degree of sulfation can be also controlled by the concentration of SO₃. Sulfation was confirmed by infrared spectroscopy as shown in Figure 1. Absorptions at 1250 and 810 cm⁻¹ of poly(GEMA) sulfate were assigned to S=O and C–O–S stretch bonds, respectively.

**Figure 2.** Anticoagulant activity of sulfated poly(GEMA), PSS, PVS, DS, and heparin.**Figure 3.** Effect of degree of sulfation of poly(GEMA) sulfate on total human blood clotting time.

The anticoagulant activity of the resulting poly(GEMA) sulfate was evaluated by total human blood clotting time using the method of Lee-White (13). In the Lee-White test, clotting time was prolonged when coagulant factors were inhibited. The results are shown in Figures 2 and 3. It is clear that poly(GEMA) sulfate prolongs the coagulation time, while, on the other hand, poly(GEMA) shows no effect. Both the concentration and the degree of sulfation of poly(GEMA) sulfate influence the coagulation time. However, the coagulation time in the presence of poly(GEMA) sulfate was not extended longer than that of heparin, which has an anticoagulant activity of 187.5 IU/mg of solid, in the same dose. As shown in Figure 2, poly(styrenesulfonic acid) (PSS) and poly(vinylsulfuric acid) (PVS), which were purchased from Nacalai Tesque, scarcely prolonged the blood clotting time in this condition. In the case of dextran sulfate (DS), which is a modified polysaccharide, the anticoagulant activity was higher than that of poly(GEMA) sulfate, though the degree of sulfation is about 1.0. As a result, the potency of anticoagulant activity of poly(GEMA) sulfate was between those of synthetic sulfated polymers and sulfated polysaccharide. These results suggest that sulfated saccharide residues play an important role in the acquisition of anticoagulant activity. The activity of heparin is much higher than that of these heparinoid polymers.

It is quite difficult to covalently immobilize heparin to polymeric materials for the preparation of antithrombogenic materials, though slow release of heparin from polymer matrix (14, 15) and the physical coating of materials with heparin (16) are considerably easy. It is also difficult to immobilize a polysaccharide such as dextran sulfate from the terminal saccharide unit and to control the chain length of the immobilized polysaccharide. Using GEMA monomer, it is possible to prepare

a hydrogel by free radical polymerization (11) and to modify polymer surfaces by surface graft polymerization (17). Sulfated poly(GEMA) with the anticoagulant activity, therefore, can be useful as a starting material for antithrombogenic biomaterials. We will compare the antithrombogenicity of poly(GEMA) sulfate with that of thrombomodulin (18, 19), which is protein C activation protein, onto endothelial cell, immobilized materials, or aramid silicone (20) resins which have hydrophobic silicone surfaces. The mechanism of the anticoagulant activity of poly(GEMA) sulfate will be reported in a further publication.

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LITERATURE CITED

- (1) Mauzac, M., and Jozefonvictz, J. (1984) Anticoagulant activity of dextran derivatives. Part I: Synthesis and characterization. *Biomaterials* 5, 301–304.
- (2) Muzzarelli, R. A. A., Tanfani, F., and Emanuelli, M. (1984) Sulfated N-(carboxymethyl)chitosans. *Carbohydr. Res.* 126, 225–231.
- (3) Nishimura, S., and Tokura, S. (1987) Preparation and antithrombogenic actives of heparinoid from 6-O-(carboxymethyl)chitin. *Int. J. Biol. Macromol.* 9, 225–232.
- (4) Doctor, V. M., Lewis, D., Coleman, M., Kemp, M. T., Marbley, E., and Sauls, V. (1991) Anticoagulant properties of semisynthetic polysaccharide sulfates. *Thromb. Res.* 64, 413–425.
- (5) Hatanaka, K., Yoshida, T., Miyahara, S., Sato, T., Ohno, F., and Uryu, T. (1987) Synthesis of new heparinoids with high anticoagulant activity. *J. Med. Chem.* 30, 810–814.
- (6) Ito, Y., Iguchi, Y., Kashiwagi, T., and Imanishi, Y. (1991) Synthesis and nontrombogenicity of polyetherurethaneurea film grafted with poly(sodium vinyl sulfonate). *J. Biomed. Mater. Res.* 25, 1347–1359.
- (7) Fougnot, C. (1986) Synthetic heparin-like materials. *Dev. Hematol. Immunol.* 14, 201–206.
- (8) Chr. Heuck, C., Schiele, U., Horn, D., Fronda, D., and Ritz, E. (1985) The role of surface charge on the accelerating action of heparin on the antithrombin III-inhibited activity of α -thrombin. *J. Biol. Chem.* 260, 4598–4603.
- (9) Kitazawa, S., Okumura, M., Kinomura, K., and Sakakibara, T. (1990) Synthesis and properties of novel vinyl monomers bearing a glycoside residues. *Chem. Lett.*, 1733–1736.
- (10) Kitazawa, S., Okumura, M., Kinomura, K., Sakakibara, T., Nakamae, K., Miyata, T., Akashi, M., and Suzuki, K. (1994) Preparation and some properties glycoside-bearing polymer. In *Carbohydrate as Organic Raw Materials* (Descotes, G., Ed.) Vol. 2, pp 115–135, VCH, Weinheim.
- (11) Fukudome, N., Suzuki, K., Yashima, E., and Akashi, M. (1994) Synthesis of nonionic and hydrogels bearing a monosaccharide residue and their properties. *J. Appl. Polym. Sci.* 52, 1759–1763.
- (12) Nakamae, K., Miyata, T., Jikihar, A., and Hoffman, A. S. (1994) Formation of poly(glucosyloxyethyl methacrylate)-Concanavalin A complex and its glucose-sensitivity. *J. Biomater. Sci. Polym. Ed.* 6 (1), 79–90.
- (13) Shapiro, G. A., Huntzinger, S. W., and Wilson, J. E. (1977) Variation among commercial activated partial thromboplastin time reagents in response to heparin. *Am. J. Clin. Pathol.* 67, 477–480.
- (14) Akashi, M., Takeda, S., Miyazaki, T., Yashima, E., Miyauchi, N., Maruyama, I., Okadome, T., and Murata, Y. (1989) Antithrombogenic poly(vinyl chloride) with heparin- and/or prostaglandin I₂-immobilized in hydrogels. *J. Bioact. Compat. Polym.* 4, 4–16.
- (15) Kwon, I. C., Bae, Y. H., and Kim, S. W. (1994) Heparin release from polymer complex. *J. Control. Rel.* 30, 155–159.
- (16) Gu, Y. J., Oevern, W., Akkerman, C., Boonstra, P. W., Huyzen, R. J., and Wildevuur, C. R. H. (1993) Heparin-coated circuits reduce the inflammatory response to cardiopulmonary bypass. *Ann. Thorac. Surg.* 55, 917–922.
- (17) Iwata, H., Kishida, A., Suzuki, M., Hata, Y., and Ikada, Y. (1988) Oxidation of polyethylene surface by corona discharge and subsequent graft polymerization. *J. Polym. Sci., Polym. Chem. Ed.* 26, 3309–3322.
- (18) Akashi, M., Maruyama, I., Fukudome, N., and Yashima, E. (1992) Immobilization of human thrombomodulin on glass beads and its anticoagulant activity. *Bioconjugate Chem.* 3, 363–365.
- (19) Kishida, A., Ueno, Y., Fukudome, N., Yashima, E., Maruyama, I., and Akashi, M. (1994) Immobilization of human thrombomodulin onto poly(ether urethane urea) for developing antithrombogenic blood-contacting materials. *Biomaterials* 15 (10), 848–852.
- (20) Furuzono, T., Yashima, E., Kishida, A., Maruyama, I., Matsumoto, T., and Akashi, M. (1993) A novel biomaterial: poly(dimethylsiloxane)-polyamide multiblock copolymer I. Synthesis and evaluation of blood compatibility. *J. Biomater. Sci. Polym. Ed.* 5, 89–98.

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ARTICLES

Binding and Transport of Benzo[a]pyrene by Blood Plasma Lipoproteins: The Possible Role of Apolipoprotein B in This Process

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The role of plasma lipoproteins as carriers in the transport of benzo[a]pyrene was assessed in *in vitro* and *in vivo* studies. Addition of [³H]benzo[a]pyrene to rat plasma resulted in binding of the xenobiotic to lipoproteins. Studies of labeled benzo[a]pyrene distribution in rat blood plasma by the method of ultracentrifugation have given the following results: high-density lipoproteins, 40%; low-density lipoproteins, 14%; very-low-density lipoproteins, 23%; other plasma proteins, 23%. Complexes of benzo[a]pyrene–lipoproteins were isolated by gel filtration with Sephadex G-25 and used for intravenous injection in rats. Biodistribution studies have shown different localization of benzo[a]pyrene in rat organs and tissues depending on lipoprotein classes. A high amount of radioactivity was bound by the liver and adrenals when all classes of lipoproteins were used, but especially with high-density lipoproteins. High levels of benzo[a]pyrene were measured in the kidneys. Equilibrium dissociation constants for complexes of benzo[a]pyrene with high-density lipoproteins and low-density lipoproteins were obtained (K_d 4.1×10^{-5} and 1.5×10^{-5} M, respectively). Binding and distribution of the protein component of lipoproteins were studied by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. More than 80% of the radioactivity recovered from the gel was localized in the area of apolipoprotein B. After isolation and purification of apolipoprotein B, the equilibrium dissociation constant for complexes of benzo[a]pyrene with apolipoprotein B was obtained, and its value indicated that apolipoprotein B might be the main protein carrier for benzo[a]pyrene.

INTRODUCTION

Blood plasma lipoproteins (LFs)¹ are involved in one of the specialized forms of biological transport. Besides the basic function of lipid transport, LPs also bind and transport a number of biologically active compounds: vitamins (1, 2), steroid and thyroid hormones (3, 4), and xenobiotics, including some drugs (5, 6). It is known that LPs are carriers in the bloodstream for many chemical carcinogens such as 2,3,7,8-tetrachloro-*p*-benzodioxin (7), 3-methylcholanthrene (8), and *p*-(dimethylamino)azobenzene (9). However, the mechanisms of carcinogen delivery to the cells and their internalization by the cell are not well understood.

The lipophilicity of transported carcinogens is considered to play the major role in the ligand binding. Most lipophilic compounds are mainly bound by LP fractions (10). Besides the lipophilicity, there are other factors which can influence the interaction between LP particles and lipophilic carcinogens. For example, dihydroxylated

derivatives of BP display an affinity for HDL higher than expected, considering the HDL lipid volume. Serum albumin plays a minor role in the binding of lipophilic carcinogens in the living organisms; 5% of unmetabolized BP associates with albumin and 95% associates with LPs (11).

Interactions of lipophilic carcinogens with polar lipids of LPs and with apolipoproteins may be involved in the mechanism of modulation of the toxicity and in the uptake of the xenobiotics by cells. However, the role of the protein component of LPs in the binding of carcinogens is still obscure. Studies on the interaction of BP and other polycyclic aromatic hydrocarbons with proteins may be very useful for understanding the molecular mechanisms of carcinogenesis.

In the present study we have obtained some quantitative characteristics of the interactions between different LP classes with BP and show a possible role of apoB in this process. In addition, the distribution of labeled BP in rat organs and tissues was investigated using different LP classes as BP carriers.

EXPERIMENTAL PROCEDURES

Isolation of Lipoproteins. Plasma LPs were isolated by sequential ultracentrifugation in potassium bromide (KBr) solution in the presence of 5 mM EDTA Na₂ (Serva, Germany) as described previously (12), using a Beckman L-75 centrifuge and 75 Ti rotor. HDL, LDL, and VLDL fractions were dialyzed overnight against 0.15 M NaCl, 5 mM Tris-HCl, pH 8.0, 5 mM EDTA Na₂, and 10 mM NaN₃ at 4 °C.

Distribution of [³H]BP among LP Fractions. [³H]BP (Amersham, England, specific activity 50 Ci/

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¹ Abbreviations: BP, benzo[a]pyrene; LP, lipoprotein; HDL, high-density lipoprotein (1.063 < density < 1.210 g/L); LDL, low-density lipoprotein (1.019 < density < 1.063 g/L); VLDL, very-low-density lipoprotein (density < 1.019 g/L); apoB, apolipoprotein B; SDS, sodium dodecyl sulfate; PAG, polyacrylamide gel; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; BSA, bovine serum albumin; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediamine-tetraacetic acid.

mmol) in 50 μ L of dimethyl sulfoxide was added to rat plasma (20 mL) before the centrifugation; the radioactivity of [^{3} H]BP in this solution was 0.05 μ Ci/mL of plasma. This solution was shaken up and incubated for 30 min, and then LP fractions were isolated as described (12). Radioactivity of each fraction was determined in a Mark-III spectrometer in toluene scintillation fluid. Counting effectiveness was 40%.

The LP fractions were further purified by chromatography with a Sephadex G-25 (Pharmacia, Sweden) column (1.6 $\times$ 32 cm) in a buffer containing 5 mM Tris-HCl, pH 8.0, and 5 mM 2-mercaptoethanol at a flow rate of 0.5 mL/min. Fractions (2 mL) were collected, and the elution profile was recorded by UV detector 2151 (LKB, Sweden). The amount of radioactivity in each fraction was determined in toluene scintillation fluid.

Biodistribution of [^{3} H]BP in Rat Tissues. Male Wistar rats weighing 160–180 g were used for *in vivo* experiments. About 1 μ Ci of [^{3} H]BP bound to LPs in 0.5 mL of a 0.15 M NaCl solution was injected into the tail vein. The procedure was performed under ether anesthesia. Rats were sacrificed 30 min after injection and perfused for 5 min with cold 0.15 M NaCl via aorta and v. porta. Pieces (100–150 mg) of required tissues were removed, weighed, and homogenized on ice in 1 mL of saline using a motor-driven Teflon Potter-Elvehjem homogenizer for 1 min at 3000 rpm. From this homogenate was loaded 100 μ L of each tissue sample onto glass microfiber filters (GF/C, Whatman, England), which were dried. Radioactivity was measured in 5 mL of toluene scintillation fluid.

Binding and Distribution of [^{3} H]BP among Apo-LP. LPs (100 μ L) were incubated with 10 μ L of [^{3} H]BP (about 1.3×10^5 cpm, in dimethyl sulfoxide) for 1 h at room temperature. Then, 10 μ L of 20% SDS (Serva, Germany) and 20 μ L of glycerol were added to each sample, followed by warming for 5 min at 90 $^{\circ}$ C. The apolipoproteins (30 μ L, about 50 μ g) were separated by SDS-PAGE (12.5%) as described by Laemmli (13). After electrophoresis, every lane of gel was divided lengthwise into two parts, one of which was stained with 0.05% Coomassie brilliant blue G-250 in 50% methanol and 10% acetic acid; the unstained part was sliced into 4-mm slices and dissolved in NCS tissue solubilizer (Amersham, England) until the samples were cleared (usually 2–3 h). The radioactivity of the gel slice samples was determined in a Mark-III liquid scintillation spectrometer. Radioactivity profiles for the gels were constructed from the radioactivity present in the gel slice samples.

Binding Analysis. The quantitative characteristics of the stability of the LP-BP bioconjugates were obtained using the cellulose circles method developed in our laboratory (14). This method was as follows: round cellulose circles prepared from Whatman 3MM (Whatman, England) were soaked with 50 μ L of dimethyl sulfoxide solution containing a constant quantity of [^{3} H]BP (1 μ g or 15 000 cpm) and increasing amounts of unlabeled BP (from 0 to 50 μ g on every circle). The circles were dried and incubated in 1 mL of LP solution in PBS (10 mM, 0.15 M NaCl, pH 7.4) or apoB in 0.02% SDS solution of PBS at 20 $^{\circ}$ C for 24 h. Control incubations of cellulose circles treated with increasing amounts of unlabeled ligands were performed in the presence of BSA. The protein concentration in these solutions was 1–3 g/L. The circles were dried, and radioactivity was determined in 5 mL of toluene scintillation fluid for every circle. The results were used for equilibrium dissociation constant (K_d) calculations.

Isolation and Purification of apoB. Rat LDLs were delipidated by extracting with a cooled methanol–

Table 1. Percentage Binding of BP to Lipoprotein Fractions (Isolated by Ultracentrifugation)

LP fractions	radioactivity (%)	plasma amounts of LPs (in %) ^a
VLDL	23	14
LDL	14	17
HDL	40	69
other plasma proteins	23	

^a According to Chapman (16).

chloroform (1:1, v/v) solution at 4 $^{\circ}$ C for 24 h and then by washing with ether alone. Then 10–15 mg of dried proteins was dissolved in 2% SDS solution, warmed for 5 min at 90 $^{\circ}$ C, and fractionated on a Sepharose 4B (Pharmacia, Sweden) column (1.6 $\times$ 100 cm) in a buffer containing 5 mM Tris-HCl, pH 8.0, 0.05% SDS, 0.01% Na₃, and 1 mM phenylmethanesulfonyl fluoride at a flow rate of 9 mL/h. The purity of the proteins obtained was evaluated by SDS-PAGE. The first peak contained both forms of apoB (B-100 and B-48).

Immunization and Separation of Antibodies to apoB. Purified apoB in 0.1% SDS solution was used for immunization of rabbits. Injections were performed subcutaneously (in multiple points of upper back part) with 0.5 mL of apoB (100 μ g) and 0.5 mL of complete Freund's adjuvant (Difco Laboratories). The same dose was repeated 10 days later in incomplete Freund's adjuvant. The last immunization was performed by intravenous injection of 100 μ g of apoB without Freund's adjuvant. Antibodies were obtained from the blood serum by repeated precipitation with 50% (NH₄)₂SO₄ followed by dialysis against PBS (10 mM sodium phosphate, 0.15 M NaCl, pH 7.4) containing 0.1% Na₃. The last purification step was ionic exchange chromatography on DEAE-Sepharose (Pharmacia, Sweden). The column (1.6 $\times$ 20 cm) was equilibrated for 1 h at room temperature with PBS (10 mM sodium phosphate, 0.15 M NaCl, pH 7.4) containing 0.1% Na₃. The antibodies were then eluted with PBS (10 mM sodium phosphate, 0.15 M NaCl, pH 7.4) containing 0.1% Na₃ at a 1 mL/min flow rate. The purified antibodies were frozen and stored at –70 $^{\circ}$ C.

Immunoblotting. Samples of rat HDL (50 μ g of protein) were fractionated by SDS electrophoresis using a 12.5% gel. After fractionation, the apolipoproteins were electrophoretically transferred to nitrocellulose membranes (Schleicher & Schuell, Germany) by a semidry method using flat coal electrodes (15). The protein transfer was carried out during 1 h at 0.8 mA/cm². After blocking with 0.5% BSA, the nitrocellulose membrane was placed into a 1000-fold diluted solution of antibody to apoB in PBS and incubated for 1 h. After washing with PBS containing 0.5% BSA, the membrane was incubated for 1 h with a 1000-fold dilution of antirabbit IgG conjugated with horseradish peroxidase (Bio-Rad) in the same solution as that used in the incubation with the first antibody, and washed thoroughly. The antigen-antibody complex was then identified by the color development after incubation of the membrane with a solution containing 1 mg/mL 3,3-diaminobenzidine, 1 mg/mL imidazole, and 1 μ L/mL 30% H₂O₂.

RESULTS AND DISCUSSION

Addition of [^{3} H]-labeled BP to rat plasma followed by ultracentrifugation has demonstrated that most of the radioactivity (about 77%) was associated with LP fractions. The greatest amount of radioactivity was found in HDLs while the smallest amount was in LDLs. The VLDL fraction had an intermediate amount of radioactivity (Table 1). After ultracentrifugation LP fractions

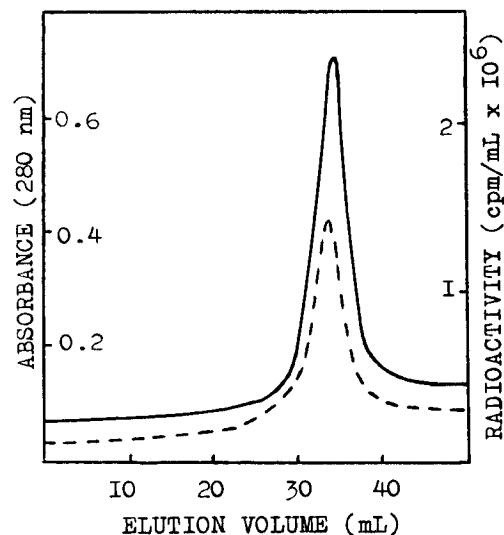


Figure 1. Elution profiles obtained with gel filtration chromatography of $[^3\text{H}]\text{BP}$ complex and HDL on Sephadex G-25, as detected by radioactivity (---) and UV absorbance at 280 nm (—). Elution was performed with 5 mM Tris-HCl buffer, pH 8.0, containing 5 mM 2-mercaptoethanol at flow rate of 0.5 mL/min. Fractions (2 mL) were collected, and the radioactivity was determined.

Table 2. Biodistribution of $[^3\text{H}]\text{BP}$ in Rats after Intravenous Injection of $[^3\text{H}]\text{BP}$ -LP Complexes (in cpm/mg Weight Tissues)^a

	VLDL	LDL	HDL
liver	27.9 ± 4.1	25.9 ± 2.6	40.8 ± 9.5
lung	6.6 ± 1.6	5.9 ± 1.0	9.6 ± 1.1
heart	3.1 ± 0.1	2.5 ± 0.2	2.3 ± 0.7
spleen	1.9 ± 0.3	3.7 ± 0.5	5.9 ± 1.0
kidney	11.5 ± 2.0	9.1 ± 1.0	26.3 ± 3.8
adrenals	24.7 ± 2.3	29.1 ± 3.7	47.5 ± 9.5
thymus	4.1 ± 0.9	3.8 ± 0.4	6.4 ± 1.3

^a Values quoted are mean ± standard deviation. Number of animals in every case was not less than five.

were passed through a Sephadex G-25 column. The BP radioactivity peak always eluted with LP fractions (Figure 1). After gel filtration, the total binding $[^3\text{H}]\text{BP}$ by LPs and the radioactivity ratio between LP fractions was practically unchanged. The low concentration of LDLs in rats, compared to HDLs and VLDLs (16), partially explains this phenomenon. Besides, both lipid and protein parts of LP particles participate in the BP binding process, and the contribution of these parts varies between different classes of LPs and depends on their ratio in LP particles and on their nature.

LPs containing labeled BP were used to transport $[^3\text{H}]\text{BP}$ in rats. BP-LP complexes were injected into the rat's tail vein. Biodistribution studies showed different $[^3\text{H}]\text{BP}$ localization in the rat's organs and tissues depending on the LP class (Table 2). It is worth mentioning that administration of $[^3\text{H}]\text{BP}$ complexed with chylomicrons under similar experimental condition resulted in a homogeneous distribution between rat organs and tissues (17). When a BP-VLDL complex was used, the $[^3\text{H}]\text{BP}$ uptake by the tissues was the greatest in liver and adrenals, followed by the kidney and the lung. Less radioactivity was found in the heart and the spleen. Earlier, we observed high uptake of $[^{125}\text{I}]\text{VLDLs}$ by liver and adrenals (18). High levels of BP were observed in adrenals when we used all classes of LPs, but especially when HDL was used. This result may be explained by the fact that cholesterol of HDL is used for steroid hormone biosynthesis in rat adrenals (19). It is probably for this reason that adrenals and testicles are the most

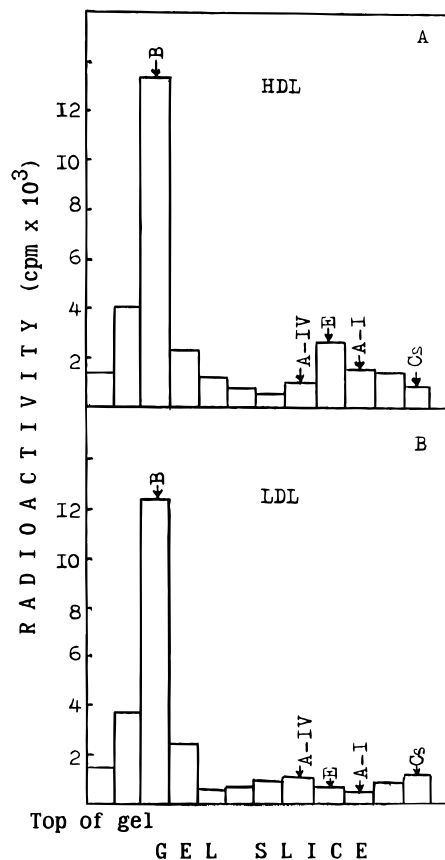


Figure 2. Distribution of $[^3\text{H}]\text{BP}$ among apolipoproteins of rat HDL (A) and LDL (B) after SDS-PAGE. LPs (100 μL) were incubated with $[^3\text{H}]\text{BP}$ (about 1.3×10^5 cpm) as described in Experimental Procedures, and 30 μL of apolipoproteins (about 50 μg total proteins) was separated by SDS-PAGE (12.5%). After electrophoresis, the gel was sliced into 4 mm sections, and the radioactivity in each slice was determined. The arrows indicate the localization of each apolipoprotein: B, apoB (M_r 340 kDa); A-IV, apoA-IV (M_r 42 kDa); E, apoE (M_r 33 kDa); A-I, apoA-I (M_r 26 kDa); Cs, apoC's, (M_r about 10–14 kDa).

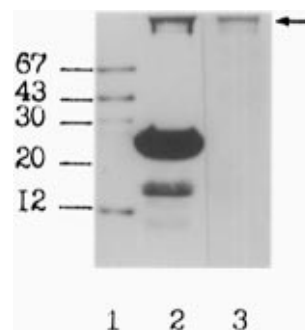


Figure 3. Demonstration of apoB in the rat HDL by immunoblotting. Lane 1 and 2, SDS-PAGE pattern of molecular weight standards and rat HDL, respectively. Lane 2 shows apolipoproteins of rat HDL containing apoB (M_r 340 kDa), apoA-I (M_r 26 kDa), and apoC's (M_r about 10–14 kDa). Lane 3 shows an immunoblot with antiserum to rat apoB after transferring proteins from gel 2 to nitrocellulose as described in the Experimental Procedures. The arrow indicates positive reactive corresponding to the apoB band.

sensitive, target for toxic action of some compounds, like chlordecon (20). The high level of radioactivity in the liver may explain the main role of this organ in both the synthesis and catabolism of all LP classes.

The high level of $[^3\text{H}]\text{BP}$ in the kidney, especially when HDL was used, is noteworthy. This is consistent with previous investigations that showed the importance of kidneys in the catabolism of apoA-I and apoE from HDLs

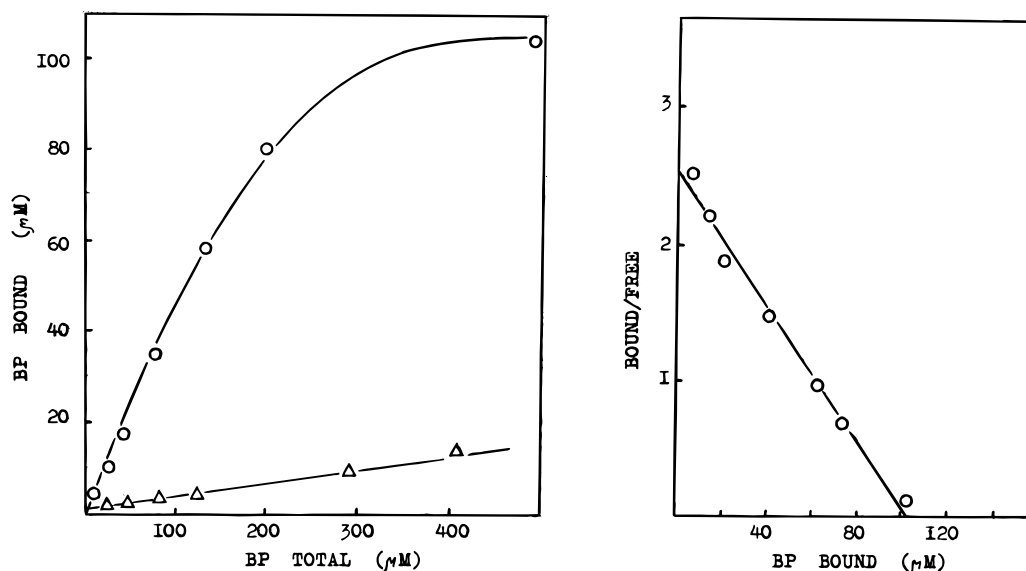


Figure 4. Saturation curve (left panel) and Scatchard plot (right panel) for binding [^3H]BP to rat LDL. Experiments were performed in the presence of the increasing amounts of unlabeled ligands as described under Experimental Procedures. Control incubations of cellulose (circles) treated with increasing amounts of unlabeled ligands with BSA showed no binding of BP (Δ). Each point represents the average of a duplicate determination. The equilibrium dissociation constants (K_d) was calculated assuming the average molecular weight of 3000 kDa for rat LDL.

(21). In the rat 39% of apoA-I is catabolized in the proximal tube of the kidney (22). On the whole, the BP radioactivity biodistribution correlates with tissue uptake of the LPs (18). The binding and distribution of BP between different protein components of rat LPs were studied by means of SDS-PAGE (Figure 2). The diagram shows the distribution of [^3H]BP among apolipoproteins of rat HDLs and LDLs (according to localization of every apolipoprotein after SDS-PAGE).

More than 80% of the radioactivity recovered from the gels was localized in the area of apoB. It is obvious that BP localization at the area of apoB may result from two main reasons: formation of stable apoB-BP complexes and covalent interaction. However, unmetabolized BP under the conditions of our experiment (see Experimental Procedures) does not show the covalent interactions with proteins (23). We can speculate only one reason to explain the localization of BP at the apoB area: that is the formation of apoB-BP complexes. The question may be raised concerning the stability of the apoB-BP complexes toward heating in SDS. The heating of LPs in SDS at 90 °C for 5 min actually results in removal of most of the lipids, but some part of lipids remains associated with the proteins. It is known that even treatment of LDLs with organic solvent (diethyl ether) resulted in just partial delipidation (24). Besides, Benvenaga and co-workers (4) have found that after SDS-PAGE radiolabeled thyroxine was still associated with the apoB-100 and its fragments (B-76 and B-21). In general, we can suppose with confidence that some part of BP remains associated with hydrophobic domains of the apoB and is sufficient for determination of radioactivity in the PAG.

The presence of apoB in the rat HDL was unexpected and was demonstrated by immunoblotting with specific antibodies to apoB (Figure 3). Our data have been confirmed by other investigators, although only with respect to the newly synthesized HDLs. ApoB-containing rat HDL particles represents one-third of the newly synthesized apoB-48 (25).

The BP binding capacity of native LDLs, HDLs, and apoB isolated from rat blood plasma was estimated quantitatively by the cellulose circles method (14). Fig-

Table 3. Equilibrium Dissociation Constants (K_d) for Complexes of BP with LDL, HDL, and Purified ApoB Obtained by the Method of Cellulose Circles (10)^a

complex	K_d , M
LDL-BP	$(1.5 \pm 0.37) \times 10^{-5}$
HDL-BP	$(4.1 \pm 1.04) \times 10^{-5}$
apoB-BP	$(2.5 \pm 0.62) \times 10^{-5}$

^a The following molecular weights and protein composition percentage were used for computation: rat LDL, M_r 3000 kDa, 20% protein; rat HDL, M_r 300 kDa, 40% protein; rat apoB, M_r 340 kDa. Each point is the average of two experiments. Control incubations of cellulose circles treated with increasing amounts of unlabeled ligands with BSA showed no binding of BP.

ure 4 shows a typical saturation curve for LDLs and a Scatchard plot analysis of binding data. Control incubations of cellulose circles treated with increasing amounts of unlabeled ligands with bovine serum albumin showed no binding of BP. Equilibrium dissociation constants for native LDLs, HDLs, and purified apoB are presented in Table 3. The fact that these values are similar support the fact that the main carrier protein for Bp may be apoB.

In summary, it seems apparent that at least one possible pathway of Bp transport into the cell is mediated by apoB-containing LPs through specific receptors for apoB. ApoB may also be involved in the binding and transport of other related compounds. Our results support, the carrier role of apolipoproteins in the blood plasma transport of BP and other hydrophobic carcinogens.

LITERATURE CITED

- (1) Blomhoff, R., Green, M. H., Berg, T., and Norum, K. R. (1990) Transport and storage of vitamin A. *Science* 250, 399–404.
- (2) Dueland, S., Pedersen, J. I., Helgerud, P., and Drevon, C. A. (1982) Transport of vitamin D₃ from rat intestine. *J. Biol. Chem.* 257, 146–150.
- (3) Panin, L. E., Polyakov, L. M., Rozumenko, A. A., and Biushkina, N. G. (1988) Transport of steroid hormones by means of blood serum lipoproteins. *Voprosy meditsinskoi khimii (Russia)* 34, 5, 56–58.
- (4) Benvenaga, S., Cahnmann, H. J., Gregg, R., and Robbins, J. (1989) Binding of thyroxine to human plasma low density

- lipoproteins though specific interaction with apoB-100. *Biochimie* 71, 263–268.
- (5) Pike, E., and Skuterud, B. (1981) The significance of lipoproteins for the serum binding of basic drugs. *Acta Pharmacol. Toxicol.* 49, 51–55.
- (6) Oliver, J., Chachaty, C., Wolf, C., Salmon, S., and Beresiat, G., (1988) Binding of spin-labelled clofibrate to lipoproteins. *Biochim. Biophys. Acta* 963, 515–524.
- (7) Shu, H. P., and Bymun, E. N. (1983) Systemic excretion of benzo(a)pyrene in the control and microsomally induced rat: the influence of plasma lipoproteins and albumin as carrier molecules. *Cancer Res.* 43, 485–490.
- (8) Shu, H. P., and Nicols, A. V. (1981) Uptake of lipophilic carcinogens by plasma lipoproteins. Structure-activity studies. *Biochim. Biophys. Acta* 665, 376–384.
- (9) Marinovitch, M., Sirtori, C. R., Galli, C. L., and Paoletti, R. (1983) The binding of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin to plasma lipoproteins may delay toxicity in experimental hyperlipidemia. *Chem. Biol. Interact.* 45, 393–399.
- (10) Benditt, E. P. (1977) The origin of atherosclerosis. *Sci. Am.* 236, 74–85.
- (11) Chen, T. C., Bradley, W. A., Gotto, A. M., and Morrisett, J. D. (1979) Binding of the chemical carcinogen, *p*-dimethylaminoazobenzene by human plasma low-density lipoproteins. *FEBS Lett.* 104, 236–240.
- (12) Hatch, F. T., and Less, R. S. (1968) Practical methods for plasma lipoprotein analysis. *Adv. Lipid Res.* 6, 2–68.
- (13) Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227, 680–685.
- (14) Polyakov, L. M., Chasovskikh, M. I., and Panin, L. E. (1991) The evaluation of benzo(a)pyrene binding with lipoproteins of the blood serum by cellulose disk method. *Ukr. Biochem. J.* 1, 101–104.
- (15) Tovey, E., and Badlo, A. (1987) Comparison of semi-dry and conventional tank-buffer electrotransfer of proteins from polyacrylamide gels. *Electrophoresis* 8, 384–387.
- (16) Chapman, M. J. (1980) Animal lipoproteins: chemistry, structure, and comparative aspects. *J. Lipid Res.* 21, 789–853.
- (17) Vauhkonen, M., Kuusi, T., and Kinnunen, P. (1980) Serum and tissue distribution of benzo(a)pyrene from intravenously injected chylomicrons in rat in vivo. *Cancer Lett.* 11, 113–119.
- (18) Polyakov, L. M. and Panin, L. E. (1985) Lipoprotein absorption by steroid producing organs in rats. *Probl. Endocrinol. (Russia)* 31, 72–75.
- (19) Gwynne, I. T., Mahaffe, D., Brever, H. B. I., and Ney, R. L. (1976) Adrenal cholesterol uptake from plasma lipoproteins: Regulation by corticotropin. *Proc. Natl. Acad. Sci. U.S.A.* 73, 4329–4333.
- (20) Soine, P. I., Blanke, R. V., Juzelian, P. S., and Schwartz, C. C. (1982) Preferential binding of chlordecone to the protein and high density lipoprotein fractions of plasma from humans and other species. *J. Toxicol. Environ. Health* 91, 109–118.
- (21) Van'T Hooft, F. M., and Van Tol, A. (1985) The sites of degradation of rat high-density-lipoprotein apolipoprotein E specifically labelled with O-(4-diaso-3-[125-I]iodobenzoyl)-sucrose. *Biochem. J.* 226, 715–721.
- (22) Glass, C. K., Pittman, R. C., Keller, G. A., and Steinberg, D. (1983) Tissue sites of apolipoprotein degradation in the rat. *J. Biol. Chem.* 258, 7161–7167.
- (23) Osborn, M. R., and Crosby, N. T. (1987) *Benzopyrenes*, 329, Cambridge University Press, Cambridge.
- (24) Krause, B. R., and Newton, R. S. (1985) Apolipoprotein changes associated with the plasma lipid-regulating activity of gemfibrozil in cholesterol-fed rats. *J. Lipid Res.* 26, 940–949.
- (25) Hussain, M. M., Zanni, E. E., Kelly, M., and Zannis, V. I. (1989) Synthesis, modification, and flotation properties of rat hepatocyte apolipoproteins. *Biochim. Biophys. Acta* 618, 347–358.

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Synthesis and Characterization of a New 5-Thiol-Protected Deoxyuridine Phosphoramidite for Site-Specific Modification of DNA[†]

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A new nucleotide analogue was developed for site-specific incorporation of a reactive thiol group into DNA. This creates a unique site for the post-synthetic modification of that nucleotide with a variety of molecular tags, such as photo-cross-linkers and fluorescent or spin-label moieties. 5'-O-(4,4'-Dimethoxytrityl)-5-[S-(2,4-dinitrophenyl)thio]-2'-deoxyuridine 3'-O-(2-cyanoethyl N,N'-diisopropylphosphoramidite) was synthesized and incorporated at internal positions in several oligonucleotides using automated DNA synthesis and standard phosphoramidite chemistry. The coupling yield of the analogue was comparable to the coupling yield for a standard phosphoramidite, and no significant differences were observed in the overall yields of the dinitrophenyl-labeled oligonucleotides compared to the corresponding unmodified oligonucleotides. Characterization of the dinitrophenyl-modified oligonucleotides included enzymatic degradation, HPLC chromatography, and gel electrophoresis. Deprotection of the mercaptan group with β -mercaptoethanol yielded an oligonucleotide containing 5-mercaptopdeoxyuridine which was then selectively modified, without purification, by reaction with 5-(iodoacetamido)fluorescein. Incorporation of the dinitrophenyl-modified oligonucleotide into double-stranded DNA was achieved using the polymerase chain reaction. Characterization of the dinitrophenyl-labeled product by immunodetection with anti-dinitrophenyl antibodies confirmed the stability of the protecting group to the thermocycling and thus established the use of this thiol-protected mercaptopdeoxyuridine phosphoramidite for preparation of site-specifically modified DNA.

INTRODUCTION

Virtually all aspects of gene expression, including DNA replication and repair, transcription, RNA processing, and translation, involve specific interactions between proteins and nucleic acids. Our understanding of the nature of these molecular interactions has increased vastly over the years due, in part, to the availability of modified nucleotides and nucleic acids to serve as molecular probes. In many cases, specific nucleotides or DNA sequences which are thought to interact directly with regulatory proteins have been identified by both genetic and biophysical methods. Frequently, these proteins have not been identified or purified, but exist in crude extracts. For the proteins which have been identified and isolated, details about the nature of their interactions with DNA, at the molecular level, are still rather limited.

Previous studies in this laboratory have utilized photo-cross-linking ribonucleoside triphosphates, 5-[(4-azidophenacyl)thio]uridine 5'-triphosphate (5-APAS-UTP) and 5-[(4-azidophenacyl)thio]cytidine 5'-triphosphate (5-APAS-CTP), to examine the RNA and protein interactions which occur during initiation, elongation, and termination of transcription in *Escherichia coli* (Dissinger and Hanna, 1990, 1991; Hanna, 1993; Hanna *et al.*, 1989, 1993; Liu and Hanna, 1995a,b; Zhang and Hanna, 1994).

These nucleoside triphosphate analogues were enzymatically incorporated at one or more positions within the nascent RNA by *E. coli* RNA polymerase. The photo-cross-linking agent in these molecules is attached at the 5 position of the mercaptopyrimidine through a thiol ether bond, and we have shown previously that modification of the pyrimidine at the 5 position does not interfere with the formation of Watson–Crick base pairs (Dissinger and Hanna, 1991).

A similar approach using photo-cross-linking studies can be used for evaluating the DNA–protein interactions which occur during transcription. To accomplish this goal, DNA which has been site-specifically modified to contain one or more photo-cross-linking groups is required. Site-specifically modified DNA can be prepared by both enzymatic and chemical methods. The enzymatic approach requires synthesis of the appropriately modified triphosphate which is then incorporated into DNA by DNA polymerase. This has been accomplished with a number of photo-cross-linking analogues, including 5-[3-[N-(p-azidobenzoyl)amino]allyl]deoxyuridine 5'-triphosphate (Bartholomew *et al.*, 1990), 4-thiodeoxythymidine 5'-triphosphate (Bartholomew *et al.*, 1994), and 5-azidodeoxyuridine 5'-triphosphate (Evans and Haley, 1987). A disadvantage to this method is that DNA polymerases have requirements with regard to substrate structure, and this can limit the types of modifications which can be present on deoxynucleoside triphosphate analogues. In addition, enzymatic incorporation requires multiple steps to achieve the single-site incorporation of the modified nucleotide. Often single-site incorporation cannot be accomplished because the method relies on DNA elongation in the presence of analogue and the absence of at least one of the four natural deoxynucleoside triphosphates. This method relies on limited elongation of the DNA to the position of the missing deoxynucleotide,

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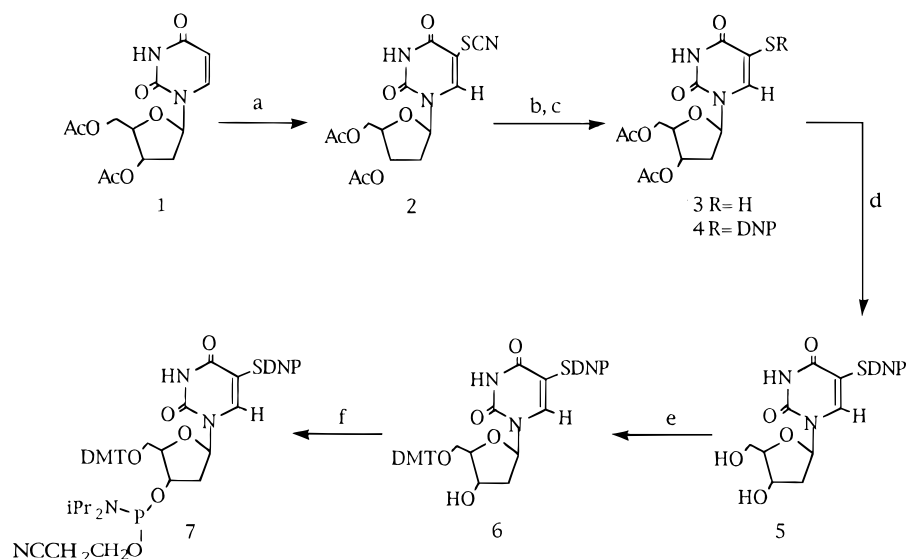


Figure 1. Synthetic pathway for the DNP-labeled phosphoramidite **7**. Reaction conditions are as follows: (a) SCNCl , $\text{CH}_3\text{CO}_2\text{H}$; (b) DTT, MeOH, EDTA; (c) 2,4-dinitrofluorobenzene, Et_3N , CH_3CN ; (d) NaOMe, MeOH; (e) $\text{DMT}^+\text{BF}_4^-$, DBMP, CH_3CN ; (f) 2-cyanoethyl *N,N,N',N'*-tetraisopropylphosphorodiamidite, tetrazole, CH_3CN .

after which analogue is removed before further elongation of the DNA (Bartholomew *et al.*, 1990). Even when HPLC-purified nucleotides are utilized in the nucleotide-deprivation step, spontaneous deamination can still lead to contamination of dCTP with dUTP and dATP with dITP. This can result in DNA polymerization beyond the desired position and incorporation of analogue at more than one position.

The chemical approach to preparation of site-specifically modified DNA involves incorporation of a modified nucleotide during the automated synthesis of an oligonucleotide. Unlike the enzymatic method, incorporation of a modified nucleotide via chemical synthesis is absolutely site-specific. The modified oligonucleotide can then be incorporated into double-stranded DNA by PCR¹ amplification or primer extension of a single-stranded DNA template. Chemical synthesis of oligonucleotides involves the polymerization of nucleoside phosphoramidites into an oligonucleotide. Because the conditions used in automated oligonucleotide synthesis are rather harsh, the direct incorporation of a photo-cross-linking azide group by this method is not possible, and a means for the postsynthetic incorporation of the azide group is required. The use of various modified phosphoramidites as masked synthons for preparing derivatized oligonucleotides at both terminal and internal sites has been reviewed (Beaucage and Iyer, 1993; Goodchild, 1990). These masked synthons allow incorporation of protected functional groups such as amines (Cruickshank and Stockwell, 1988; Levina *et al.*, 1993; Telser *et al.*, 1989), carboxylic acids (Wang and Bergstrom, 1993), thiols (Bradley and Hanna, 1992; Goodwin and Glick, 1993; Kuijpers and van Boeckel, 1993; Kumar, 1993b), and thiocarbonyls (Coleman and Kesicki, 1994; Waters and Connolly, 1992; Xu *et al.*, 1995). Once incorporated, the analogues are deprotected and modified postsyntheti-

cally. Convertible nucleoside phosphoramidites, monomers containing leaving groups (MacMillan and Verdine, 1991), have also been used to incorporate cross-linking (Webb and Matteucci, 1986; Zheng *et al.*, 1995) and photo-cross-linking groups (Zheng *et al.*, 1995) by postsynthetic substitution of the leaving group with a nucleophile such as NaN_3 . None of the analogues reported previously, however, has the versatility of the analogue described here, which contains a sulfur substitution directly on a non-base-pairing position of the base. We have developed this phosphoramidite analogue to serve as a masked synthon for placement of a 5-thiol-protected mercaptopyrimidine within an oligonucleotide. Once this modified nucleotide is incorporated into an oligonucleotide or nucleic acid, the protecting group can be removed to unmask the reactive thiol, making it available for further derivatization with thiol-specific agents.

Previously, we reported the synthesis and utilization of 5-(thiocyanato)deoxyuridine phosphoramidite for placement of the sulfur directly on the base (Bradley and Hanna, 1992). However, the synthesis of this compound varies in both yield and purity. We now describe the synthesis of 5'-O-(4,4'-dimethoxytrityl)-5-[S-(2,4-dinitrophenyl)thio]-2'-deoxyuridine 3'-O-(2-cyanoethyl *N,N'*-diisopropylphosphoramidite) (**7**, Figure 1), which is more stable than 5-(thiocyanato)deoxyuridine phosphoramidite. Although this analogue was initially designed for modification with photo-cross-linking agents, a variety of functional groups can be attached to the reactive thiol. This method has the advantage that an analogue-tagged oligonucleotide can be synthesized and stored for long periods without removal of the protecting group. When needed, an aliquot can then be deprotected and modified. We describe here a method for site-specific modification of DNA with this analogue, which provides a tool for identification and isolation of DNA binding proteins and detailed analysis of their interaction with specific nucleotides.

EXPERIMENTAL PROCEDURES

Materials. Unless otherwise stated, starting materials for the chemical synthesis of the phosphoramidite were obtained from Aldrich, Sigma, Fluka, or Fisher Scientific and were used without further purification. Anhydrous HOAc was prepared by fractional freezing

¹ Abbreviations: DNP, 2,4-dinitrophenyl; 5-IAF, 5-(iodoacetamido)fluorescein; CIP, calf intestinal alkaline phosphatase; TEAA, triethylammonium acetate; TEAB, triethylammonium bicarbonate; DMT, dimethoxytrityl; RP HPLC, reverse phase high-pressure liquid chromatography; PCR, polymerase chain reaction; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; DMTCl, dimethoxytrityl chloride; $\text{DMT}^+\text{BF}_4^-$, dimethoxytrityl tetrafluoroborate; BME, β -mercaptoethanol; HR FAB MS, high-resolution fast atom bombardment mass spectroscopy.

(Nagamachi *et al.*, 1974). Anhydrous CH_2Cl_2 was distilled from phosphorus pentoxide. Anhydrous CH_3CN was purchased from Cruachem. Dowex 50W x4 was purchased from Bio-Rad. Analytical thin-layer chromatography was performed on Whatman silica gel 60 plates with fluorescent indicator. Column chromatography was performed with silica gel 60A from American Scientific Products. Reagents for the oligonucleotide syntheses were obtained from Cruachem. Standard exocyclic amine protecting groups were used: benzoyl for dA and dC and isobutyryl for dG. Solvents utilized for the reverse phase chromatographic purification of the oligonucleotides were of HPLC grade. The thiol modifying agent, 5-IAF, was purchased from Molecular Probes (Eugene, Oregon).

Buffers. The buffers used were as follows: buffer A, 100 mM TEAA (pH 7.1); buffer B, 20 mM TEAA (pH 7.1); buffer C, 50 mM Tris-HCl (pH 4.9), 1 mM MgCl_2 , 0.1 mM ZnCl_2 ; buffer D, 50 mM Tris-HCl (pH 9.0), 1 mM MgCl_2 , 0.1 mM ZnCl_2 , 0.5 mM spermidine; buffer E, 7 M urea, 0.05% (w/v) xylene cyanol; buffer F, 5% (v/v) glycerol, 0.04% (w/v) xylene cyanol, 0.04% (w/v) bromophenol blue.

Enzymes and Antibodies. Nuclease P1 was obtained from Pharmacia Biotech. CIP was supplied by Promega. T4 Polynucleotide Kinase was purchased from New England Biolabs. AmpliTaq Polymerase was obtained from Perkin-Elmer. Rabbit polyclonal anti-DNP antibody was purchased from Sigma. Anti-rabbit Ig, horseradish peroxidase-linked whole antibody was obtained from Amersham.

Analytical Methods. Melting points were determined on a Mel-Temp melting point apparatus and are uncorrected. ^1H NMR and ^{31}P NMR spectra were recorded on Varian XL-300 and -500 spectrometers, respectively. Chemical shifts are reported in parts per million (δ) relative to internal tetramethylsilane or to external 85% phosphoric acid. UV spectra were measured on either a LKB Biochem Ultraspec II or a Beckman DU 7500 spectrophotometer. IR spectra were recorded with a Perkin-Elmer 710B infrared spectrometer. FAB MS were recorded on a VG analytical ZAB-E spectrometer. Unless otherwise stated, all chemical reactions were performed under a nitrogen atmosphere.

HPLC analyses were performed with a Beckman LC system which was equipped with a 126 solvent delivery module and a 168 diode array detector and was controlled with Beckman System Gold Software. A reverse phase C18 column (Beckman Ultrasphere ODS, 250×4.6 mm i.d.) and guard column (Upchurch, $1 \text{ cm} \times 4.3$ mm i.d.) were utilized with both analytical and semipreparative separations. Sample loops of 1 mL and $50 \mu\text{L}$ were used for semipreparative and analytical analyses, respectively. All HPLC analyses were performed using a gradient solvent system composed of triethylammonium buffers and acetonitrile at a flow rate of 1 mL/min. Unless otherwise stated, UV absorption was monitored at a wavelength of 260 nm.

3',5'-O-Diacetyl-5-(thiocyanato)-2'-deoxyuridine (2). Anhydrous HOAc (150 mL) was added to a three-neck round-bottom flask equipped with a vacuum adapter and Teflon needle. Dry Cl_2 was slowly bubbled through the Teflon needle into the reaction vessel for a brief period. A 5 mL aliquot of the Cl_2 /HOAc solution was removed and subjected to iodometric titration to determine the amount of Cl_2 added (2.9 g, 41 mmol) (Flaschka *et al.*, 1969). KSCN (4.4 g, 45 mmol), previously dried overnight at 100°C , was added to the reaction vessel. The reaction mixture was allowed to stir at room temperature for 30 min prior to the addition of 3',5'-O-diacetyl-2'-

deoxyuridine (**1**)² (1.1 g, 3.5 mmol). After the mixture was stirred for an additional 1.5 h, cyclohexene (10 mL) was added. The mixture was then stirred for 15 min and filtered; the filtrate was concentrated under vacuum. Residual HOAc was removed azeotropically with toluene, and the residue was triturated with petroleum ether. The petroleum ether was decanted and the residue subjected to column chromatographic purification [petroleum ether: EtOAc (2:1–1:3)] which yielded a semisolid. Recrystallization from EtOH:EtOAc provided **2** as a white solid (1.05 g; 82%); mp $154\text{--}156^\circ\text{C}$; IR (KBr) 2140 cm^{-1} (SCN stretch); ^1H NMR (CDCl_3) δ 9.12 (bs, 1H, N_3H), 8.18 (s, 1H, H_6), 6.14 (dd, $J = 7.8, 5.7 \text{ Hz}$, 1H, H_1), 5.13 (m, 1H, H_3), 4.24 (m, 1H, H_5), 4.19–4.15 (m, 2H, H_4' and H_5'), 2.32 (ddd, $J = 14.4, 5.7, 2.1 \text{ Hz}$, 1H, H_2), 2.13–2.07 (m, 7H, 2 CH_3 and H_2); UV (MeOH) 270 nm.

3',5'-O-Diacetyl-5-mercapto-2'-deoxyuridine (3). *Caution: reaction should be performed in fume hood.* EDTA (25 mL; 0.1 M, pH 7.7) and DTT (293 mg, 1.90 mmol) were added to a solution of thiocyanate **2** (207 mg, 0.561 mmol) in MeOH (30 mL). The reaction mixture was allowed to stir at room temperature for 35 min and was then filtered. The filtrate was reduced to half by rotary evaporation, made acidic with 10% (v/v) H_2SO_4 , and extracted with CH_2Cl_2 . The CH_2Cl_2 extract was concentrated under reduced pressure, and the residue was triturated with H_2O to provide **3** as a white solid (148 mg; 77%). TLC analysis showed a yellow spot upon development with 2,2'-dithiobis(5-nitropyridine) (Grasetti and Murray, 1969): mp³ = $159\text{--}163^\circ\text{C}$; IR (KBr) 2530 cm^{-1} (SH stretch); ^1H NMR (CDCl_3) δ 8.70 (bs, 1H, N_3H), 7.67 (s, 1H, H_6), 6.30 (dd, $J = 8.2, 5.8 \text{ Hz}$, 1H, H_1), 5.22 (m, 1H, H_3), 4.43–4.25 (m, 3H, H_4' and H_5'), 3.60 (s, 1H, SH), 2.50 (ddd, $J = 14.4, 5.8, 2.2 \text{ Hz}$, 1H, H_2), 2.21–2.10 (m, 7H, 2 CH_3 and H_2); UV (0.1 M EDTA w/ DTT, pH 7.76) 260, 334 nm.

3',5'-O-Diacetyl-5-[S-(2,4-dinitrophenyl)thio]-2'-deoxyuridine (4). 2,4-Dinitrofluorobenzene (57 mg, 0.31 mmol) and Et_3N (200 μL) were added to a solution of mercaptan **3** (86 mg, 0.25 mmol) in anhydrous CH_3CN (10 mL). The reaction mixture was allowed to stir at room temperature for 1.5 h before the solvent was removed by rotary evaporation. The crude material was purified using column chromatography [hexanes:EtOAc (4:1–1:1)]. The product was obtained as a yellow solid (113 mg, 89%); IR (KBr) $1530, 1340 \text{ cm}^{-1}$ (NO_2 stretch); ^1H NMR (CDCl_3) δ 9.14 (d, $J = 2.4 \text{ Hz}$, 1H, H_3 of DNP), 8.40 (bs, 1H, N_3H), 8.31 (dd, $J = 9.1, 2.5 \text{ Hz}$, 1H, H_5 of DNP), 8.23 (s, 1H, H_6), 7.33 (d, $J = 9 \text{ Hz}$, 1H, H_6 of DNP), 6.32 (dd, $J = 7.8, 5.7 \text{ Hz}$, 1H, H_1), 5.25 (m, 1H, H_3), 4.44–4.25 (m, 3H, H_4' and H_5'), 2.55 (m, 1H, H_2), 2.27 (m, 1H, H_2), 2.13 (s, 3H, CH_3), 2.10 (s, 3H, CH_3); UV (MeOH) 264, 323 nm.

5-[S-(2,4-Dinitrophenyl)thio]-2'-deoxyuridine (5). NaOMe (25% solution in MeOH; 82 μL ; 0.36 mmol) was added to a solution of diacetate **4** (92 mg, 0.18 mmol) in anhydrous MeOH (6 mL), and the reaction mixture was maintained at room temperature. After 2 h, the mixture was treated with Dowex 50W x4 and filtered. The filtrate was reduced by rotary evaporation. The product was obtained as a yellow solid following column chromato-

² 3',5'-O-Diacetyl-2'-deoxyuridine was prepared by reaction of deoxyuridine with acetic anhydride in pyridine for an 82% yield: mp = $106\text{--}108^\circ\text{C}$ (Lin and Gao, 1983).

³ The broad melting point range may indicate the presence of 5,5''-dithiobis(3',5'-diacetyldeoxyuridine). On the average, 7% disulfide analogue was present in the sample as determined by UV absorbance (λ_{max} 335 nm) in the presence and absence of DTT (Bardos and Kalman, 1966).

graphic purification of the reaction mixture [EtOAc:petroleum ether (1:4–1:0)] (62 mg; 81%): IR (KBr) 1530, 1340 cm^{-1} (NO_2 stretch); ^1H NMR (CD_3OD) δ 9.08 (d, J = 2.4 Hz, 1H, H_3 of DNP), 8.85 (s, 1H, H_6), 8.39 (dd, J = 9, 2.4 Hz, 1H, H_5 of DNP), 7.64 (d, J = 9 Hz, 1H, H_6 of DNP), 6.33 (t, J = 6.3 Hz, 1H, $\text{H}_{1'}$), 4.45 (m, 1H, H_3), 4.00 (m, 1H, H_4), 3.84 (dd, J = 12, 3 Hz, 1H, H_5), 3.75 (dd, J = 12, 3 Hz, 1H, H_5), 2.50–2.33 (m, 2H, H_2); UV (0.1 M TEAA, pH 7.1 with 57% CH_3CN) 262, 334 nm.

5'-O-(Dimethoxytrityl)-5-[S-(2,4-dinitrophenyl)-thio]-2'-deoxyuridine (6). Anhydrous CH_3CN (6 mL) was added to a mixture of compound **5** (42 mg, 0.10 mmol), $\text{DMT}^+\text{BF}_4^-$ (57 mg, 0.14 mmol),⁴ and 2,6-di-*tert*-butyl-4-methylpyridine (DBMP) (35 mg, 0.18 mmol). The resulting red solution was heated to reflux. After 1.5 h, a second portion of $\text{DMT}^+\text{BF}_4^-$ (26 mg, 0.064 mmol) and DBMP (13 mg, 0.067 mmol) was added and the reaction mixture was maintained at reflux for an additional 8 h. The CH_3CN was removed by rotary evaporation. CH_2Cl_2 and 5% aqueous NaHCO_3 were added to the residue. The mixture was stirred briefly, and the resulting two layers were separated. The NaHCO_3 layer was re-extracted twice with additional CH_2Cl_2 . The organic layers were combined, dried (Na_2SO_4), and reduced. The product was purified using column chromatography [CH_2Cl_2 :MeOH:pyridine (99.5:0.5–98.5:2:0.5)] (27 mg; 37%): ^1H NMR (CDCl_3) δ 9.02 (d, J = 2.1 Hz, 1H, H_3 of DNP), 8.46 (s, 1H, H_6), 8.07 (dd, J = 9, 2.4 Hz, 1H, H_5 of DNP), 7.35–7.09 (m, 10H, H_6 of DNP and Ar), 6.75–6.64 (m, 4H, Ar), 6.41 (dd, J = 9, 2.4 Hz, 1H, $\text{H}_{1'}$), 4.70 (m, 1H, H_3), 4.16 (m, 1H, H_4), 3.73 (2s, 6H, 2 CH_3), 3.38 (d, J = 7.5 Hz, 2H, H_5), 2.64 (m, 1H, H_2), 2.44 (m, 1H, H_2); UV (0.1 M TEAA, pH 7.1 with 82% CH_3CN) 264, 324 nm; HR FAB MS calcd for $\text{C}_{36}\text{H}_{32}\text{N}_4\text{O}_{11}\text{S}$ (M)⁺ 728.1788, found 728.1802 (1.9 ppm).

5'-O-(4,4'-Dimethoxytrityl)-5-[S-(2,4-dinitrophenyl)-thio]-2'-deoxyuridine 3'-O-(2-cyanoethyl *N,N'*-diisopropylphosphoramidite) (7). Compound **6** (13 mg, 18 μmol) was dried under vacuum in the reaction vessel. Anhydrous CH_3CN (0.3 mL) was added, and the reaction vessel was transferred to an atmosphere bag filled with dry N_2 . A mixture of 2-cyanoethyl *N,N,N',N'*-tetraisopropylphosphorodiamidite (5.5 μL , 17 μmol) and tetrazole (1.3 mg, 18 μmol) in anhydrous CH_3CN (50 μL) was added. The reaction mixture was maintained at room temperature for 30 min. An additional mixture of 2-cyanoethyl *N,N,N',N'*-tetraisopropylphosphorodiamidite (2.3 μL , 7.2 μmol) and tetrazole (0.6 mg, 8.3 μmol) in anhydrous CH_3CN (25 μL) was added, and the reaction mixture was stirred for 30 min. CH_2Cl_2 (20 mL) and 5% (w/v) aqueous NaHCO_3 (15 mL) were added to the mixture; the organic layer was removed and re-extracted with water, dried (Na_2SO_4), and evaporated. The residue was subjected to column chromatographic purification [CH_2Cl_2 :MeOH:pyridine (99.5:0.5–98.5:1.0:0.5)] to obtain the phosphoramidite **7** (8.6 mg, 52%). The yellow product was analyzed by analytical RP HPLC using a gradient of buffer A and acetonitrile [70% (v/v) CH_3CN to 80% in 3 min; 80% CH_3CN to 100% in 15 min]. UV absorption was monitored at wavelengths of 254 and 330 nm. The diastereomers eluted at 11.09 and 12.49 min. UV scans of the diastereomers showed absorption peaks at 264 and 327 nm. Phosphorus NMR indicated the presence of two diastereomers [^{31}P NMR (CDCl_3) δ 149.12, 149.27 ppm].

Synthesis and Purification of Oligonucleotides. Oligonucleotides were synthesized on an Applied Biosystems 392 DNA Synthesizer using the standard β -cyano-

Table 1. Sequence and Characterization Data for the Synthetic Oligonucleotides

No.	Sequence ^a	ASWY (%) ^b	Overall Yield (%) ^c	RT (min) ^d
A	GTA TGT A	97.5	62.1	9.8
B	GTA T*GT A	98.1	50.1	11.6
C	GAC TCA ACG ATG GGT TAA TTC G	98.0	40.2	9.6
D	GAC TCA ACG AT*G GGT TAA TTC G	97.9	41.0	10.0
E	GGG TAA ATT TGA CTC AAC GAT GGG	97.6	21.9	9.7
F	GGG T*AA ATT TGA CTC AAC GAT GGG	97.2	28.1	10.5

^a T* denotes the position of the thiol-modified nucleoside.

^b ASWY = average stepwise yield for nucleotide addition. ^c Overall yield = A_{260} of purified oligonucleotide/ A_{260} of crude oligonucleotide $\times$ 100%. ^d Retention time of purified oligonucleotide during RP HPLC analysis.

ethyl-protected phosphoramidite method. A series of three modified oligonucleotides were prepared on a 40 nmol scale using a Trityl On method. The thiol-protected analogue **7** was manually dissolved into anhydrous acetonitrile at a concentration of 0.027 M, and the solution was attached to the spare port on the DNA synthesizer. As the concentration of **7** was lower than the concentration of the commercial phosphoramidites used in these syntheses (0.05 M), the coupling time for the modified analogue was increased by 120 s. Syntheses of the corresponding unmodified oligonucleotides were also performed. The sequences of the oligonucleotides are shown in Table 1.

The oligonucleotides were removed from the column by treatment with concentrated NH_4OH (1 mL). Deprotection of the exocyclic amines was then accomplished by treatment of the oligonucleotides with an additional 1 mL of fresh concentrated NH_4OH at room temperature for 44 h. The NH_4OH solution was concentrated using a Savant Speed Vac without heat. Small portions of Et_3N were periodically added to the concentrating solution to maintain a basic pH. The crude oligonucleotides were redissolved in 10 mM TEAB (pH 7.1) and purified as the DMT ethers using semipreparative RP HPLC. Two different gradients of buffer B and acetonitrile were utilized. The shorter oligonucleotides (7 and 22 nucleotides) were purified with an elution gradient of CH_3CN from 5% (v/v) to 30% in 15 min then 30% to 100% in 5 min. The 24-mer oligonucleotides were purified with an elution gradient of CH_3CN from 5% to 30% in 18 min then 30% to 100% in 5 min. The appropriate fractions were evaporated to dryness using a Jouan concentrator without heat. Following purification of the oligonucleotides, the DMT group was removed by treatment with 80% (v/v) HOAc (200 μL) at room temperature for 20 min. The oligonucleotides were ethanol precipitated and redissolved into 300 μL of Nanopure water and stored at -20°C . The purity of the oligonucleotides was analyzed by analytical RP HPLC (Figure 2A, oligonucleotides A and B shown). Elution of the oligonucleotides was performed using a linear gradient of CH_3CN (5 to 20% in 15 min) with buffer B. The purified oligonucleotides were also analyzed by 25% polyacrylamide–7 M urea gel (acrylamide/methylenebis(acrylamide) = 19/1) (Figure 3A, oligonucleotides C–F shown).

Enzymatic Digestion with Nuclease P1 and CIP.

Oligonucleotides A and B were subjected to enzymatic digestion. The pure oligonucleotides were incubated with Nuclease P1 (2 U) at 37°C for 6 h in a total volume of 50 μL of buffer C. Five microliters of 10X buffer D and 43 μL of H_2O were added to the reaction. CIP (2 U) was added, and the pH of the reaction was adjusted with 5% (v/v) NaOH (0.8 μL). The reaction mixture was incubated overnight at 37°C . To recover the nucleosides, 11 μL of 3 M NaOAc (pH 5.3) and 250 μL of absolute EtOH were

⁴ Synthesized by the method of Bleasdale *et al.* (1990).

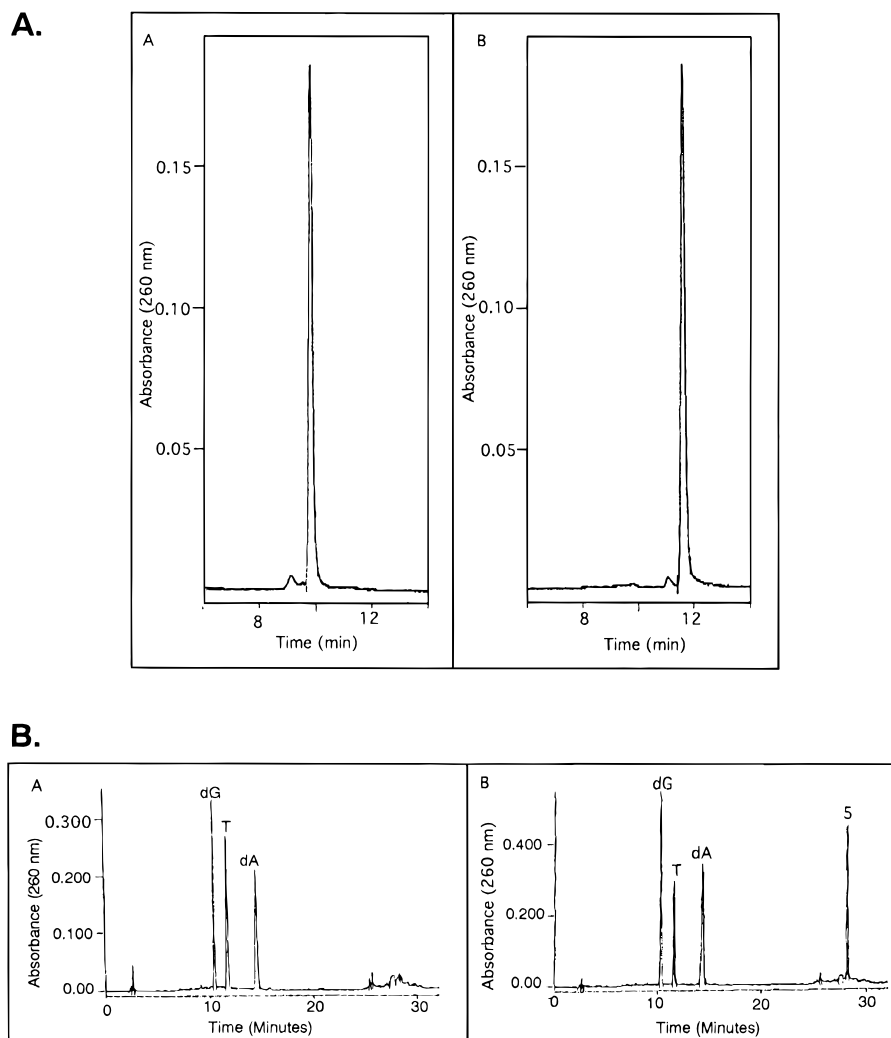


Figure 2. (A, top) RP HPLC chromatograms of oligonucleotides A and B. The purified 7-mers were analyzed by analytical HPLC on an Ultrasphere ODS column eluted with a 15 min linear gradient from 5% to 20% (v/v) acetonitrile in 20 mM TEAA (pH 7.1). (A) Oligonucleotide A, GTA TGT A, eluted at 9.8 min. (B) DNP-labeled oligonucleotide B, GTA T*GT A, eluted at 11.6 min. (B, bottom) RP HPLC profile of enzymatic degradation of oligonucleotides A and B. The 7-mers were subjected to enzymatic treatment with Nuclease P1 and CIP. The reaction mixture was analyzed by analytical HPLC on an Ultrasphere ODS column eluted with a linear gradient of acetonitrile in 100 mM TEAA (pH 7.1) from 0% to 5% (v/v) in 5 min, 5% to 6% (v/v) in 15 min, 6% to 50% (v/v) in 5 min, and finally 50% to 80% (v/v) in 15 min. (A) Oligonucleotide A reaction. (B) DNP-labeled oligonucleotide B. 5 represents [(dinitrophenyl)-thio]deoxyuridine.

added to the reaction mixture. The mixture was stored at -80°C for 4 h and then centrifuged at 14 000 rpm for 20 min. The supernatant fraction was removed and concentrated. The resulting residue was redissolved into 35 μL of buffer A and analyzed by analytical RP HPLC (Figure 2B). The elution gradient consisted of buffer A with an increasing concentration of CH_3CN (0% to 5% in 5 min, 5% to 6% in 15 min, 6% to 50% in 5 min, and finally 50% to 80% in 15 min). The UV absorption was monitored at 260 and 330 nm. Commercial nucleosides and monophosphates of dA, dC, dG, and T were utilized as standards. Synthetic 5-[S-(2,4-dinitrophenyl)thio] deoxyuridine (5) was also utilized as a standard. An enzymatic digestion without oligonucleotide was performed to identify background peaks arising from the buffer and enzymes.

Deprotection and Alkylation of a DNP-Modified Oligonucleotide. Oligonucleotide F (100 pmol) was radiolabeled by reaction with T4 Polynucleotide Kinase using $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. The labeled oligonucleotide F was isolated by ethanol precipitation and redissolved into 30 μL of H_2O . The oligonucleotide (4.5 μL) was treated with 1.4 M BME either at 45°C for 4 h or at room temperature for 12 h in a 20 μL solution of 20 mM Tris-HCl (pH 8.2).

Following this deprotection to remove the DNP group, one half of the reaction mixture was precipitated with ethanol while the other half of the mixture was subjected to alkylation conditions with 5-IAF. The corresponding unmodified oligonucleotide E was treated identically to serve as a control.

To recover the deprotected oligonucleotides by ethanol precipitation, 0.36 M NaOAc [pH 5.6 (100 μL)], 1 M MgCl_2 (1.2 μL), and Nanopure water (10 μL) were added to the reaction mixture (10 μL) to provide a final volume of 121 μL . Absolute ethanol (300 μL) was added, and the mixture was cooled at -80°C . The oligonucleotides were pelleted by centrifuging at 14 000 rpm for 20 min. The supernatant was removed, and the pellet was washed with cold 95% (v/v) ethanol. The mixture was chilled for 20 min in a dry ice/ethanol bath and spun at 14 000 rpm for 20 min. Following the removal of the supernatant, the recovered oligonucleotides were dissolved into 20 μL of Nanopure water. Mixtures of oligonucleotides (4.5 μL), 100 mM DTT (0.5 μL), and buffer E (5 μL) were prepared and analyzed by electrophoresis on a 25% polyacrylamide-7 M urea gel (acrylamide/methylenebis(acrylamide) = 19/1) (Figure 3B).

Alkylation of the deprotected thiol moiety was ac-

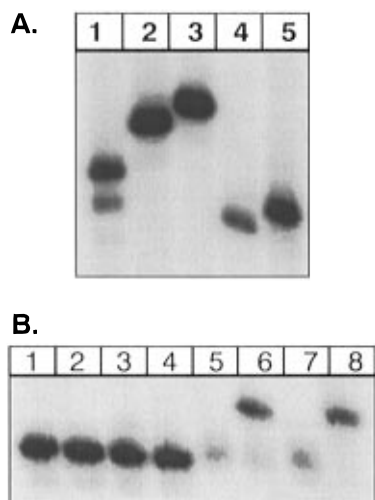


Figure 3. (A) Gel electrophoretic analysis of DNP-labeled oligonucleotides. The [^{32} P]-labeled oligonucleotides C–F were analyzed by electrophoresis on a 25% polyacrylamide–7 M urea gel (acrylamide/methylenebis(acrylamide) = 19:1). Lane 1: commercial 24-mer marker, CGC GGA ATT CTC ATG CAT TGC CCA. Lane 2: 24-mer, oligonucleotide E. Lane 3: DNP-labeled 24-mer, oligonucleotide F. Lane 4: 22-mer, oligonucleotide C. Lane 5: DNP-labeled 22-mer, oligonucleotide D. (B) Gel electrophoretic analysis of the deprotection and alkylation reactions. Oligonucleotides E and F were reacted with 1.4 M BME. Half of these reactions were alkylated with 5-IAF. The treated oligonucleotides were analyzed by electrophoresis on a 25% polyacrylamide–7 M urea gel (acrylamide/methylenebis(acrylamide) = 19:1). Lanes 1: oligonucleotide E + 1.4 M BME at room temperature. Lane 2: lane 1 reaction + 5-IAF. Lane 3: oligonucleotide E + 1.4 M BME at 45 °C. Lane 4: lane 3 reaction + 5-IAF. Lane 5: oligonucleotide F + 1.4 M BME at room temperature. Lane 6: lane 5 reaction + 5-IAF. Lane 7: oligonucleotide F + 1.4 M BME at 45 °C. Lane 8: lane 7 reaction + 5-IAF.

complished by the addition of 15 μ L of 20 mM Tris-HCl (pH 4.8) to 10 μ L of the deprotection reaction (pH 8.2). Following this addition, the pH of the alkylation reaction was 7 (as judged by pH paper). One microliter of 10 mM 5-IAF in DMF was added, and the reaction mixture was maintained at room temperature overnight. The oligonucleotides were recovered by ethanol precipitation and redissolved into 20 μ L of Nanopure water. The alkylated oligonucleotides (4.5 μ L) were mixed with 100 mM DTT (0.5 μ L) and buffer E (5 μ L) and analyzed by electrophoresis on a 25% polyacrylamide–7 M urea gel (acrylamide/methylenebis(acrylamide) = 19/1) (Figure 3B).

DNA Synthesis via PCR using a DNP-Modified Oligonucleotide. PCR reactions were performed on a Perkin-Elmer Cetus Gene Amp PCR System 9600. The DNA fragment containing the bacteriophage λ PR' promoter, 6S gene, and tR' terminator, with the λ *qut* site (Yang *et al.*, 1987), was amplified from plasmid pHA100 (Zhang and Hanna, 1995) using either oligonucleotide E or F and the universal T7 primer (Stratagene). The DNA product was purified by excision of the band from an agarose gel and extraction of the DNA using the commercially available GENE CLEAN kit (BIO 101 Inc., La Jolla, CA). The PCR products were dissolved into equal volumes of buffer F and analyzed by electrophoresis on a 1.5% agarose gel. Detection of DNA was achieved by addition of ethidium bromide to the electrophoresis buffer (Figure 4A).

Immunodetection of DNP-Labeled DNA. Dot blot experiments were performed manually. The DNP-labeled PCR product and the corresponding unlabeled DNA fragment (1 pmol each) were in 10X SSC solution (1.5 M NaCl, 0.15 M $\text{Na}_3\text{Citrate}$, pH 7). Samples (13 μ L) were applied to a pre-wetted charged nylon membrane

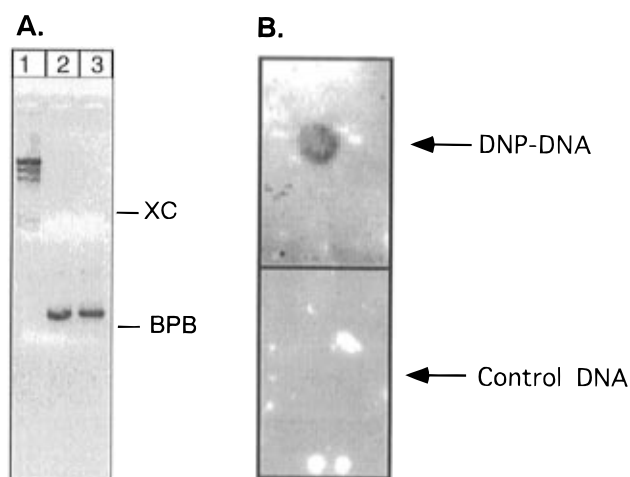


Figure 4. Analysis of PCR products. (A) PCR products were analyzed by electrophoresis on a 1.5% agarose gel. DNA was detected with ethidium bromide. Lane 1: λ Hind III markers. Lane 2: PCR product using oligonucleotide E as a primer. Lane 3: PCR product using DNP-labeled oligonucleotide F as a primer. XC represents xylene cyanol and BPB represents bromophenol blue. (B) Dot Blot immunodetection of DNP-labeled DNA. PCR products were attached to a nylon membrane and incubated with a primary antibody to DNP and a secondary antibody linked to horseradish peroxidase. Chemiluminescence was used for detection. Upper panel: PCR product using DNP-labeled oligonucleotide F as a primer. Lower panel: PCR product using oligonucleotide E as a primer.

(Zeta Probe GT; Bio-Rad). The membranes were air-dried, rinsed briefly with 2X SSC, and heated at 80 °C under vacuum for 30 min. The membranes were blocked by overnight incubation at 4 °C in TBS-T (20 mM Tris HCl, 500 mM NaCl, 0.4% (v/v) Tween 20, pH 7.6) containing 10% (w/v) dried milk. The membranes were then washed with TBS-T two times briefly, one time for 15 min, and two times for 5 min. The membranes were incubated with a 1:1500 dilution of anti-DNP antibodies for 1 h, washed as described above, and then incubated with a 1:10 000 dilution of horseradish peroxidase-linked whole anti-rabbit Ig for 1 h. Membranes were then washed one time for 15 min and four times for 5 min with TBS-T. Detection of the antibodies was carried out using the ELC chemiluminescence method (Amersham). The membranes were incubated with the detection agents for 1 min before exposure to X-ray film for 2 min (Figure 4B).

RESULTS

The synthesis and characterization of a novel deoxyuridine phosphoramidite **7** containing a protected thiol group at the 5 position of the mercaptopyrimidine ring is described (Figure 1). This DNP-labeled analogue was site-specifically incorporated internally into a series of oligonucleotides. Significant differences in overall synthetic yields were not observed between oligonucleotides made with normal deoxynucleoside phosphoramidites and those which contained the analogue, indicating that the modified phosphoramidite is a suitable reagent for automated oligonucleotide synthesis. Enzymatic digestion of the DNP-labeled oligonucleotide established the stability of the modified pyrimidine to the conditions for chemical synthesis and purification of oligonucleotides (Figure 2B). Selective deprotection of the 5-thiol moiety with BME and subsequent modification of the unmasked thiol with 5-IAF were verified by gel electrophoresis of the oligonucleotides (Figure 3B). Incorporation of the DNP-labeled oligonucleotide into double-stranded DNA was achieved using PCR. Isolation and characterization

of the PCR products included gel electrophoresis and immunodetection with anti-DNP antibodies (Figure 4A and B).

Synthesis. Synthesis of the deoxyuridine phosphoramidite analogue **7** was carried out following the sequence of reactions outlined in Figure 1. Compounds **2–7** have not been previously described in the literature. The method chosen for incorporation of the thiol group required thiocyanation of the pyrimidine ring. Initially, this reaction was performed on deoxyuridine, and the reaction conditions utilized were similar to those previously described (Bradley and Hanna, 1992; Nagamachi *et al.*, 1974; Torrence *et al.*, 1968). Although literature reports indicate yields of 54% for the thiocyanation of deoxyuridine, this was difficult to achieve. The yield for this reaction varied considerably and was at times as low as 9%. A major side product in this reaction was isolated and identified by NMR and IR as 5'-O-acetyl-5-(thiocyanato)deoxyuridine, indicating that, along with thiocyanation, acetylation of the sugar ring had occurred.

To circumvent the problems associated with the reaction described above, 3',5'-O-diacetyl-2'-deoxyuridine (**1**) was utilized as the starting material.² Protection of the sugar hydroxyls by acetylation prior to thiocyanation results in higher yields (95%) for the reaction (Nagamachi *et al.*, 1974). However, removal of the acetyl protecting groups could not be accomplished using standard basic conditions required for de-esterification without destruction of the thiocyanato group. Therefore, a synthetic approach was developed which required reduction of the thiocyanato compound to the mercapto analogue prior to removal of the acetyl groups (Figure 1). Thiocyanation of the 3',5'-O-diacetyl-2'-deoxyuridine (**1**) was achieved by following the literature procedure described above except for one slight variation. The crude material was extracted with petroleum ether to remove nonpolar impurities prior to column chromatographic purification. The pure product was obtained in 82% yield as a white solid.

Reduction of the thiocyanato derivative **2** to the mercapto analogue **3** was achieved by reaction with DTT in a solvent mixture of MeOH and 0.1 M EDTA (pH = 7.8). This reaction is a variation of a reported procedure (Lin *et al.*, 1988) for the reduction of 3'-azido-2',3'-dideoxy-5-(thiocyanato)uridine; this mercapto analogue however was not isolated. Purification of **3** was achieved by acidification of the reaction mixture followed by concentration of the mixture. The residue was extracted with CH₂Cl₂ and H₂O. The presence of the acetyl groups ensured that the product was organic soluble. To remove residual DTT, the organic layer was reduced and the residue triturated with water to crystallize the product as well as to dissolve residual DTT. Mercaptan **3** was obtained as a white solid in 75% yield.

The mechanism for the reduction of the thiocyanato requires displacement of the cyano group by DTT. Since no attempt was made to remove the cyanide ion before acidification of this reaction mixture, every step of the isolation of **3** from acidification to trituration with H₂O was performed in a fume hood. This synthesis of **3** was designed for the preparation of both 5-(alkyldithio)- and 5-(alkylthio)- or 5-(arylthio)pyrimidine analogues. To prevent degradation of the 5-alkyldithio compounds by DTT, purification of intermediate **3** was necessary. This is not the case with formation of 5-(alkylthio)pyrimidines (Lin *et al.*, 1988) and in recent syntheses of **4**, this purification step has been eliminated, resulting in a slight decrease in yield.

The DNP protecting group has been employed in oligonucleotide synthesis as a protecting group for 6-mer-

captopurine and 6-thioguanine phosphoramidites (Xu *et al.*, 1992a,b) and as a functional moiety on a nonnucleoside phosphoramidite (Grzybowski *et al.*, 1993). These studies indicated that the DNP group was stable during automated DNA chemical synthesis. Conversion of mercaptan **3** into the 2,4-DNP analogue **4** was achieved by reaction with 2,4-dinitrofluorobenzene in anhydrous CH₃CN with Et₃N present as a catalyst (Xu *et al.*, 1992b). The product was isolated as a yellow solid in 88% yield. Attachment of the thiol protecting group prior to removal of the acetyl moieties ensured that the product was soluble in organic solvents, which aided in the purification of the material. More importantly, protection of the sulfur inhibits the oxidation of the analogue to the disulfide compound, a potential reaction of 5-mercaptopyrimidines (Kalman and Bardos, 1967).

Another method for preparing 5-arenethiol ethers of deoxyuridine has been previously described. Bergstrom *et al.* have prepared a series of (alkylthio)- and (arylthio)-deoxyuridine analogues using a palladium-mediated reaction between 5-(chloromercurio)-2'-deoxyuridine and the appropriate disulfide (Bergstrom *et al.*, 1991). Although this method offers a simple route to arenethiol ether analogues, it was not selected for study. Development of a more versatile intermediate such as **3** which can be easily converted into various types of analogues including disulfides was desired for future studies. Additionally, the synthetic utility of the method has not been fully evaluated and therefore formation of the desired dinitrophenyl analogue **5** was uncertain.

De-esterification of nucleosides is commonly performed under basic conditions using NH₃ or NaOMe (Kini *et al.*, 1989; Trivedi and Bruns, 1989). Literature reports suggest that the DNP group is marginally stable to nucleophiles such as NaOMe, the stability of this group being strongly dependent on the reaction conditions (Greene, 1981). Successful removal of the acetyl groups was achieved by reaction of the diacetyl analogue **4** with NaOMe in MeOH without an apparent loss or modification of the DNP group as judged by NMR and IR. Following the deacetylation, the diol **5** was isolated as a yellow solid in 81% yield. An initial attempt to deacetylate **4** under mild reductive methods was performed (Brown *et al.*, 1982; Soai and Ookawa, 1986). However, reaction with LiBH₄ in a mixture of Et₂O and MeOH did not result in the formation of **5**, possibly due to the poor solubility of the starting material.

Incorporation of the DMT protecting group at the 5' position of nucleosides is typically accomplished by reaction with DMTCl in the presence of pyridine or dimethylpyridine (Chaudhary and Hernandez, 1979). However, as utilization of these conditions with other 5-thiol-modified nucleosides was not successful (unpublished results), this reaction was not attempted for the synthesis of **6**. Conversion to the 5'-dimethoxytrityl analogue **6** was achieved by reaction of **5** with DMT⁺BF₄⁻ in the presence of DBMP in CH₃CN at reflux. This reagent, DMT⁺BF₄⁻, has only recently been utilized in the preparation of 5'-protected nucleosides (Bleasdale *et al.*, 1990; Lakshman *et al.*, 1992).

The proposed mechanism for dimethoxytritylation with DMTCl and pyridine requires formation of a (dimethoxytrityl)pyridinium salt which then undergoes reaction with the 5' hydroxyl group (Chaudhary and Hernandez, 1979). Formation of the pyridinium salt is considered the limiting step. The reagent DMT⁺BF₄⁻ serves as a source for the dimethoxytrityl cation. By preforming the cation prior to reaction, the slow step in the dimethoxytritylation has been eliminated and the ether should be more easily formed. Although **6** was obtained using this

reagent, the reaction was performed under reflux conditions. It is unclear why the reaction requires such stringent conditions, but presumably steric hindrance plays some part. Modification of other bases has been reported to inhibit dimethoxytritylation possibly due to steric hindrance (Cosstick and Douglas, 1991). In addition, the acidity of the N-3 proton is changed by the incorporation of a mercapto moiety at the 5 position of deoxyuridine (Bardos and Kalman, 1966). As incorporation of the DMT group is accomplished under basic conditions, partial deprotonation at the N-3 probably occurs. This generation of a second nucleophilic site could be complexing with the DMT cation and preventing reaction with the primary alcohol.

Silica gel column chromatography was employed to isolate **6**. Due to the acidic nature of the silica gel, a small percentage of pyridine was added to the eluting solvent to prevent loss of the acid-sensitive DMT group. Initially, Et₃N was used in the solvent system; however, triethylammonium salts of **6** were isolated as determined by NMR. This was not observed during the synthesis of the standard 5'-(dimethoxytrityl)-2'-deoxyuridine. Switching to pyridine, a weaker base, in the eluting solvent system eliminated this problem. A satisfactory high-resolution FAB mass spectrum was obtained for this DMT intermediate **6**.

The dimethoxytrityl analogue **6** was isolated as a yellow solid in yields ranging from 35 to 48%, making formation of the dimethoxytrityl ether the lowest yielding reaction in this synthetic sequence. However, approximately 20% of the starting material **5** was converted into the 3',5'-bis(dimethoxytrityl) analogue, which was isolated and converted back into **4** for recycling into the synthesis.

The final reaction in the preparation of **7** is the incorporation of the phosphoramidite moiety at the 3' position of the nucleoside. This is commonly accomplished by reaction of the appropriately protected nucleoside with 2-cyanoethyl *N,N*-diisopropylchlorophosphoramidite in the presence of *N,N*-diisopropylethylamine (DIEPA) (Sinha *et al.*, 1984). Utilization of these conditions did not result in the desired product and starting material was re-isolated. However, a standard reaction with 5'-(dimethoxytrityl)deoxyuridine was successful. A second method for incorporation of the phosphoramidite moiety which utilizes the reagent 2-cyanoethyl *N,N,N',N'*-tetraisopropylphosphorodiamidite in the presence of tetrazole in CH₃CN was evaluated (Barone *et al.*, 1984). Unlike the basic reaction with DIEPA, these slightly acidic conditions provided the DNP-labeled phosphoramidite **7**. RP HPLC analysis of **7** indicated the presence of a pair of closely eluting peaks (11.0 and 12.4 min) representative of the expected 1:1 mixture of diastereomers. The overall purity of the sample was determined to be greater than 96%,⁵ and no starting material was observed in the chromatogram. Similar results were observed using silica gel TLC analysis [CH₂Cl₂:MeOH:Et₃N (96:3:1)]. The phosphorous NMR spectrum showed two characteristic peaks associated with the diastereomeric phosphoramidites (149.12 and 149.27 ppm). An additional peak appeared in the NMR at 14.3 ppm. On the basis of the chemical shift of this peak, the impurity is presumed to be a hydrolysis product (Scremin *et al.*, 1994). No attempt was made to remove this material prior to oligonucleotide synthesis as it is not reactive in the oligonucleotide chemistry. A high-resolution FAB mass spectrum could not be obtained for **7** due to its

instability during analysis. This was not surprising as the standard deoxyuridine phosphoramidite displayed a high degree of instability during high-resolution FAB MS analysis; however, a small molecular ion peak was identified in the spectrum. Evaluation of **7** using low-resolution mass spectroscopy showed a fragment (*m/z* = 407) consistent with the loss of the DMT and the phosphoramidite moieties. As a similar fragmentation pattern was observed for the standard deoxyuridine phosphoramidite under these experimental conditions, the information supports the proposed structure.

Syntheses and Purification of Oligonucleotides.

Internal incorporation of the phosphoramidite analogue **7** into a series of oligonucleotides has been accomplished using an automated DNA synthesizer. The natural oligonucleotides with corresponding sequences were prepared as standards. The sequences for the oligonucleotides which are listed in Table 1 were chosen for two reasons: the 7-mer was designed for characterization purposes while the 22-mer and the 24-mer were developed to study the DNA-protein interactions during transcription of the bacteriophage λ 6S operon. The concentration of the phosphoramidite solution of **7** was diluted 2-fold as compared to the normal phosphoramidites (dA, dG, T, and dC) utilized in these DNA syntheses. This dilution was performed to conserve material. The coupling time for the reaction involving the modified phosphoramidite was increased by 120 s. Under these reaction conditions, the coupling yield for the modified phosphoramidite was >96%, which was comparable to the coupling yields of the other standard phosphoramidites. The average stepwise yields for all six oligonucleotide syntheses were determined automatically by the ABI synthesizer using a trityl cation assay. These yields are listed in Table 1.

Selective removal of the exocyclic amine protecting groups in the presence of the DNP moiety was achieved by treatment with NH₄OH at room temperature for 44 h. Typically, this deprotection is performed at elevated temperatures of 55 °C for 8 h (ABI technical bulletin #13, 1987). However, studies involving reaction of the DNP-labeled nucleoside **5** with concentrated NH₄OH at 55 °C indicated that the analogue was not stable to these conditions but was stable at room temperature (data not shown). As successful removal of standard exocyclic amine protecting groups at room temperature has been reported for other oligonucleotides containing modified phosphoramidites, these conditions were utilized (Sheardy and Seeman, 1986). Although these initial studies were performed with the standard exocyclic amine protecting groups, phosphoramidites containing a wide variety of protecting groups which can be efficiently removed under mild conditions are now commercially available. These will be investigated for compatibility with phosphoramidite **7**.

The crude oligonucleotides were purified as the DMT ethers by RP HPLC. Removal of the DMT group under standard acidic conditions did not affect the DNP moiety. Following removal of the DMT group, the oligonucleotides were analyzed for purity by both analytical RP HPLC and gel electrophoresis as shown in Figures 2A and 3A, respectively. The oligonucleotides showed one major product in both analyses. The retention times of the DNP-labeled oligonucleotides were greater than the corresponding natural oligonucleotides in the HPLC analyses due to the lipophilic nature of the dinitrophenyl group. The RP HPLC retention times for the purified oligonucleotides are listed in Table 1. The overall yields of the pure material which were calculated by dividing the optical density of pure oligonucleotide by the optical

⁵ HPLC data were quantified using a simple area percent method.

density of crude DMT-labeled oligonucleotides times 100% are found in Table 1 (Kuimelis and Nambiar, 1994). The yields for the corresponding normal and DNP-labeled oligonucleotides were comparable.

Enzymatic Digestion of the Seven-Nucleotide Oligonucleotide. To establish that the DNP group is stable to the conditions of automated DNA synthesis, deprotection, and purification, a DNP-labeled oligonucleotide was enzymatically digested into its corresponding nucleosides and evaluated by RP HPLC. To observe the single DNP-labeled nucleoside present in the enzymatic digest, the short-chain oligonucleotide B was used for this analysis. The sequence chosen for this seven-nucleotide oligonucleotide did not contain deoxycytidine. Deoxycytidine coelutes with 5-mercaptopdeoxyuridine,⁶ a potential side product arising from the loss of the DNP group, in the gradient system utilized in the analysis. Deoxycytidine was therefore omitted from the sequence to eliminate uncertainty in identifying the nucleoside peaks.

Initial degradation of the oligonucleotide B and the corresponding normal oligonucleotide A was attempted by concurrent digestion with snake venom phosphodiesterase (SVP) and CIP (Kuimelis and Nambiar, 1994). This method proved useful in the digestion of the natural oligonucleotide but did not appear to fully degrade the DNP-labeled material. This is consistent with other studies in which SVP has failed to function with some modified oligonucleotides and nucleotides (Gao *et al.*, 1992; Hanna *et al.*, 1989, 1993). Substitution of the exonuclease SVP with an endonuclease Nuclease P1 resulted in complete digestion. Due to a difference in the pH requirements of these enzymes for activity, this digestion was accomplished in two consecutive enzymatic reactions. First, Nuclease P1 cleaved the phosphodiester bonds to provide the 5'-monophosphates which were then subjected to treatment with CIP. The resulting nucleosides were isolated and analyzed by RP HPLC. The results are shown in Figure 2B.

This enzymatic degradation provided the correct ratios of unmodified and modified nucleotides. For oligonucleotide A, the ratio of dA, dG, and T was 1.9:2.1:2.9. For oligonucleotide B, the nucleoside 5 is also expected in the reaction mixture providing a ratio of dA, dG, T, and 5 of 1.9:2.1:1.9:1.1. Chemically synthesized 5 was utilized as a standard. Both the enzymatically obtained and the chemically obtained nucleoside 5 eluted with the same retention time during RP HPLC analysis. In addition, these compounds provided identical UV spectra with absorption peaks appearing at 262 and 334 nm. Finally, no unidentifiable peaks were observed in the HPLC chromatograms of the enzymatic digests and only one peak representing nucleoside 5 appeared in the HPLC chromatogram monitored at 330 nm. In this analysis, absorption at 330 nm is associated with the presence of either the (dinitrophenyl)thiol ether or the 5-mercaptopyrimidine moiety. If the DNP-labeled analogue had undergone modification during automated DNA synthesis, deprotection, or purification, extraneous peaks were predicted to appear in this chromatogram.

Deprotection and Alkylation of a DNP-Modified Oligonucleotide. Removal of the DNP moiety was accomplished by reaction of oligonucleotide F with 1.4 M BME in Tris buffer (pH 8) (Shaltiel, 1967). The reaction was performed under two different reaction conditions: room temperature for 12 h and 45 °C for 4 h. Oligonucleotide E was reacted under the same condi-

tions to serve as a standard. No attempt was made to isolate the deprotected oligonucleotide prior to modification with 5-IAF. The pK_a of BME is 9.6 while the pK_a of 5-mercaptopdeoxyuridine is 5.0 (Bardos and Kalman, 1966). Modification of a thiol by reaction with haloacetyl compounds such as 5-IAF requires formation of the thiolate anion. By lowering the pH of the reaction mixture, the selectivity for modification of the oligonucleotide is enhanced over that of BME, a potential side reaction. The oligonucleotide products in both the deprotection and modification reactions were isolated by ethanol precipitation and analyzed by electrophoresis using a 25% polyacrylamide-7 M urea gel. The results are shown in Figure 3B. Lanes 1-4 represent reactions utilizing oligonucleotide E. No difference is observed in these four lanes, indicating the natural oligonucleotide structure is stable to the conditions needed for deprotection and alkylation of the thiol. Lanes 5 and 7 represent reactions of oligonucleotide F with BME at room temperature and 45 °C, respectively, while lanes 6 and 8 depict the corresponding alkylation reactions which were carried out at room temperature. No apparent mobility shift is observed between the natural oligonucleotide (lanes 1-4) and lanes 5 and 7, which is consistent with our observations that a 20-mer RNA containing 5-SH-UTP comigrates in this gel system with a 20-mer containing no analogue (He *et al.*, 1995). In contrast, a substantial difference is observed following modification with fluorescein, consistent with other reports of gel mobility shifts of fluorescein-labeled oligonucleotides (Clegg *et al.*, 1992; Mergny *et al.*, 1994). In fact, the selection of 5-IAF for the alkylation experiments instead of a thiol-modifying photo-cross-linking reagent such as *p*-azidophenacyl bromide (APB) was due in part to the ease in which the reaction results could be monitored by gel electrophoresis. The conditions for alkylation of the oligonucleotide with 5-IAF however should prove useful with other haloacetyl derivatives such as APB. We have specifically labeled the thiol group in an RNA molecule containing 5-SH-UMP with APB, and conditions for DNA labeling should be similar (He *et al.*, 1995).

As the mobility shift associated with the DNP label is small (Figure 3A), it is possible that a mixture of DNP-labeled and 5-mercapto-labeled oligonucleotides may appear as one band on a 25% polyacrylamide-7 M urea gel. Therefore, one cannot determine the extent to which deprotection has occurred. However, the appearance of a new band in lanes 6 and 8 (Figure 3B) with the subsequent disappearance of the band seen in lanes 5 and 7 indicates that the oligonucleotide underwent deprotection and alkylation. Although deprotection does occur with time at room temperature, the reduced time required to deprotect at 45 °C makes this the preferred method.

PCR Reaction using a DNP-Modified Oligonucleotide. The DNP-labeled oligonucleotide was incorporated into double-stranded DNA using PCR. Plasmid pHA100 served as the template for this reaction. A 492 base pair segment of this plasmid was amplified using the universal T7 primer and either oligonucleotide E or F. The products isolated from the reaction were analyzed on a 10% polyacrylamide-7 M urea gel (data not shown) and a 1.5% agarose gel (Figure 4A). Differences in mobility were not observed between the standard PCR product and the DNP-labeled PCR product in these gel analyses, and no difference in yield was observed.

Immunodetection of DNP-Labeled Oligonucleotides and DNA. To ensure the stability of the DNP protecting group during the PCR reaction, the products described above were subjected to immunodetection

⁶ 5-Mercaptopdeoxyuridine was synthetically prepared in this lab by reduction of 5-(thiocyanato)deoxyuridine (Nagamachi *et al.*, 1974).

studies using rabbit polyclonal anti-DNP antibodies. The natural PCR product served as a negative control. The DNP group was detected using a chemiluminescence method, and only the DNP-labeled nucleic acid provided a signal (Figure 4B).

DISCUSSION

Development of the 5-DNP-labeled phosphoramidite **7** resulted from our ongoing investigations into the molecular mechanism of transcription. Many of our studies investigating the macromolecular interactions occurring during transcriptional regulation have involved photo-cross-linking. Photo-cross-linking groups have been incorporated into RNA, and upon photoactivation, the RNA has been covalently attached to adjacent macromolecules (i.e., proteins, DNA, or RNA) with which it has direct contact. Much of our work has utilized two photo-cross-linking ribonucleotide analogues, 5-APAS-UTP and 5-APAS-CTP, which can be incorporated enzymatically in place of uridine and cytidine, respectively, into the nascent RNA by a variety of RNA polymerases (Dissinger and Hanna, 1990; Hanna *et al.*, 1989, 1993). However, many of the questions remaining about the regulation of transcription involve interactions between proteins and DNA. To study these interactions, a method for the site-specific placement of a photo-cross-linking group within a DNA template was needed. The DNP-labeled phosphoramidite **7** was designed with this goal in mind.

The structure and utility of the 5-DNP-modified phosphoramidite is unique. Use of a phosphoramidite analogue provides an efficient method for the site-specific modification of an oligonucleotide by chemical synthesis. The selection of the 5 position as a site for modification allows for both internal and terminal incorporation without disruption of the normal Watson-Crick base pairing. Thus a thiol group can be introduced at a single site or numerous sites within an oligonucleotide. The thiol is a reactive functional group which is capable of selective deprotection and derivatization in the presence of typical functional moieties found in nucleic acids such as amines and alcohols. Several types of thiol-modifying reagents are available which generate a variety of thiol linkages ranging from the very stable thiol ether to the easily cleavable disulfide bond. These allow for modification of the unmasked thiol with an assortment of molecular probes. The sulfur atom is a linker of minimal size between the modifying reagent and the oligonucleotide. This single atom linker provides flexibility because probes can be positioned very close to, or at a distance from, the pyrimidine base. If desired, the distance between the molecular tag and the oligonucleotide can be easily varied by adjusting the structure of the modifying reagent, many of which are commercially available, rather than that of the oligonucleotide.

Although other thiol-labeled analogues for incorporation into oligonucleotides via automated syntheses have been reported [reviewed in Beaucage and Iyer (1993) and Goodchild (1990)], none possess the diversity and flexibility of our 5-thiopyrimidine probe. Some thiol-labeled analogues can be incorporated only at the 5' and 3' ends of oligonucleotides (Kuijpers and van Boeckel, 1993; Kumar, 1993a,b). However, many nucleic acid-protein interactions involve internal nucleic acid sequences and cannot be studied with these probes. Modifications along the phosphate backbone using a thiol tether attached to the phosphate utilizes a mixture of phosphoramidite and hydrogen phosphonate chemistry, which requires part of the synthesis to be performed manually (Fidanza and McLaughlin, 1992). In addition, the oligonucleotide product contains diastereomers which can be difficult to

separate. Modification using phosphorothioates suffers from similar problems. To ensure stereochemical purity, dinucleotides containing phosphorothioate diester linkages are first prepared. The resulting diastereomers are separated by chromatographic procedures, converted to phosphoramidites, and then incorporated into oligonucleotides (Fidanza *et al.*, 1994). Both of these phosphate modifications make incorporation of multiple labels difficult.

Other analogues involving modifications at the C5 position of deoxyuridine have been previously reported. The thiol-containing analogue, 5-(thiocyanato)deoxyuridine phosphoramidite, provided a 5-mercaptodeoxyuridine moiety within the oligonucleotide following reduction of the thiocyanate (Bradley and Hanna, 1992). However, the thiocyanato moiety displayed variable stability during synthesis of both the nucleotide and the oligonucleotide and therefore this compound did not represent an ideal analogue for incorporation of 5-thiol-modified nucleotides. The syntheses of a series of phosphoramidites containing alkanethiol tethers at the C5 position of deoxyuridine has been reported (Goodwin and Glick, 1993). The thiol groups in these analogues are attached to the ring by either a three-, four-, or five-carbon chain. The presence of the carbon chains makes the minimal distance between the molecular probes and the oligonucleotide greater than that which can be achieved with our analogue. This increase in distance can be undesirable in studies which probe the immediate environmental contacts between macromolecules. In addition, these compounds represent alkanethiol analogues which have a different reactivity for modification than the 5-mercaptopyrimidine analogue. This is due to an increase in acidity of 5-mercaptopyrimidines ($pK_a \sim 5-5.6$) over alkanethiol moieties ($pK_a \sim 8-10$). Phosphoramidites containing alkanethiol tethers at the N3 position of thymidine have also been prepared, but the position of this modification results in a disruption in the Watson-Crick base pairing. These analogues have been used mainly for preparing disulfide cross-links in DNA for studies involving stem loop and triple-helical structures (Glick, 1991; Goodwin *et al.*, 1994).

Several 5-modified deoxyuridine phosphoramidites are commercially available which contain functional groups (i.e., carboxylic acids or alkylamines) for postmodification following incorporation into the oligonucleotide.⁷ Likewise, alkanethiol ethers of 5-mercaptodeoxyuridine containing protected carboxylic acids and alkylamines have also been described (Bergstrom *et al.*, 1991). The protected functional groups in these analogues are not attached directly to the ring but are positioned at the end of carbon chains. Additionally, these groups are not as easily modified as mercaptans: the functional groups formed during postmodification are limited, and an easily cleavable group is not available. Thiol-containing phosphoramidites for incorporating 4-thiothymidine (Clivio *et al.*, 1992; Xu *et al.*, 1992c), 4-thiothymidine (Clivio *et al.*, 1992; Coleman and Kesicki, 1994), 2-thiothymidine (Connolly and Newman, 1989; Kuimelis and Nambiar, 1994), 6-thiothymidine (Christopherson and Broom, 1991; Waters and Connolly, 1992; Xu *et al.*, 1992b), and 6-thioinosine (Clivio *et al.*, 1992; Xu *et al.*, 1992a) have been reported.⁷ These analogues can occupy internal positions within an oligonucleotide and can serve as photochemical cross-linkers. However, they cannot be

⁷ Carboxy-dT and amino modifiers C2dT and C6dT are available from Glen Research Sterling, VA. Thiol-containing phosphoramidites for incorporating 4-thiopyrimidines and 6-thiopyrimidines are also available from Glen Research.

further modified without disrupting Watson–Crick base pairing and, therefore, as photo-cross-linking probes are only useful for evaluating interactions which occur directly with the nucleotide base. In addition, the deprotection of oligonucleotides containing these thiol-modified nucleobases must be carefully monitored to prevent conversion of these analogues to the corresponding oxygen and nitrogen derivatives.

Analogue 7 can be used for site-specific modification of DNA at internal and external positions within the DNA sequence, and molecular probes can be placed at variable distances from the DNA backbone. The DNP group is stable to the conditions of PCR amplification and can therefore be easily incorporated into DNA. The commercial availability of antibodies to the DNP group allows the direct use of the DNP-labeled oligonucleotides as immunodetection probes (Grzybowski *et al.*, 1993). More importantly, functionalization of the thiol following deprotection can be varied to allow attachment of a wide variety of functional groups. These include photoaffinity or fluorescent tags for investigation of molecular interactions, reporter groups for purification or detection purposes, and peptides or drugs for therapeutic agents.

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LITERATURE CITED

- Bardos, T. J., and Kalman, T. I. (1966) Spectrophotometric and Chemical Studies of 5-Mercaptouracil, 5-Mercaptodeoxyuridine, and Their S-Substituted Derivatives *J. Pharm. Sci.* 55, 606–610.
- Barone, A. D., Tang, J.-Y., and Caruthers, M. H. (1984) *In situ* Activation of bis-Dialkylaminophosphines—A New Method for Synthesizing Deoxyoligonucleotides on Polymer Supports. *Nucleic Acids Res.* 12, 4051–4061.
- Bartholomew, B., Kassavetis, G. A., Braun, B. R., and Geiduschek, E. P. (1990) The Subunit Structure of *Saccharomyces cerevisiae* Transcription Factor IIIC Probed with a Novel Photocrosslinking Reagent. *EMBO J.* 9, 2197–2205.
- Bartholomew, B., Braun, B. R., Kassavetis, G. A., and Geiduschek, E. P. (1994) Probing Close DNA Contacts of RNA Polymerase III Transcription Complexes with the Photoactive Nucleoside 4-Thiooxythymidine. *J. Biol. Chem.* 269, 18090–18095.
- Beaucage, S. L., and Iyer, R. P. (1993) The Functionalization of Oligonucleotides Via Phosphoramidite Derivatives. *Tetrahedron* 49, 1925–1963.
- Bergstrom, D. E., Beal, P., Jenson, J., and Lin, X. (1991) Palladium-mediated Synthesis of C-5 Pyrimidine Nucleoside Thioethers from Disulfides and Mercurinucleosides. *J. Org. Chem.* 56, 5598–5602.
- Bleasdale, C., Ellwood, S. B., and Golding, B. T. (1990) 4,4'-Dimethoxytrityl and 4-Monomethoxytrityl Tetrafluoroborate: Convenient Reagents for the Protection of Primary Alcohols Including Sugars. *J. Chem. Soc., Perkin Trans. 1* 803–804.
- Bradley, D. H., and Hanna, M. M. (1992) Synthesis and Utility of 5-Thiocyanato Deoxyuridine and Uridine Phosphoramidites as Masked Synthons. *Tetrahedron Lett.* 33, 6223–6226.
- Brown, H. C., Narasimhan, S., and Choi, Y. M. (1982) Selective Reduction. 30. Effect of Cation and Solvent on the Reactivity of Saline Borohydrides for Reduction of Carboxylic Esters. Improved Procedures for the Conversion of Esters to Alcohols by Metal Borohydrides. *J. Org. Chem.* 47, 4702–4708.
- Chaudhary, S. K., and Hernandez, O. (1979) A Simplified Procedure for the Preparation of Triphenylmethylethers. *Tetrahedron Lett.* 95–98.
- Christopherson, M. S., and Broom, A. D. (1991) Synthesis of Oligonucleotides Containing 2'-Deoxy-6-thioguanosine at a Predetermined Site. *Nucleic Acids Res.* 19, 5719–5724.
- Clegg, R. M., Murchie, A. I. H., Zechel, A., Carlberg, C., Diekmann, S., and Lilley, D. M. J. (1992) Fluorescence Resonance Energy Transfer Analysis of the Structure of the Four-Way DNA Junction. *Biochemistry* 31, 4846–4856.
- Clivio, P., Fourrey, J.-L., Gasche, J., Audic, A., Favre, A., Perrin, C., and Woisard, A. (1992) Synthesis and Purification of Oligonucleotides Containing Sulfur Substituted Nucleobase: 4-Thiouracil, 4-Thiothymine and 6-Mercaptopurine. *Tetrahedron Lett.* 33, 65–68.
- Coleman, R. S., and Kesicki, E. A. (1994) Synthesis and Postsynthetic Modification of Oligodeoxynucleotides Containing 4-Thio-2'-deoxyuridine (d^SU). *J. Am. Chem. Soc.* 116, 11636–11642.
- Connolly, B. A., and Newman, P. C. (1989) Synthesis and Properties of Oligonucleotides Containing 4-Thiothymine, 5-Methy-2-pyrimidinone-1- β -D-(2'-deoxyribose) and 2-Thiothymidine. *Nucleic Acids Res.* 17, 4957–4974.
- Cosstick, R., and Douglas, M. E. (1991) Synthesis of a Dinucleoside Monophosphate Analogue Containing 6-N-(2-Aminoethyl)-2'-Deoxyadenosine. A Novel Approach to Sequence Specific Crosslinking in Synthetic Oligonucleotides. *J. Chem. Soc., Perkin Trans. 1* 1035–1040.
- Cruickshank, K. A., and Stockwell, D. L. (1988) Oligonucleotide Labelling: A Concise Synthesis of a Modified Thymidine Phosphoramidite. *Tetrahedron Lett.* 29, 5221–5224.
- Dissinger, S., and Hanna, M. M. (1990) Active Site Labeling of *Escherichia coli* Transcription Elongation Complexes with 5-((4-Azidophenyl)thio)uridine 5'-Triphosphate. *J. Biol. Chem.* 265, 7662–7668.
- Dissinger, S., and Hanna, M. M. (1991) RNA-Protein Interactions in a Nus A-containing *Escherichia coli* Transcription Complex Paused at an RNA Hairpin. *J. Mol. Biol.* 219, 11–25.
- Evans, R. K., and Haley, B. E. (1987) Synthesis and Biological Properties of 5-Azido-2'-deoxyuridine 5'-Triphosphate, a Photoactive Nucleotide Suitable for Making Light-Sensitive DNA. *Biochemistry* 26, 269–276.
- Fidanza, J. A., and McLaughlin, L. W. (1992) Use of a Thiol Tether for the Site-Specific Attachment of Reporter Groups to DNA. *J. Org. Chem.* 57, 2340–2346.
- Fidanza, J. A., Ozaki, H., and McLaughlin, L. W. (1994) Functionalization of Oligonucleotides by the Incorporation of Thiol-Specific Reporter Groups. *Protocols for Oligonucleotide Conjugates. Synthesis and Analytical Techniques* (Agrawal, S., Ed.) pp 121–144, Humana Press, Totowa.
- Flaschka, H. A., Barnard, A. J., and Sturrock, P. E. (1969) *Quantitative Analytical Chemistry, Short Introduction to Practice*, Barnes & Noble, Inc., New York.
- Gao, H., Fathi, R., Gaffney, B. L., Goswami, B., Kung, P.-P., Rhee, Y., Jin, R., and Jones, R. A. (1992) 6-O-(Pentafluorophenyl)-2'-deoxyguanosine: A Versatile Synthon for Nucleoside and Oligonucleotide Synthesis. *J. Org. Chem.* 57, 6954–6959.
- Glick, G. D. (1991) Synthesis of a Conformationally Restricted DNA Hairpin. *J. Org. Chem.* 56, 6746–6747.
- Goodchild, J. (1990) Conjugates of Oligonucleotides and Modified Oligonucleotides: A Review of Their Synthesis and Properties. *Bioconjugate Chem.* 1, 165–187.
- Goodwin, J. T., and Glick, G. D. (1993) Incorporation of Alkylthiol Chains at C-5 of Deoxyuridine. *Tetrahedron Lett.* 34, 5549–5552.
- Goodwin, J. T., Osborne, S. E., Swanson, P. C., and Glick, G. D. (1994) Synthesis of a Disulfide Cross-linked DNA Triple Helix. *Tetrahedron Lett.* 35, 4527–4530.
- Grassetti, D. R., and Murray, J. F., Jr. (1969) The Use of 2,2'-Dithiobis(5-nitropyridine) as a Selective Reagent for the Detection of Thiols. *J. Chromatogr.* 41, 121–123.
- Greene, T. W. (1981) *Protective Groups in Organic Synthesis*, John Wiley & Sons, New York.
- Grzybowski, J., Will, D. W., Randall, R. E., Smith, C. A., and Brown, T. (1993) Synthesis and Antibody-mediated Detection of Oligonucleotides Containing Multiple 2,4-Dinitrophenyl Reporter Groups. *Nucleic Acids Res.* 21, 1705–1712.

- Hanna, M. M. (1993) Photocrosslinking Analysis of Protein-RNA Interactions in *E. coli* Transcription Complexes. *Cell. Mol. Biol. Res.* 39, 393–399.
- Hanna, M. M., Dissinger, S., Williams, B. D., and Colston, J. E. (1989) Synthesis and Characterization of 5-[4-(Azidophenacyl)thio]uridine 5'-Triphosphate, a Cleavable Photo-Cross-Linking Nucleotide Analogue. *Biochemistry* 28, 5814–5820.
- Hanna, M. M., Zhang, Y., Reidling, J. C., Thomas, M. J., and Jou, J. (1993) Synthesis and Characterization of a New Photocrosslinking CTP Analog and its Use in Photoaffinity Labeling *E. coli* and T7 RNA Polymerases. *Nucleic Acids Res.* 21, 2073–2079.
- He, B., Riggs, D. L., and Hanna, M. M. (1995) Preparation of Probe-Modified RNA with 5-Mercapto-UTP for Analysis of Protein-RNA Interactions. *Nucleic Acids Res.* 23, 1231–1238.
- Kalman, T. I., and Bardos, T. J. (1967) The Autoxidation of 5-Mercaptouracil and 5-Mercaptodeoxyuridine. *J. Am. Chem. Soc.* 89, 1171–1175.
- Kini, G. D., Robins, R. K., and Avery, T. L. (1989) Synthesis and Antitumor Activity of Ribavirin Imidates. A New Facile Synthesis of Ribavirin Amidine (1- β -D-Ribofuranosyl-1,2,4-triazole-3-carboxamide Hydrochloride). *J. Med. Chem.* 32, 1447–1449.
- Kuijpers, W. H. A., and van Boeckel, C. A. A. (1993) A New Strategy for the Solid-Phase Synthesis of 5'-Thiolated Oligodeoxynucleotides. *Tetrahedron* 49, 10931–10944.
- Kuimelis, R. G., and Nambiar, K. P. (1994) Synthesis of Oligodeoxynucleotides Containing 2-Thiopyrimidine Residues—A New Protection Scheme. *Nucleic Acids Res.* 22, 1429–1436.
- Kumar, A. (1993a) A Rapid Solid Phase Method for the Synthesis of 3'-Thiol Group Containing Oligonucleotides. *Nucleosides Nucleotides* 12, 729–736.
- Kumar, A. (1993b) A Versatile Solid Phase Method for the Synthesis of Masked 3'-Thiol Group Containing Oligonucleotides. *Nucleosides Nucleotides* 12, 1047–1059.
- Lakshman, M. K., Sayer, J. M., Yagi, H., and Jerina, D. M. (1992) Synthesis and Duplex-Forming Properties of a Nona-nucleotide Containing an N⁶-Deoxyadenosine Adduct of a Bay-Region Diol Epoxide. *J. Org. Chem.* 57, 4585–4590.
- Levina, A. S., Tabatadse, D. R., Khalimskaya, L. M., Prichodka, T. A., Shishkin, G. V., Alexandrova, L. A., and Zarytova, V. P. (1993) Oligonucleotide Derivatives Bearing Reactive and Stabilizing Groups Attached to C5 of Deoxyuridine. *Bioconjugate Chem.* 4, 319–325.
- Lin, T.-S., and Gao, Y.-S. (1983) Synthesis and Biological Activity of 5-(Trifluoromethyl)- and 5-(Pentafluoroethyl)-pyrimidine Nucleoside Analogues. *J. Med. Chem.* 26, 598–601.
- Lin, T.-S., Guo, J.-Y., Schinazi, R. F., Chu, C. K., Xiang, J.-N., and Prusoff, W. H. (1988) Synthesis and Antiviral Activity of Various 3'-Azido Analogues of Pyrimidine Deoxyribonucleosides Against Human Immunodeficiency Virus (HIV-1, HTLV-III/LAV). *J. Med. Chem.* 31, 336–340.
- Liu, K., and Hanna, M. M. (1995a) NusA Contacts Nascent RNA in *Escherichia coli* Transcription Complexes. *J. Mol. Biol.* 247, 547–558.
- Liu, K., and Hanna, M. M. (1995b) NusA Interferes with Interactions Between the Nascent RNA and the C-terminal Domain of the α Subunit of RNA Polymerase in *Escherichia coli* Transcription Complexes. *Proc. Natl. Acad. Sci. U.S.A.* 92, 5012–5016.
- MacMillan, A. M., and Verdine, G. L. (1991) Engineering Tethered DNA Molecules by the Convertible Nucleoside Approach. *Tetrahedron* 47, 2603–2616.
- Mergny, J.-L., Bourtine, A. S., Garestier, T., Belloc, F., Rougee, M., Vallychev, N. V., Koshkin, A. A., Bourson, J., Lebedev, A. V., Valeur, B., Thuong, N. T., and Helene, C. (1994) Fluorescence energy transfer as a probe for nucleic acid structures and sequences. *Nucleic Acids Res.* 22, 920–928.
- Nagamachi, T., Fourrey, J.-L., Torrence, P. F., Waters, J. A., and Witkop, B. (1974) Synthesis, Chemistry, and Biological Activity of 5-Thiocyanatopyrimidine Nucleosides as Potential Masked Thiols. *J. Med. Chem.* 17, 403–406.
- Scremin, C. L., Zhou, L., Srinivasachar, K., and Beaucage, S. L. (1994) Stepwise Regeneration and Recovery of Deoxyribo-nucleoside Phosphoramidite Monomers during Solid-Phase Oligonucleotide Synthesis. *J. Org. Chem.* 59, 1963–1966.
- Shaltiel, S. (1967) Thiolytic of Some Dinitrophenyl Derivatives of Amino Acids. *Biochem. Biophys. Res. Commun.* 29, 178–183.
- Sheardy, R. D., and Seeman, N. C. (1986) The Synthesis of a Deoxyoligonucleotide Incorporating 5-Iododeoxyuridine. *J. Org. Chem.* 51, 4301–4303.
- Sinha, N. D., Biernat, J., McManus, J., and Koster, H. (1984) Polymer Support Oligonucleotide Synthesis XVIII: Use of β -cyanoethyl-N,N-dialkylamino-N-morpholino Phosphoramidite of Deoxynucleosides for the Synthesis of DNA Fragments Simplifying Deprotection and Isolation of the Final Product. *Nucleic Acids Res.* 12, 4539–4557.
- Soai, K., and Ookawa, A. (1986) Mixed Solvents Containing Methanol as Useful Reaction Media for Unique Chemoselective Reductions with Lithium Borohydride. *J. Org. Chem.* 51, 4000–4005.
- Telser, J., Cruickshank, K. A., Morrison, L. E., and Netzel, T. L. (1989) Synthesis and Characterization of DNA Oligomers and Duplexes Containing Covalently Attached Molecular Labels: Comparison of Biotin, Fluorescein, and Pyrene Labels by Thermodynamic and Optical Spectroscopic Measurements. *J. Am. Chem. Soc.* 111, 6966–6976.
- Torrence, P. F., Waters, J. A., and Witkop, B. (1968) 2'-Deoxy-5-(thiocyanato)uridine [1-(2-deoxy- β -D-erythropentofuranosyl)-5-(thiocyanato)uracil]. *Synthetic Procedures in Nucleic Acid Chemistry* (Zorbach, W. W., Tipson, R. S., Eds.) pp 367–370, Interscience, New York.
- Trivedi, B. K., and Bruns, R. F. (1989) C2,N⁶-Disubstituted Adenosines: Synthesis and Structure–Activity Relationships. *J. Med. Chem.* 32, 1667–1673.
- Wang, G., and Bergstrom, D. E. (1993) Synthesis of Oligonucleotides Containing N²-(5-Carboxypentyl)-2'-deoxyguanosine and 5-[2-(4'-Methyl-2,2'-dipyrid-4-yl-carboxamido)ethylthio]-2'-deoxyuridine. *Tetrahedron Lett.* 34, 6721–6724.
- Waters, T. R., and Connolly, B. A. (1992) Straightforward Synthesis of 6-Thiodeoxyguanosine and Its Incorporation into Oligodeoxynucleotides. *Nucleosides Nucleotides* 11, 985–998.
- Webb, T. R., and Matteucci, M. D. (1986) Hybridization Triggered Cross-linking of Deoxyoligonucleotides. *Nucleic Acids Res.* 14, 7661–7674.
- Xu, Y.-Z., Zheng, Q., and Swann, P. F. (1992a) Synthesis and Duplex Stability of Oligodeoxynucleotides Containing 6-Mercaptopurine. *Tetrahedron Lett.* 33, 5837–5840.
- Xu, Y.-Z., Zheng, Q., and Swann, P. F. (1992b) Synthesis by Post-Synthetic Substitution of Oligomers Containing Guanine Modified at the 6-Position with S-, N-, O-derivatives. *Tetrahedron* 48, 1729–1740.
- Xu, Y.-Z., Zheng, Q., and Swann, P. F. (1992c) Synthesis of DNA Containing Modified Bases by Postsynthetic Substitution. Synthesis of Oligomers Containing 4-Substituted Thymine: O⁴-Alkylthymine, 5-Methylcytosine, N⁴-(Dimethylamino)-5-methylcytosine, and 4-Thiothymine. *J. Org. Chem.* 57, 3839–3845.
- Xu, Y.-Z., Zheng, Q., and Swann, P. F. (1995) Preparation of Oligodeoxynucleotides Containing 6-Methylthiopurine Residues by Chemical Synthesis or Specific Methylation. *Nucleosides Nucleotides* 14, 929–933.
- Yang, X. J., Hart, C. M., Grayhack, E. J., and Roberts, J. W. (1987) Transcription Antitermination by Phage Lambda Gene Q Protein Requires a DNA Segment Spanning the RNA Start Site. *Genes Dev.* 1, 217–226.
- Zhang, Y., and Hanna, M. (1994) NusA Changes the Conformation of *Escherichia coli* RNA Polymerase at the Binding Site for the 3' End of the Nascent RNA. *J. Bacteriol.* 176, 1787–1789.
- Zhang, Y., and Hanna, M. M. (1995) Expression and Functional Characterization of *Escherichia coli* NusA and Lambda Q as Glutathione-S-Transferase Fusion Proteins. *Protein Expression Purif.* 6, 625–631.
- Zheng, Q., Xu, Y.-Z., and Swann, P. F. (1995) An Improved Post-Synthetic Substitution Approach for Synthesis of Oligodeoxynucleotides Containing Labile 4-Substituted Thymines. *Nucleosides Nucleotides* 14, 939–942.

Optimizing the Targeted Chemical Nuclease Activity of 1,10-Phenanthroline–Copper by Ligand Modification

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Our interest in improving the efficiency of targeted scission reagents has prompted us to study the influence of ring substituents on the nuclease activity of 1,10-phenanthroline–copper conjugated to oligonucleotides and DNA-binding proteins. Since methyl substitution at all but the 2 and 9 positions enhances the copper-dependent chemical nuclease activity of 1,10-phenanthroline, we have compared the reactivity of conjugates prepared from 5-(aminomethyl)-1,10-phenanthroline (MOP) to those of conjugates prepared from 5-amino-1,10-phenanthroline (amino-OP). Tethering MOP derivatives to the *Escherichia coli* Fis protein enhances DNA scission several-fold at the weaker cleavage sites initially observed with conjugates prepared from amino-OP. However, scission efficiency is not increased at the stronger cleavage sites, or when scission is targeted to single-stranded DNA by a complementary oligonucleotide. These results are consistent with a change in the rate-determining step for cleavage associated with the differential accessibility of the DNA-bound coordination complex to solvent and reductant. Although the free *bis* cuprous complex of 2,9-dimethyl-1,10-phenanthroline (neocuproine) is redox-inactive, an oligonucleotide tethered to neocuproine through C5 of the phenanthroline ring efficiently cleaves a complementary DNA sequence. These results establish that the nucleolytic species in *targeted* scission is the 1:1 cuprous complex and suggest that the oxidative reaction proceeds through a copper–oxo intermediate rather than a metal-coordinated peroxy species. However, substituents at the 2 and 9 positions of the ligand will often hinder close approach of the phenanthroline–copper moiety to the oxidatively sensitive ribose as shown by the preference of the oligonucleotide-targeted chimera for cleavage of single-stranded regions and the failure of neocuproine–DNA-binding protein chimeras and a C2-tethered chimera to cleave DNA.

INTRODUCTION

The generation of new functional biomolecules has been the focus of current research with catalytic antibodies (1), ribozymes (2), *in vitro* selection schemes (3), and site-directed mutagenesis (4–6). Functionalization of biomolecules by conjugation with the cleavage reagent 1,10-phenanthroline–copper(I) constitutes another direction in the development of reactive molecules with designed specificities (7–9). This redox-active coordination complex has proven especially useful in the development of highly specific chemical nucleases and proteases (9) since it does not generate diffusible hydroxyl radicals but rather reacts via a copper–oxo species (10).

Several factors contribute to the specificity of chimeric reagents prepared with 1,10-phenanthroline–copper. Preeminent among these are the orientation and proximity of the redox-active copper ion to the oxidatively sensitive site (11). However, the intrinsic reactivity of the copper–oxo intermediate is also likely to play a role in the specificity and efficiency of the chimeric scission reagents. Since substituents on the phenanthroline (OP) ring modulate the redox activity of the chelates (12), we initiated a study to determine if the cleavage efficiency and selectivity can be improved by derivatization of the 1,10-phenanthroline ligand. In the first series of experiments, our goal was to synthesize more efficient cleavage reagents by inserting a methylene group between the 1,10-phenanthroline ring system and the iodoacetamido

substituent used for covalent attachment of OP to the targeting biomolecule. Our prior observation that the 1:2 cuprous complexes of 5-methyl- and 5,6-dimethyl-1,10-phenanthroline were more active as catalysts in the oxidation of thiols and in the untargeted cleavage of DNA than (OP)₂Cu⁺ motivated this modification of the ligand (12).

We similarly reasoned that methyl substituents at phenanthroline carbons 2 and 9 might enhance the reactivity of the 1:1 cuprous complex of phenanthroline–DNA-binding ligand chimeras. Therefore, oligonucleotides were alkylated with 2,9-dimethyl-5-(iodoacetamido)-1,10-phenanthroline and then used to cleave complementary DNA sequences. Although 2,9-dimethyl-1,10-phenanthroline does not form a redox-active 2:1 complex because steric clash between the ortho methyl groups inhibits the redox cycling through the square-planar cupric complex, this kinetic barrier is not relevant in targeted scission where only a single ligand is linked to a carrier (13–15).

Our results indicate that insulation of the phenanthroline ring system from the amido group by a methylene spacer does not increase the nucleolytic activity when oligonucleotides are the targeting vehicles. However, substantial rate enhancements are observed in protein-targeted scission at certain cleavage sites, particularly those at which only weak cleavage is observed without the methylene spacer. In contrast, at sites at which strong cleavage is observed without the insulating methylene group, insertion of the methylene group does not enhance scission. The observation of rate enhancement at a specific site may depend on whether reduction or oxidation of the copper is the rate-determining step in

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Table 1

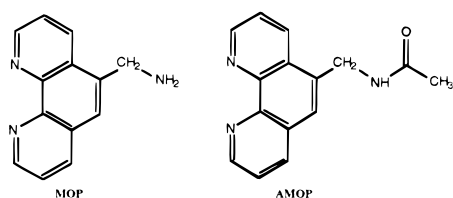
X-OP	chemical redox activity: ^a half-life (min)		nuclease activity of the 2:1 complex: ^b concentration at 50% cleavage (μ M)
	2:1 copper complex	1:1 copper complex	
1,10-phenanthroline (OP)	12	57	19
5-CH ₃ -OP	2.6	30	7.4
5-CH ₃ CONH-OP	21	80	57
5-CH ₃ CONHCH ₂ -OP	7.8	44	25

^a Half-lives for the oxidation of mercaptopropionic acid (MPA) by the 2:1 and 1:1 copper complexes of X-OP (8 μ M:2 μ M or 2 μ M:2 μ M X-OP:CuSO₄). ^b Cleavage of a 141 bp long Lac UV-5 promoter (−96 to +45) by (X-OP)₂Cu⁺.

reaction at that site. Our results further indicate that chimeras prepared from 2,9-dimethyl-1,10-phenanthroline [neocuproine (NC)] and oligonucleotides are competent site-directed cleavage reagents. Therefore, the steric factors that inhibit the redox chemistry of the 2:1 neocuproine–cuprous complex [(NC)₂Cu⁺] are not present in the 1:1 complex linked to a targeting ligand.

RESULTS

Insulation of the Amido Moiety from 1,10-Phenanthroline. (a) *Untargeted Bis Complexes.* The methylene group of 5-(aminomethyl)-1,10-phenanthroline (MOP) (16) inhibits any electronic interaction of an amido moiety, prepared from MOP, with the 1,10-phenanthroline ring system. Before preparation of phenanthroline-linked chimeras for site-directed scission, the catalytic oxidation of thiols and the oxidative cleavage of duplex DNA by the 2:1 and 1:1 complexes of 5-(acetamidomethyl)-1,10-phenanthroline (AMOP) and copper(I) was studied to confirm the anticipated enhanced reactivity of these coordination complexes compared to those of 5-acetamido-1,10-phenanthroline. The disappearance of the free, titratable sulfhydryl group of 3-mercaptopropionic acid (MPA) according to the method of Ellman (17) provides a convenient assay of the redox activity of the copper chelates. Both the 1:1 and 2:1 cuprous chelates prepared from 5-AcNHCH₂OP (AMOP) have half-lives for the oxidation of MPA to the disulfide that are 2–3-fold shorter than those of the corresponding chelates prepared from 5-acetamido-1,10-phenanthroline (5-AcNHOP) (Table 1).



This enhanced redox activity is also reflected in the nuclease activity of the untargeted 2:1 1,10-phenanthroline–cuprous complex toward a 141-base pair (bp) ³²P 5'-end-labeled DNA probe containing the *lac* UV-5 promoter. Quantification of the disappearance of the DNA parent band as a function of concentration of the cleaving agent (Table 1) indicates that (AMOP)₂Cu⁺ is a more potent nuclease than (5-AcNHOP)₂Cu⁺ and approaches the nucleolytic activity of (OP)₂Cu⁺. The scission patterns of the different chelates are the same for this DNA segment. These results indicate that the binding specificity of the tetrahedral cuprous chelates does not depend on substituents at the C5 position of the ligand.

(b) *Oligonucleotide-Targeted Scission.* The simplest test system to assay the intrinsic reactivity of a tethered coordination complex involves the targeted scission of a single-stranded DNA by a complementary OP-linked deoxyoligonucleotide (18). In the first series of experiments, insertion of the methylene group insulated the

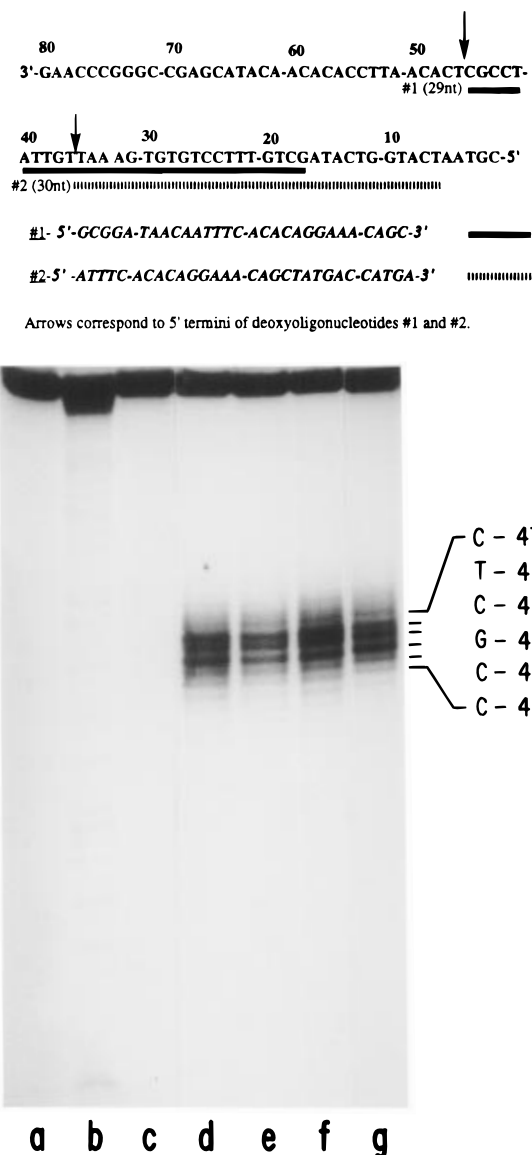


Figure 1. Targeted scission of a single-stranded DNA by an oligonucleotide derivatized with a series of 5'-substituted 1,10-phenanthrolines: (top) oligonucleotide sequences; (bottom) lane a, 80-mer single-stranded DNA probe; lane b, Maxam–Gilbert G+A digest; lanes c–g, probe treated with CuSO₄ and MPA and hybridized to no OP–oligonucleotide (lane c), AOP–oligonucleotide (lane d), AMOP–oligonucleotide (lane e), AAOP–oligonucleotide (lane f), or AGMOP–oligonucleotide (lane g).

1,10-phenanthroline nuclease from the tethering amido group. The sequences of the relevant oligonucleotides are presented in the top panel of Figure 1. No significant effect of insulating the 1,10-phenanthroline from the amido group is apparent from the cleavage data (bottom panel of Figure 1). For example, the cleavage efficiency of the 50-mer oligonucleotides derivatized with IAMOP was virtually identical to that observed with IAOP. The reactivities of oligonucleotides derivatized with IAAOP

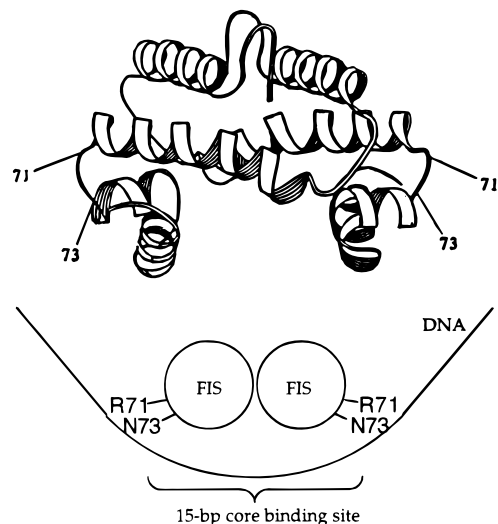
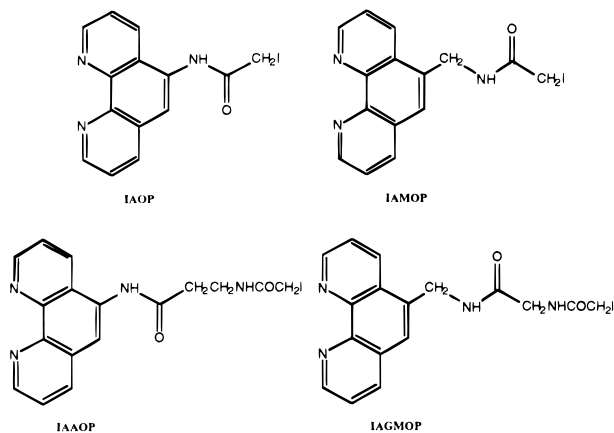


Figure 2. (Top) Ribbon diagram of Fis dimer showing the positions of the two amino acids modified with derivatives of 1,10-phenanthroline. (Bottom) Schematic representation showing the binding of Fis dimer to DNA.

and IAGMOP were also compared. Chimeras prepared from these two 1,10-phenanthroline derivatives have isomeric tethers of the same length. The patterns of reactivity were virtually identical, indicating that there is no advantage in oligonucleotide-targeted scission either by lengthening the tether to the phenanthroline ring system or by inserting the methylene spacer.



(c) Protein-Targeted Scission. Since the oxidative chemical nuclease activity of 1,10-phenanthroline-copper proceeds by a multiple step pathway that includes the reduction of the cupric complex ion, followed by the reductive cleavage of hydrogen peroxide to generate the copper-oxo species directly responsible for scission (19), it is possible that the limiting step in the cleavage chemistry of the targeted and untargeted reactions differs or depends on the carrier used for directing the cleavage. Since proteins are effective targeting ligands of the chemical nuclease activity, we investigated the influence of the intervening methylene group in this method of site-specific scission.

This question was addressed with the *Escherichia coli* Fis protein (top panel of Figure 2), a 98-amino acid homodimer with four α -helices per monomer that functions in a number of biological systems, including recombination, transcription, and DNA replication, yet lacks a well-defined consensus sequence (20, 21). The helix-turn-helix C and D subunits of each monomer comprise the DNA binding domain, and binding induces a consid-

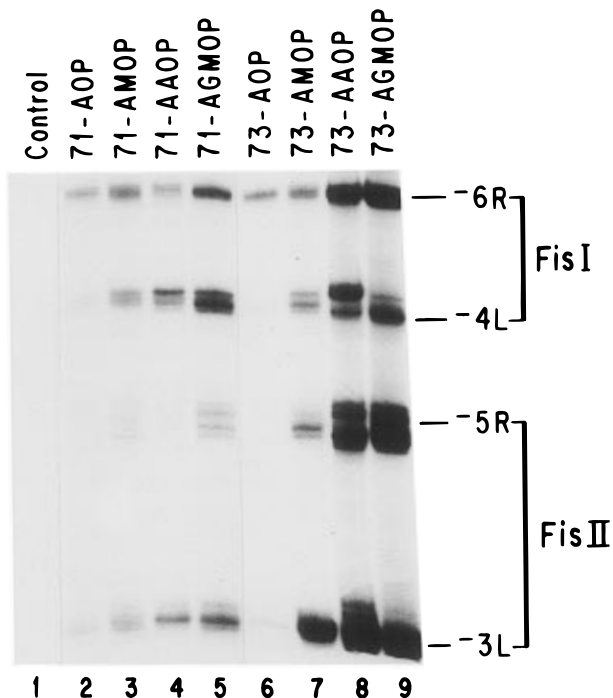


Figure 3. Single-stranded cleavage of the proline permease promoter by bound Fis-OP-Cu conjugates. The numbers on the right refer to the positions of the flanking DNA. Fis binding sites are defined by a 15 bp core with the sequences denoted as -1L to -10L for the left flank and -1R to -10R for the right flank. The parent bands are not shown. AOP = $\text{CH}_2\text{C}(\text{O})\text{NH}-\text{OP}$. AMOP = $\text{CH}_2\text{C}(\text{O})\text{NHCH}_2-\text{OP}$. AAOP = $\text{CH}_2\text{C}(\text{O})\text{NHCH}_2-\text{CH}_2\text{C}(\text{O})\text{NH}-\text{OP}$. AGMOP = $\text{CH}_2\text{C}(\text{O})\text{NHCH}_2\text{C}(\text{O})\text{NHCH}_2-\text{OP}$.

erable degree of DNA bending (22). Four phenanthroline derivatives, IAMOP, IAOP, IAGMOP, and IAAOP, were linked to the Fis mutants R71C and N73C. In the Fis-DNA model, the guanidino group of the Arg-71 side chain points toward, but does not directly contact, the DNA flanking the 15-base pair binding site. The Asn-73 side chain contacts the phosphates on the edge of the binding site (Figure 2B).

Since the difference in the length of the tethers in the Fis-AMOP and Fis-AOP conjugates is one methylene unit, the change in the scission efficiency may not be due to differences in the intrinsic reactivity of the coordination complex. However, the isomeric Fis-AMGOP and Fis-AAOP chimeras have tethers of the same length and chemical composition. As a result, any change in reactivity will reflect insulation of the carboxamide from the aromatic system of the 1,10-phenanthroline ligand.

Figure 3 shows single-stranded cleavage by the Fis-OP-Cu conjugates at two known binding sites on a 266-base pair DNA from the promoter region (-157 to +109) of the *E. coli* proline permease. A dramatic enhancement of the scission upon insertion of the methylene group is evident upon comparison of lane 2 to lane 3 and lane 6 to lane 7. For example, conjugate 73-AOP cleaves weakly only in the right flanking sequence of the Fis binding site I, while 73-AMOP shows strong cleavage in both flanking regions of binding sites I (-48 to -34) and II (-88 to -74). Since Fis-OP-Cu conjugates 71-AAOP, 71-AMGOP, 73-AAOP, and 73-AMGOP have isomeric tethers of identical length, the insulating effect of the methylene group (compare lane 4 to lane 5 and lane 8 to lane 9) is responsible for the observed enhancements. Scission is enhanced by a factor of 1.7–3.2 at most sites (Table 2).

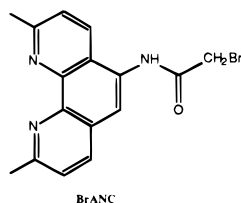
However, scission efficiency is not always enhanced. For sites that are efficiently cleaved by chimeras prepared from 5-(iodoacetamido)-1,10-phenanthroline, in-

Table 2. Ratios of DNA Scission by Fis–OP Conjugates with Isomeric Tethers

	scission state			
	–6R	–4L	–5R	–3L
71-AGMOP:71-AAOP	2.4	1.8	3.2	1.7
73-AGMOP:73-AAOP	2.7	0.8	1.7	0.7

sulation of the ring system by a methylene derivative does not yield increased scission. Indeed, a slight decrease is observed at the two sites most efficiently cleaved by the chimera prepared with N73C and IAAOP (Table 2). The insulated OP derivatives only enhance scission at the weaker scission sites of these conjugates.

Targeted Scission by Chimeras Prepared with a Neocuproine Analog. Substituents at phenanthroline carbons 2 and 9 inhibit the oxidative activity of the copper chelates with stoichiometry of two ligands per metal ion. However, *targeted* scission with chimeric nucleases incorporating 1,10-phenanthroline–copper reflects the reactivity of the 1:1 complex. The lack of reactivity of the free 2:1 complex of 2,9-dimethyl-1,10-phenanthroline [neocuproine (NC)], therefore, does not necessarily mean the 1:1 complex is inactive. Given the enhanced reactivity of copper complexes composed of methyl-substituted phenanthrolines (12), chimeric nucleases prepared with the neocuproine derivative (BrANC) could be more reactive than the parent phenanthroline. To test this idea, the same oligonucleotide-targeting system described in the top panel of Figure 1 was used to assay the cleavage competence of NC-linked oligonucleotides.



The results (Figure 4) clearly indicate that oligonucleotides linked to phenanthroline (lanes a and b) and neocuproine (lane e) are directly comparable in scission yield, although the patterns of scission are not identical. Although NC–oligonucleotides cleave within the double-stranded region, they cleave more efficiently in the less sterically constrained single-stranded regions than do the AOP-linked conjugates. Central to this comparison is that chimeras prepared with NC-linked oligonucleotides cleave by the same reaction mechanism as OP-linked oligonucleotides. The principal criterion for similarity (or identity) of mechanism is that the cleavage reaction is dependent on the cuprous ion and hydrogen peroxide. The absolute requirement for a reducing agent (e.g., 3-mercaptopropionic acid or ascorbic acid) indicates the importance of cuprous ion, and the inhibition of scission by catalase reflects the importance of hydrogen peroxide (23).

In all the OP conjugates described thus far, the carrier was linked through phenanthroline C5. The cleavage efficiency of 1,10-phenanthroline derivatives in which the tether was placed at positions 2 or 4 was also explored. An oligonucleotide linked to OP by a 2-aminomethyl substituent (using the phenanthroline derivative 2-IA-MOP) did not lead to measurable cleavage in the target DNA even in the presence of added hydrogen peroxide (Figure 4, lane f). Since this analog should be comparable in intrinsic reactivity to the 5-aminomethyl derivative, linkage through the 2 position must inhibit the close approach of the copper–oxo species to the C1'H of the

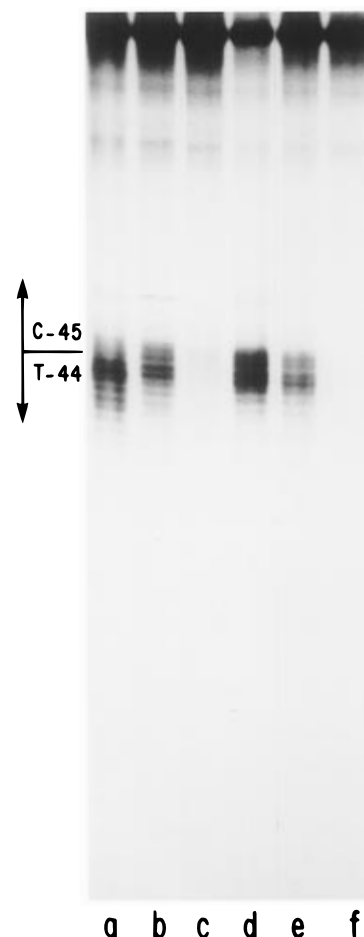
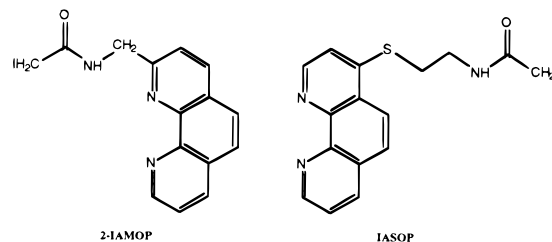


Figure 4. Targeted scission of a single-stranded DNA by oligonucleotides tethered to neocuproine via C5 and to 1,10-phenanthroline via C2, C4, and C5: lane a, AOP–oligonucleotide; lane b, AAOP–oligonucleotide; lane c, ASOP–oligonucleotide; lane d, 5-glycylneocuproine–oligonucleotide (see Experimental Section); lane e, ANC–oligonucleotide; lane f, 2-AMOP–oligonucleotide.

deoxyribose, the presumed oxidative target of the nuclease activity. Because of its inactivity, further chimeras were not prepared with this analog. Modified oligonucleotides were also prepared with the derivative IASOP. As in the case of the C2-substituted conjugates, no cleavage was observed (Figure 4, lane c).



Targeting the Template Strand of the *E. coli* RNA Polymerase–Lac UV-5 Open Complex. The most novel reactivity of the chemical nuclease bis(1,10-phenanthroline)–copper(I) is its cleavage of the single-stranded template strand of catalytically competent open transcription complexes formed between promoters and RNA polymerases (24–27). Although hyperreactivity of the nuclease at several prokaryotic and eukaryotic transcription start sites has been reported (28, 29), we have studied the *E. coli* lac UV-5 promoter and *E. coli* RNA polymerase most extensively. The single-stranded template strand is also accessible to sequence-specific

hybridization to complementary oligonucleotides, and we have demonstrated that OP-linked 5'-UGGAA-3' specifically cleaves the template strand of the *E. coli* RNA polymerase-lac UV-5 open complex at positions -4 to -6 (30). Since NC exhibited preferential cleavage of single-stranded regions of DNA in the experiments summarized in Figure 4, we examined the targeted scission reaction by NC-linked UGGAA. The NC-UGGAA conjugate caused little detectable scission of the template strand. This result suggests that the 2,9-dimethyl groups block the steric approach to the single-stranded template strand of the open complex. This potential steric interference, albeit different in origin from that responsible for the lack of oxidative activity of the 2:1 coordination complex, (NC)₂Cu⁺, suggests that the neocuproine derivative will not be as generally useful as other OP derivatives in targeted scission. Moreover, neocuproine linked to Fis N71C did not cleave Fis binding sites (data not shown), again indicating that the methyl groups at the 2 and 9 positions may block the access of the copper-oxo group to the oxidatively sensitive C1'H of the deoxyribose in bent DNA. While these experiments suggest the limitations of NC in the preparation of targeted scission reagents, they confirm the view that diffusible reactive species are not being generated by this redox-active coordination complex.

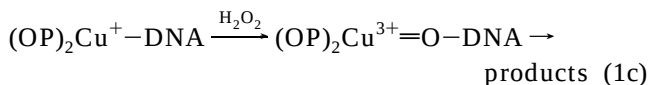
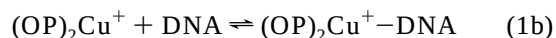
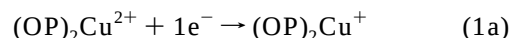
DISCUSSION

The chimeras prepared by tethering a series of 1,10-phenanthroline derivatives to oligonucleotides and proteins have provided valuable insights into the mechanism of DNA scission by 1,10-phenanthroline-copper(I) linked to a targeting ligand. The studies with 2,9-dimethyl-1,10-phenanthroline (neocuproine) have been instructive in two contexts. First, they have provided definitive, confirmatory evidence that the nucleolytic species formed upon tethering 1,10-phenanthroline to a ligand is active as the 1:1 complex. If the cleavage reaction always required assistance from a second ligand, these conjugates would be cleavage-inactive. The preferential reactivity of neocuproine-oligonucleotide conjugates toward single-stranded DNA, however, indicates that steric constraints continue to influence the reactivity but arise from the interference of the ortho methyl groups with the target rather than with a second NC ligand.

The second context in which the studies with NC are informative is that their competence as scission reagents suggests that the oxidative reaction proceeds through a copper-oxo intermediate rather than through a metal-coordinated peroxy species. Methyl substituents at phenanthroline carbons 2 and 9 would disfavor a metal-coordinated peroxy species. Yet there are no differences in the extent of DNA cleavage by the NC- and OP-oligonucleotide chimeras. As a result, this mechanism is excluded.

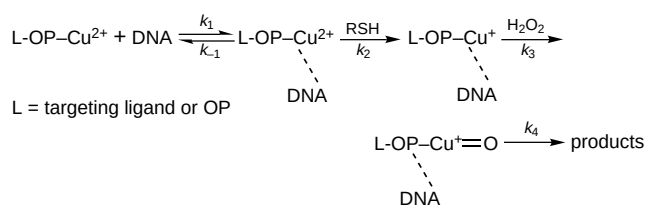
Despite the enhanced reactivity in the untargeted scission of DNA by (5-AcNHCH₂OP)₂Cu⁺ as compared to that of (5-AcNHOP)₂Cu⁺, site-specific scission reagents incorporating the 5-(carboxamidomethyl)-1,10-phenanthroline unit are not routinely more efficient than chimeras prepared with the carboxamido group directly attached to the phenanthroline ring system. The explanation for this dilemma may reside in the multistep reaction pathway of the chemical nuclease. The first step in the kinetic sequence is the reduction of the cupric complex to the corresponding cuprous complex, which then binds to the DNA and reductively cleaves hydrogen peroxide to generate the oxo species directly responsible for DNA cleavage (31). Previous work has suggested that there is an obligatory order in the reaction sequence (12).

Since the 2:1 complex (OP)₂Cu²⁺ intercalates into DNA and is not accessible to reduction (32), the only kinetically viable pathway for the formation of the cuprous complex is the reduction of the freely diffusible cupric complex.



In untargeted scission, the reductive cleavage of hydrogen peroxide may be the slow step at the low concentrations of *in situ*-generated hydrogen peroxide. The immeasurably fast rate of DNA cleavage observed when millimolar concentrations of hydrogen peroxide are added to the reaction mixture supports this observation (14). The aminomethyl derivatives would react more quickly because the electron-releasing methylene group stabilizes the cupric complex and therefore facilitates the formation of the higher valent cupric ion formed by hydrogen peroxide reduction. In untargeted scission, scission is more efficient because the slow step of the reaction (i.e., reduction of hydrogen peroxide, eq 1c) is enhanced.

In targeted scission, on the other hand, the 1:1 OP-Cu complex may be bound tightly to DNA, in which case the cupric complex must be reduced at its specific site even though the negatively charged DNA may hinder the approach of the anionic 3-mercaptopropionate. The failure to see an enhanced rate of cleavage in targeted scission may result from a difference in the rate-determining step in untargeted and targeted scission. In targeted scission, the slow step of the cleavage reaction may be reduction of the DNA-bound cupric complex (e.g., *k*₂, RSH). This first and rate-limiting step would not be enhanced by the 5-methylene substituent. Chimeras prepared with the 5-methylene derivatives will only show rate enhancement when the metal ion can be easily reduced and the reductive cleavage of peroxide (e.g., *k*₃, H₂O₂) limits the overall rate of scission. This is likely to occur at weak sites of scission because in these cases the copper ion does not approach as closely to the oxidatively sensitive bonds of the deoxyribose and therefore is more accessible to solvent and reductant.



In summary, these studies indicate that the efficiency of targeted scission is primarily a function of the orientation of the OP-Cu to the oxidatively sensitive C1'H of the deoxyribose (33). Modification of the phenanthroline leads to changes in the reactivity of these conjugates but not always in a predictable manner. These studies demonstrate two important mechanistic features of these chimeras. (a) Only a single phenanthroline ligand in the coordination complex is essential for scission activity, and (b) a metal-coordinated peroxy intermediate is not involved in the scission. The important contribution of orientation to scission efficiency underscores the fact that the oxidative species responsible for scission is not freely diffusible. The variable reactivity of the 5-aminomethyl OP derivatives in the Fis-targeted scission indicates that

the rate of formation of the cuprous complex may be rate-limiting in the overall reaction especially at strong scission sites where the metal ion may not be freely accessible to diffusible reductants such as 3-mercaptopropionic acid or ascorbic acid.

EXPERIMENTAL SECTION

Synthesis of Neocuproine Derivatives. *5-Nitro-neocuproine.* Neocuproine (GFS Chemicals) (2.0 g) in a mixture of concentrated H_2SO_4 (15 mL) and fuming HNO_3 (9 mL) was heated to reflux for 3–5 h. The reaction mixture was then added to ice and enough solid NaOH to give an alkaline pH. The product was extracted twice with equal volumes of CHCl_3 , the CHCl_3 was removed on a rotary evaporator, and the product was recrystallized from a mixture of CHCl_3 and hexanes, thereby yielding pure 5-nitro-neocuproine (0.55 g, 27%): ^1H NMR (CDCl_3 , 200 MHz) δ 2.98 (s, 3H), 3.00 (s, 3H), 7.63 (d, $J = 8.4$ Hz, 1H), 7.67 (d, $J = 8.8$ Hz, 1H), 8.28 (d, $J = 8.2$ Hz, 1H), 8.61 (s, 1H), 8.92 (d, $J = 8.8$ Hz, 1H).

5-Aminoneocuproine. 5-Nitro-neocuproine (0.5 g) was suspended in ethanol (40 mL), $(\text{NH}_4)_2\text{S}$ (80 mL, 11% w/v in ethanol) was added, and the mixture was heated to reflux for 3–5 h. The solvent was removed on a rotary evaporator, water was added, and the pH was adjusted to 14 with aqueous KOH. The product was extracted three times with equal volumes of CHCl_3 . After removal of the CHCl_3 , the product was recrystallized from a mixture of CHCl_3 and hexanes, thereby yielding 5-aminoneocuproine (0.3 g, 60%): ^1H NMR (CDCl_3 , 200 MHz) δ 2.85 (s, 3H), 2.92 (s, 3H), 6.89 (s, 1H), 7.36 (d, $J = 8.4$ Hz, 1H), 7.49 (d, $J = 8.4$ Hz, 1H), 7.98 (d, $J = 8.2$ Hz, 1H), 8.16 (d, $J = 8.6$ Hz, 1H).

5-(Bromoacetamido)neocuproine (BrANC). 5-Amino-neocuproine (0.3 g) was dissolved in CHCl_3 (10 mL), and a layer of aqueous KOH (0.5 M, 10 mL) was added on top. Bromoacetyl bromide (150 mL, 1.3 molar equiv, Aldrich Chemical Co.) was added via a Pasteur pipette to the organic layer on the bottom. The reaction mixture was allowed to stir overnight at 25 °C. The reaction was then analyzed by TLC, and additional bromoacetyl bromide was added if necessary until complete conversion to the acylated product was observed. Next, the solution was shaken vigorously, the clear organic phase containing the product was removed, the CHCl_3 was removed on a rotary evaporator, and the product was recrystallized from hexanes: ^1H NMR (CDCl_3 , 200 MHz) δ 2.82 (s, 3H), 2.90 (s, 3H), 4.28 (s, 2H), 7.42 (d, $J = 10$ Hz, 1H), 7.47 (d, $J = 8.6$ Hz, 1H), 8.10 (d, $J = 8.2$ Hz, 1H), 8.19 (s, 1H), 8.40 (d, $J = 12$ Hz, 1H).

Synthesis of 5-(Aminomethyl)-1,10-phenanthroline and Its Derivatives. *5-(Bromomethyl)-1,10-phenanthroline.* 5-Methyl-1,10-phenanthroline (367.1 mg, 1.892 mmol, GFS Chemicals), benzoyl peroxide (50.2 mg, 0.11 molar equiv), and *N*-bromosuccinimide (355.5 mg, 1.056 molar equiv) were added to dry benzene (19 mL) in a round-bottomed flask, and the mixture was illuminated, under an atmosphere of argon, with a 300 W incandescent light bulb placed about 5 in. from the flask. The temperature within the flask was maintained at 50–60 °C, and the reaction was allowed to proceed for 2 h. TLC and ^1H NMR analyses of the supernatant at the end of the 2 h period indicated that no unreacted 5-methyl-1,10-phenanthroline remained and that the major product was the desired bromide. The reaction mixture was cooled on ice for at least 1 h and then filtered to remove a reddish tar and the succinimide which precipitated during cooling. The solids were rinsed twice with benzene. The benzene was removed from the filtrate on a

rotovap, and the resulting bromide was used without further purification: ^1H NMR (CDCl_3 , 200 MHz) δ 9.24 (dd, $J = 4.7, 1.6$ Hz, 1H), 9.20 (dd, $J = 4.4, 1.6$ Hz, 1H), 8.54 (dd, $J = 8.4, 1.5$ Hz, 1H), 8.19 (dd, $J = 8.0, 1.6$ Hz, 1H), 7.84 (s, 1H), 7.72 (dd, $J = 8.4, 4.4$ Hz, 1H), 7.62 (dd, $J = 8.2, 4.4$ Hz, 1H), 4.94 (s, 2H).

5-(Aminomethyl)-1,10-phenanthroline (AMOP). Ethanol (16 mL) and concentrated aqueous ammonia (4 mL) were added to the crude 5-(bromomethyl)-1,10-phenanthroline, and the mixture was stirred at 22 °C for 2 h. Next, the ethanol and excess ammonia were removed on a rotovap. Dichloromethane was added to the residue, and then 0.1 N HCl was added until the aqueous layer had a pH of about 4. The aqueous layer containing the desired product was extracted several times with dichloromethane, then the pH was adjusted to about 12 with 1 N NaOH, and the product was extracted seven to ten times with dichloromethane. The dichloromethane extract was dried over Na_2SO_4 , and the volatiles were removed on a rotovap, thereby giving the crude product as a yellow oil. The product was purified by flash chromatography on silica gel (2 cm inside diameter $\times$ 13 cm, 98:2:0.5 CHCl_3 : CH_3OH :isopropylamine) to afford 5-(aminomethyl)-1,10-phenanthroline as a light yellow oil (138.9 mg, 35% from 5-methyl-1,10-phenanthroline): ^1H NMR (CD_3OD , 200 MHz) δ 9.06 (dd, $J = 4.4, 1.4$ Hz, 1H), 9.02 (dd, $J = 4.4, 1.5$ Hz, 1H), 8.59 (dd, $J = 8.4, 1.3$ Hz, 1H), 8.37 (dd, $J = 8.1, 1.4$ Hz, 1H), 7.87 (s, 1H), 7.76 (dd, $J = 8.5, 4.4$ Hz, 1H), 7.71 (dd, $J = 8.3, 4.4$ Hz, 1H), 4.35 (s, 2H); ^{13}C NMR (CD_3OD , 50 MHz) δ 152.9, 152.8, 149.0, 148.5, 140.5, 139.9, 135.8, 132.1, 130.8, 127.3, 127.2, 126.8, 45.7; HRMS (EI) m/e 209.0958 (M^+ , calculated for $\text{C}_{13}\text{H}_{11}\text{N}_3$ 209.0953).

5-(Iodoacetamidomethyl)-1,10-phenanthroline (IAMOP). Iodoacetic acid (8.6 mg, 1.1 molar equiv) and 1,3-dicyclohexylcarbodiimide (DCC, 9.0 mg, 1.05 molar equiv) were added to 5-(aminomethyl)-1,10-phenanthroline (8.7 mg, 0.042 mmol) in a round-bottomed flask. Chloroform (1.5–1.6 mL) was added, and the reaction flask was degassed and filled with Ar. The reaction mixture was allowed to stir at 22 °C overnight (14–20 h). The solid which had precipitated was removed by filtration. The clear, pale yellow filtrate was extracted three times with 0.01 N HCl. Next, the aqueous extract was washed once with deuteriochloroform. Then, the pH of the aqueous layer was raised to about 9 or 10 by adding saturated aqueous NaHCO_3 dropwise. The product was then extracted into deuteriochloroform (4 $\times$ 2 mL). The clear, pale yellow extract was dried over anhydrous Na_2SO_4 , which was removed by filtration. The volume of the solution was measured, and the purity and yield of the 5-(iodoacetamidomethyl)-1,10-phenanthroline were determined by ^1H NMR using an internal standard (e.g., benzene). The yield is approximately 80%, and the purity is >95%. The product is stable for a few days in chloroform solution if stored at 4 °C but eventually decomposes to give a chloroform-insoluble tan- to purple-colored solid. For derivatization of nucleic acid oligomers or proteins, the CDCl_3 solution of 5-(iodoacetamidomethyl)-1,10-phenanthroline was sufficiently concentrated to be added directly to a solution of the ligand to be derivatized in buffer–dimethylformamide: ^1H NMR (CD_3OD , 200 MHz) δ 9.11 (dd, $J = 4.4, 1.5$ Hz, 1H), 9.08 (dd, $J = 4.4, 1.6$ Hz, 1H), 8.61 (dd, $J = 8.4, 1.5$ Hz, 1H), 8.43 (dd, $J = 8.1, 1.6$ Hz, 1H), 7.95 (s, 1H), 7.81 (dd, $J = 8.4, 4.3$ Hz, 1H), 7.76 (dd, $J = 8.0, 4.4$ Hz, 1H), 4.95 (s, 2H), 3.79 (s, 2H); ^{13}C NMR (CDCl_3 , 90 MHz) δ 167.5, 150.2, 150.0, 145.8, 145.7, 135.9, 132.2, 131.9, 127.6, 126.9, 126.1, 123.2, 123.1, 42.0, –0.66; HRMS (EI) m/e 377.0026 (M^+ , calculated for $\text{C}_{15}\text{H}_{12}\text{N}_3\text{OI}$ 377.0025).

5-[*N*-(Phthaloyl)glycylamidomethyl]-1,10-phenanthroline. *N*-Phthaloylglycine (88.1 mg, 1.1 molar equiv) and DCC (89 mg, 1.1 molar equiv) were added to 5-(aminomethyl)-1,10-phenanthroline (82 mg, 0.39 mmol) in a 10 mL round-bottomed flask equipped with a septum and a magnetic stirring bar. Chloroform (3.9 mL) was added, and the reaction mixture was allowed to stir under an inert atmosphere at 22 °C overnight (16 h). The chloroform was removed on a rotovap, and NMR analysis of the CD₃OD soluble portion of the resulting residue indicated that all the amine had reacted. The crude 5-[*N*-(phthaloyl)glycylamidomethyl]-1,10-phenanthroline was used without purification.

5-(Glycylamidomethyl)-1,10-phenanthroline. Ethanol (8 mL) and hydrazine (36.7 mL, 97%, 3.0 molar equiv) were added to the crude 5-[*N*-(phthaloyl)glycylamidomethyl]-1,10-phenanthroline, and the reaction mixture was allowed to reflux under an atmosphere of argon for 30 min, at which time TLC analysis indicated all the 5-[*N*-(phthaloyl)glycylamidomethyl]-1,10-phenanthroline had reacted to give a more polar product. Next, the volatiles were removed on a rotovap, and the resulting off-white solid was triturated with 0.01 N HCl. The white solid was removed from the aqueous layer containing the product by filtration and was washed well with 0.01 N HCl and water. Then, the pH of the combined aqueous washes was adjusted to about 12 with 1 N NaOH, and the water was removed on a rotovap from a 30 °C water bath. The resulting solid was washed thoroughly with methanol to dissolve the product. A small amount of silica gel was added to the methanol extract, and then the methanol was removed on a rotovap. The resulting powder (the crude product adsorbed onto silica gel) was poured onto a prepacked flash column (2 cm inside diameter × 4 cm), and the product was eluted with 150 mL of 100:3:1 CHCl₃:CH₃OH:isopropylamine, followed by 100 mL of 100:6:2 CHCl₃:CH₃OH:isopropylamine. 5-(Glycylamidomethyl)-1,10-phenanthroline was obtained as a light yellow foam in 56% overall yield (58.3 mg): ¹H NMR (CD₃OD, 200 MHz) δ 9.04 (m, 2H), 8.58 (dd, *J* = 8.4, 1.5 Hz, 1H), 8.36 (dd, *J* = 8.2, 1.7 Hz, 1H), 7.82 (s, 1H), 7.74 (m, 2H), 4.93 (s, 2H), 3.41 (s, 2H); ¹³C NMR (CD₃OD, 90 MHz) δ 171.6, 151.0, 150.8, 146.9, 146.5, 137.7, 134.2, 133.9, 129.7, 128.7, 127.1, 124.9, 124.6, 43.6, 41.6; HRMS (EI) *m/e* 266.1168 (M⁺, calculated for C₁₅H₁₄N₄O 266.1168).

5-[*N*-(2-Iodoacetyl)glycylamidomethyl]-1,10-phenanthroline (IAGMOP). Iodoacetic acid (6.8 mg, 1.1 molar equiv) and DCC (7.0 mg, 1.05 molar equiv) were added to 5-(aminomethyl)-1,10-phenanthroline (8.6 mg, 0.032 mmol) in chloroform (1.5–1.6 mL), and the reaction mixture was allowed to stir under argon at 22 °C overnight (14–20 h). The solid which had precipitated (DCU) was removed by filtration, and the clear, pale yellow filtrate was extracted three times with 0.01 N HCl (1–2 mL per extraction). Next, the aqueous extract was washed once with deuteriochloroform. Then, the pH of the aqueous layer was raised to about 9 or 10 by adding saturated aqueous NaHCO₃ dropwise. The product was then extracted into deuteriochloroform (4 × 2 mL). The clear, pale yellow extract was dried over anhydrous Na₂SO₄, which was removed by filtration. The volume of the solution was measured, and the purity and yield of the 5-[*N*-(2-iodoacetyl)glycylamidomethyl]-1,10-phenanthroline were determined by ¹H NMR using an internal standard (e.g., benzene). The yield is approximately 73%, and the purity is >95%. The product is stable for a few days in chloroform solution if stored at 4 °C but eventually decomposes. For derivatization of nucleic acid oligomers or proteins, the CDCl₃ solution of 5-[*N*-(2-

iodoacetyl)glycylamidomethyl]-1,10-phenanthroline was sufficiently concentrated to be added directly to a solution of the ligand to be derivatized in buffer–dimethylformamide: ¹H NMR (CD₃OD, 200 MHz) δ 9.08 (dd, *J* = 4.4, 1.4 Hz, 1H), 9.05 (dd, *J* = 4.4, 1.6 Hz, 1H), 8.59 (dd, *J* = 8.4, 1.6 Hz, 1H), 8.42 (dd, *J* = 8.1, 1.6 Hz, 1H), 7.88 (s, 1H), 7.78 (dd, *J* = 8.4, 4.3 Hz, 1H), 7.75 (dd, *J* = 7.9, 4.4 Hz, 1H), 4.95 (s, 2H), 3.96 (s, 2H), 3.79 (s, 2H).

Modification of Oligonucleotides with OP Derivatives. The oligonucleotide was first 5'-labeled with thiophosphate by allowing the nucleotide (~5 nmol) to react with [γ -³⁵S] ATP (11.5 nmol, specific activity of 6.6 Ci/mmol) and T4 polynucleotide kinase in 100 μ L of kinase buffer at 37 °C for 2 h. After phenol/chloroform extraction, the labeled product was desalted by passage through a Sephadex G-50 spin column. An aliquot (5 μ L) was saved as a reference, while the remainder was immediately derivatized. Typically, a 30-fold excess of an iodoacetamido derivative of OP in aqueous DMF was used, and the reaction was carried out at 37 °C for 2 h. The modified product was electrophoresed on a 15% denaturing polyacrylamide gel along with the γ -³⁵S-labeled but underivatized reference oligonucleotide. The OP-derivatized product, which moved more slowly than the reference, was excised, eluted, and precipitated with ethanol.

The neocuproine-derivatized oligonucleotide was also prepared from 5-glycylneocuproine. The 5'-phosphorylated oligonucleotide was first converted into its imidazolide by reaction with [3-(dimethylamino)propyl]carbodiimide (0.12 M) in imidazole hydrochloride buffer (100 μ L, 0.1 M, pH 6.1) at room temperature for 2 h as reported previously (18, 34). 2,6-Lutidine (6 μ L) was then added, followed by 5-glycylneocuproine (6 mg). This mixture was heated at 50 °C for 2 h. The product was purified as described above.

Catalytic Oxidation of 3-Mercaptopropionic Acid. Each redox reaction mixture contained 1 mM MPA in 50 mM phosphate (pH 8.0) at 22 °C. At each time point, a 30 μ L aliquot was diluted with 970 μ L of a solution containing 0.25 mM dithiobis(2-nitrobenzoic acid) and 5 mM EDTA in 50 mM phosphate (pH 8.0). The absorbance of the 4-mercapto-2-nitrobenzoic acid produced was measured at 412 nm.

Kinetics of DNA Scission by Phenanthroline-Copper Complexes. Cleavage reactions were carried out in 40 mM Tris/50 mM KCl/10 mM MgCl₂, 5.8 mM MPA, and pH 7.9 buffer for 4.0 min at 37 °C. The reactions were quenched with EDTA, and the DNA was precipitated, redissolved in loading buffer, and electrophoresed in a 12% denaturing polyacrylamide gel. The relative amounts of uncut parent band were measured using a Molecular Dynamics Phospho-Imager 445SI.

Cleavage of Single-Stranded DNA by Oligonucleotide-1,10-Phenanthroline-Copper(I) Chimeras. The single-stranded substrate was synthesized on a Pharmacia Gene Assembler, 5'-labeled with γ -³²P and T4 kinase, and purified using a 10% denaturing polyacrylamide gel. In a typical cleavage experiment, a solution of substrate (0.1 pmol) and OP-derivatized oligonucleotide (1–5 pmol) in 20 μ L of buffer (50 mM, pH 7.5 Tris/50 mM NaCl/5 mM MgCl₂) was denatured by heating to 90 °C for 5 min and then allowed to reanneal at room temperature for 1 h. The cleavage reaction was initiated by addition of aqueous CuSO₄ (2 μ L, 200 μ M) and MPA (2 μ L, 58 mM). After 2 h at 37 °C, the reaction was quenched with neocuproine. Products were analyzed on a 8% denaturing polyacrylamide gel.

Cleavage of the *E. coli* Proline Permease Promoter by Fis Protein-1,10-Phenanthroline-Cop-

per(I) Chimeras. The Fis-1,10-phenanthroline conjugates were incubated with ^{32}P end-labeled DNA probe in 20 mM Tris (pH 7.5)/80 mM NaCl/50 mM CuSO_4 for 10 min at 22 °C. MPA was added to a concentration of 3 mM to initiate cleavage. The reaction was quenched with neocuproine after 1 h, loading buffer was added, and the cleavage products were separated by denaturing PAGE (12%). The relative intensities of the bands were determined using a Molecular Dynamics Phospho-Imager 445SI.

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LITERATURE CITED

- (1) Lerner, R. A., Benkovic, S. J., and Schultz, P. G. (1991) At the Crossroads of Chemistry and Immunology: Catalytic Antibodies. *Science* 252, 659–667.
- (2) Thorn, S. N.; Daniels, R. G.; Auditor, M.-T. M., and Hilvert, D. (1995) Large rate accelerations in antibody catalysis by strategic use of haptenic charge. *Nature* 373, 228–230.
- (3) Szostak, J. W. (1992) *In vitro* genetics. *TIBS* 17, 89–93.
- (4) Malcolm, B. A., Rosenberg, S., Corey, M. J., Allen, J. S., Baetselier, A. d., and Kirsch, J. D. (1989) Site-Directed Mutagenesis of the Catalytic Residues Asp-52 and Glu-35 of Chicken Egg White Lysozyme. *Proc. Natl. Acad. Sci. U.S.A.* 86, 133–137.
- (5) Sprang, S., Standing, T., Fletterick, R. J., Stroud, R. M., Finer-Moore, J., Xuong, N. H., Hamlin, R., Rutter, W. J., and Craik, C. S. (1987) Three-dimensional Structure of Asn¹⁰² Mutant of Trypsin: Role of Asp¹⁰² in Serine Protease Catalysis. *Science* 237, 905–909.
- (6) Wagner, C., and Benkovic, S. (1990) Site directed mutagenesis: a tool for enzyme mechanism dissection. *Trends Biotechnol.* 8, 263–270.
- (7) Sigman, D. S., Bruice, T. W., Mazumder, A., and Sutton, C. L. (1993) Targeted Chemical Nucleases. *Acc. Chem. Res.* 26, 98–104.
- (8) Pan, C. Q., Landgraf, R., and Sigman, D. S. (1994) DNA Binding Proteins as Site-Specific Nucleases. *Mol. Microbiol.* 12, 335–342.
- (9) Wu, J., Perrin, D. M., Sigman, D. S., and Kaback, H. R. (1995) Helix packing of lactose permease in *Escherichia coli* studied by site-directed chemical cleavage. *Proc. Natl. Acad. Sci. U.S.A.* 92, 9186–9190.
- (10) Sigman, D. S., Mazumder, A., and Perrin, D. M. (1993) Chemical Nucleases. *Chem. Rev.* 93, 2295–2316.
- (11) Bruice, T. W., Wise, J., Rosser, D. S. E., and Sigman, D. S. (1991) Conversion of Lambda Phage Cro into an Operator-Specific Nuclease. *J. Am. Chem. Soc.* 113, 5446–5447.
- (12) Thederahn, T. B., Kuwabara, M. D., Larsen, T. A., and Sigman, D. S. (1989) Nuclease Activity of 1,10-Phenanthroline-copper: Kinetic Mechanism. *J. Am. Chem. Soc.* 111, 4941–4946.
- (13) Graham, D. R., Marshall, L. E., Reich, K. A., and Sigman, D. S. (1980) Cleavage of DNA by Coordination Complexes. Superoxide Formation in the Oxidation by 1,10-Phenanthroline-Cuprous Complexes by Oxygen—Relevance to DNA-cleavage Reaction. *J. Am. Chem. Soc.* 102, 5419–5421.
- (14) Marshall, L. E.; Graham, D. R.; Reich, K. A., and Sigman, D. S. (1981) Cleavage of Deoxyribonucleic Acid by the 1,10-Phenanthroline-Cuprous Complex. Hydrogen Peroxide Requirement and Primary and Secondary Structure Specificity. *Biochemistry* 20, 244–250.
- (15) Reich, K. A., Marshall, L. E., Graham, D. R., and Sigman, D. S. (1981) Cleavage of DNA by the 1,10-Phenanthroline-Copper Ion Complex. Superoxide Mediates the Reaction Dependent on NADH and Hydrogen Peroxide. *J. Am. Chem. Soc.* 103, 3582–3584.
- (16) Kimura, E., Bu, X., Shionoya, M., Wada, S., and Maruyama, S. (1992) New Nickel(II) Cyclam (Cyclam = 1,4,8,11-Tetraazacyclotetradecane) Complex Covalently Attached to $\text{Ru}(\text{phen})_3^{2+}$ (phen = 1,10-Phenanthroline). A New Candidate for the Catalytic Photoreduction of Carbon Dioxide. *Inorg. Chem.* 31, 4542–4546.
- (17) Ellman, G. L., Courtney, K. D., Andres, V., and Featherstone, R. M. (1961) A New and Rapid Colorimetric Determination of Acetylcholinesterase Activity. *Biochem. Pharmacol.* 7, 89–95.
- (18) Chen, C.-h. B., and Sigman, D. S. (1986) Nuclease Activity of 1,10-Phenanthroline-copper: Sequence-specific Targeting. *Proc. Natl. Acad. Sci. U.S.A.* 83, 7147–7151.
- (19) Pan, C. Q., Feng, J., Finkel, S. E., Landgraf, R., Johnson, R., and Sigman, D. S. (1994) Structure of the *Escherichia coli* fis protein-DNA complex probed by protein conjugated with 1,10-phenanthroline copper(I) complex. *Proc. Natl. Acad. Sci. U.S.A.* 91, 1721–1725.
- (20) Finkel, S. E., and Johnson, R. C. (1992) The Fis protein: it's not just for DNA inversion anymore. *Mol. Microbiol.* 6, 3257–3265.
- (21) Yuan, H. S., Finkel, S. E., Feng, J.-A., Kaczor-Grzeskowiak, M., Johnson, R. C., and Dickerson, R. E. (1991) The molecular structure of wild-type and a mutant Fis protein: Relationship between mutational changes and recombinational enhancer function or DNA binding. *Proc. Natl. Acad. Sci. U.S.A.* 88, 9558–9562.
- (22) Feng, J.-A., Yuan, H. S., Finkel, S. E., Johnson, R. C., Kaczor-Grzeskowiak, M., and Dickerson, R. E. (1992) The Interaction of Fis Protein with Its DNA-Binding Sequences. *Proteins*, Vol. 2, pp 1–9, Adenine Press, Guilderland, NY.
- (23) Sigman, D. S., Graham, D. R., D'Aurora, V., and Stern, A. M. (1979) Oxygen-dependent Cleavage of DNA by the 1,10-Phenanthroline-cuprous Complex. Inhibition of *Escherichia coli* DNA Polymerase I. *J. Biol. Chem.* 254, 12269–12272.
- (24) Spassky, A., and Sigman, D. S. (1985) Nuclease Activity of 1,10-Phenanthroline-Copper Ion. Conformational Analysis and Footprinting of the *lac* Operon. *Biochemistry* 24, 8050–8056.
- (25) Kuwabara, M. D., and Sigman, D. S. (1987) Footprinting DNA-Protein Complexes in Situ following Gel Retardation Assays Using 1,10-Phenanthroline-Copper Ion: *Escherichia coli* RNA Polymerase-*lac* Promoter Complexes. *Biochemistry* 26, 7234–7238.
- (26) Thederahn, T., Spassky, A., Kuwabara, M. D., and Sigman, D. S. (1990) Chemical Nuclease Activity of 5-Phenyl-1,10-phenanthroline-Copper Ion Detect Intermediates in Transcription Initiation by *E. coli* RNA Polymerase. *Biochem. Biophys. Res. Commun.* 168, 756–762.
- (27) Mazumder, A., Perrin, D. M., Watson, K. H., and Sigman, D. S. (1993) A transcription inhibitor specific for unwound DNA in RNA polymerase-promoter open complexes. *Proc. Natl. Acad. Sci. U.S.A.* 90, 8140–8144.
- (28) Frantz, B., and O'Halloran, T. V. (1990) DNA Distortion Accompanies Transcriptional Activation by the Metal-Responsive Gene-Regulatory Protein MerR. *Biochemistry* 29, 4747–4751.
- (29) Buratowski, S., Sopta, M., Greenblatt, J., and Sharp, P. A. (1991) RNA Polymerase II-associated Proteins are Required for a DNA Conformation Change in the Transcription Initiation Complex. *Proc. Natl. Acad. Sci. U.S.A.* 88, 7509–7513.
- (30) Perrin, D. M., Mazumder, A., Sadeghi, F., and Sigman, D. S. (1994) Hybridization of a complementary ribooligonucleotide to the transcription start site of the *lac* UV-5-*Escherichia coli* RNA polymerase open complex. Potential for gene-specific inactivation reagents. *Biochemistry* 33, 3848–3854.
- (31) Sigman, D. S. (1986) Nuclease Activity of 1,10-Phenanthroline-Copper Ion. *Acc. Chem. Res.* 19, 180–186.
- (32) Graham, D. R., and Sigman, D. S. (1984) Zinc Ion in *E. coli* DNA Polymerase: A Reinvestigation. *Inorg. Chem.* 23, 4188–4191.
- (33) Goynes, T. E., and Sigman, D. S. (1987) Nuclease Activity of 1,10-Phenanthroline-Copper Ion. Chemistry of Deoxyribose Oxidation. *J. Am. Chem. Soc.* 109, 2846–2848.
- (34) Chu, B. C. F., Wahl, G. M., and Orgel, L. E. (1983) Derivatization of Unprotected Polynucleotides. *Nucleic Acids Res.* 11, 6513–6529.

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Tc-99m-Labeled Tropenes as Dopamine Transporter Imaging Agents

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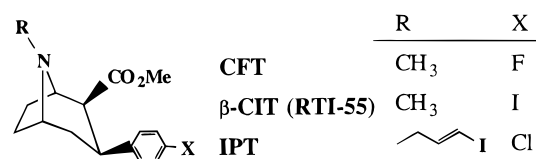
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The development of novel Tc-99m-labeled tropane derivatives as dopamine transporter imaging agents is reported. A series of neutral and lipophilic conjugated complexes, containing *N*-(alkylthiolato)-tropane, aminobis(ethylthiolato), and a [^{99m}Tc]TcO³⁺ center core, were prepared and evaluated as central nervous system (CNS) dopamine transporter imaging agents in rats. One of the compounds, [^{99m}Tc]technetium, [methyl 3-(4-chlorophenyl)-8-(2-mercaptoethyl)-8-azabicyclo[3.2.1]octane-2-carboxylato-S][[2,2'-(methylimino)bis[ethanethiolato]](2-)-*N,S,S'*]oxo (**25**), displayed low initial uptake in rat brain (0.1% at 2 min post iv injection); the striatal/cerebellar (ST/CB) ratio reached 3.50 at 60 min after an iv injection. The specific uptake can be blocked by pretreating rats with a competing dopamine transporter binding agent, β -CIT (RTI-55, *N*-methyl-2 β -carbomethoxy-3 β -(4-iodophenyl)tropane; iv, 1 mg/kg), which reduced the regional brain uptake ratio (ST/CB) to 1.0. In contrast, the specific uptake in striatum was not affected by pretreating rats with a noncompeting ligand, haldol (iv, 1 mg/kg). *In vitro* autoradiography of rat brain sections exhibited elevated labeling in striatum, major islands of Calleja, and olfactory tubercle regions, where dopamine neurons are known to be concentrated. This series of compounds is the first example of technetium-99m labeled CNS receptor-specific imaging agents and may provide a convenient source of short-lived imaging agents for routine diagnosis of CNS abnormality in conjunction with single photon emission computed tomography.

Central nervous system (CNS) receptor function has been successfully evaluated *in vivo* (1–3) using C-11 ($T_{1/2}$ = 20 min, β^+) or F-18 ($T_{1/2}$ = 120 min, β^+) labeled agents for positron emission tomography (PET) imaging and I-123 ($T_{1/2}$ = 13 h, 159 keV) labeled agents for single photon emission computed tomography (SPECT) imaging. Despite tremendous success in developing new techniques using PET and SPECT for imaging CNS receptors, their use in routine procedures is hampered by the limited supply of the three isotopes mentioned above, all of which are produced by cyclotron. The development of receptor-specific agents that could be labeled with technetium-99m ($T_{1/2}$ = 6 h, 140 keV) (4, 5), the most widely used radionuclide in diagnostic nuclear medicine, would be highly desirable, since Tc-99m is readily produced by a Mo-99/Tc-99m generator and its medium γ -ray energy emission (140 keV) is suitable for gamma camera detection. Over 85% of the routine nuclear medicine procedures currently performed use radiopharmaceuticals based on Tc-99m. In the past 10 years, significant progress has been made in defining technetium chemistry using the chemical level of Tc-99 ($T_{1/2}$ = 2.1×10^5 years) (4). Improvements in the understanding of technetium chemistry have significantly enhanced the development of a new generation of technetium radiopharmaceuticals that will potentially benefit millions of patients who receive Tc-99m agents for routine nuclear medicine diagnosis.

Radioactive ligands selective for dopamine transporters are useful in evaluating changes in dopamine function *in vivo* and *in vitro*, especially for patients with Parkin-

sonian disease (PD), which is characterized by a selective loss of dopamine neurons in the basal ganglia and substantia nigra. Recent publications describing [¹¹C]-CFT (WIN 35,428) and [¹²³I]- β -CIT (RTI-55) suggest a strong correlation between the decrease in localization in the anterior putamen area and PD symptoms (6–10). These results provide an impetus for further development of these agents for diagnosing and monitoring the treatment of PD patients. Currently, [¹²³I]- β -CIT is being



widely investigated for this purpose. However, one of the drawbacks of [¹²³I]- β -CIT is the length of time (>18 h) required to reach optimal uptake ratio in the target area (semiequilibrium state), the basal ganglia (BG), vs the nontarget area, the frontal cortex (CX). Because of the need for agents with faster equilibrium times, new ligands such as [¹²³I]IPT [*N*-(3-iodopropen-2-yl)-2 β -carbomethoxy-3 β -(4-chlorophenyl)tropane] (11–13) and [¹²³I]- β -CIT-FP (14), both of which reach equilibrium in less than 1 h, will play an important role as the future agents of choice. However, since I-123 is a cyclotron-produced isotope that costs about \$30/mCi, a similar agent labeled with Tc-99m, which costs less than \$1/mCi and is readily available on demand, will be highly desirable for routine clinical study.

The potential of developing Tc-99m-labeled agents for receptor imaging is well recognized (4, 15). Several recent papers demonstrated that it is possible to incorporate [^{99m}Tc^vO]³⁺N₂S₂ [bis(aminoethanethiol) (BAT)] into potential receptor-selective imaging agents for muscarinic receptors (16), vesamicol sites (17), and steroid hormone receptors (18–23). Recently, new compounds composed of mixed ligands—aminobis(ethanethiol) and mono-thiol—complexed with a [^{99m}Tc]Tc^vO]³⁺ center core (“three

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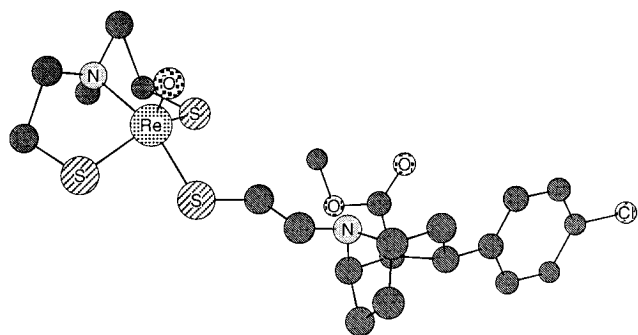


Figure 1. X-ray crystallographic structure of rhenium complex containing a tropane moiety, **23** (27).

plus one complexes") that showed high brain uptake and retention in animals were reported (24–26). These neutral Tc-99m-labeled three plus one complexes for brain imaging, as well as heterodimeric aminothiols plus aminothiols complexes ("two plus two complexes") designed as Tc-99m steroid analogs (18, 19), are quite stable *in vivo* and *in vitro*. To develop Tc-99m-based dopamine transporter imaging agents, we have synthesized monothiol derivatives of tropane, **19–22**, and prepared new Tc-99m complexes containing a $[\text{Tc}^{\text{VO}}]^{3+}$ center core and the mixed ligands bis(aminoethanethiol) and tropane-thiol. The rhenium complex, **23**, has been studied using X-ray crystallography (27) as a surrogate for the Tc-99m complex. Complex **23** shows a Re(V) center with a square pyramidal environment; the oxygen occupies the apical position and the *N*-methyl group is in an anti position to the metal-oxo function (Figure 1), which is similar to that reported for other three plus one complexes with a $\text{S}_1\text{-NS}_2\text{-Tc-core}$ (24).

This paper describes examples of a series of Tc-99m-labeled tropane derivatives, and particularly compound **25**, [$^{99\text{m}}\text{Tc}$](technetium, [methyl 3-(4-chlorophenyl)-8-(2-mercaptoethyl)-8-azabicyclo[3.2.1]octane-2-carboxylato-S][[2,2'-(methylimino)bis[ethanethiolato]](2-)-*N,S,S'*-oxo), developed as potential dopamine transporter imaging agents.

EXPERIMENTAL PROCEDURES

Reagents used in the syntheses were purchased from Aldrich (Milwaukee, WI) or Fluka (Ronkonkoma, NY) and were used without further purification unless otherwise indicated. Anhydrous Na_2SO_4 was used as a drying agent. Reaction yields are reported without attempts at optimization. Thin layer chromatography was performed on EM Science (Gibbstown, NJ) precoated (0.2 mm) silica gel 60 plates, and the spots were detected with iodine vapor and/or UV light. Silica gel 60 (70–230 mesh), obtained from EM Science (Gibbstown, NJ), was used for column chromatography. ^1H NMR spectra were obtained on a Bruker spectrometer (Bruker AC 300). All samples prepared for NMR analysis were dissolved in CDCl_3 , purchased from Aldrich. Chemical shifts are reported as δ values with chloroform or TMS as the internal reference. Coupling constants are reported in hertz. The multiplicity is defined by s (singlet), d (doublet), t (triplet), br s (broad signal), dt (doublet of triplet), and m (multiplet). IR spectra were recorded with a Mattson Polaris FT-IR spectrometer and are reported in cm^{-1} . Melting points were determined on a Meltemp (Cambridge, MA) apparatus and are uncorrected. Elemental analyses were performed by Atlantic Microlabs (Norcross, GA). HRMS was performed by the Nebraska Center for Mass Spectroscopy, University of Nebraska (Lincoln, NE).

Compounds **7**, **8**, **10** (28), and **12** were prepared according to the literature methods. Satisfactory elemen-

tal analyses (within 0.4%) could not be obtained for the thiols and dithiols, and hence HRMS data for those compounds are reported.

Preparation of *N*-[(2-(4'-Methoxybenzylthio)-2-methylpropyl)] 2-(4'-Methoxybenzylthio)-2-methylpropionamide (3**).** 2-(4'-Methoxybenzylthio)-2-methylpropionic acid (2.4 g, 10 mmol) and SOCl_2 (7 mL) in CHCl_3 (50 mL) were heated at reflux for 3 h. The reaction mixture was allowed to cool to room temperature and concentrated *in vacuo*. The resulting oil, **1**, was dried under a high vacuum, redissolved in CH_2Cl_2 (20 mL), and cooled to -20°C . A solution of [2-(4'-methoxybenzylthio)-2-methylpropanamine (**2**) and Et_3N (1.7 mL) in CH_2Cl_2 (20 mL) was then added, and the resulting mixture was stirred at room temperature for 12 h. Water (50 mL) was added, and the product, **3**, was extracted with CH_2Cl_2 (2×25 mL). The CH_2Cl_2 layer was dried (Na_2SO_4) and concentrated *in vacuo* to obtain a viscous oil, which was chromatographed on silica (50% EtOAc/hexane) to obtain the title compound, **3**, as a waxy solid: yield 63%; IR (CHCl_3) 1664 cm^{-1} ; ^1H NMR (300 MHz) δ 1.33 (s, 12H), 2.56 (s, 2H), 3.69 (s, 4H), 3.76 (s, 6H), 6.80 and 7.25 (d, $J = 8.67$ Hz, 4H each); HRMS (FAB), m/z 447.1902 calcd for $\text{C}_{24}\text{H}_{33}\text{NO}_3\text{S}_2$, $M^+ + 1$, 448.1994.

Preparation of *N,N*-Bis[(2-(4'-methoxybenzylthio)-2-methylpropyl)]amine (4**).** Borane/THF (100 mL, 1 M solution in THF) was added to a solution of compound **3** (4.4 g, 10 mmol) in THF (100 mL), and the resulting mixture was heated at reflux under N_2 for 12 h. The reaction mixture was cooled in an ice bath, and water (20 mL) was carefully added. The resulting solution was concentrated *in vacuo* to obtain a viscous oil that was suspended in 6 N HCl (50 mL). This mixture was heated at reflux for 1 h. After the reaction mixture was cooled in an ice bath, it was basified with concentrated NH_4OH and the product, **4**, was extracted with CH_2Cl_2 and purified on silica (EtOAc): yield 63%; mp $58\text{--}60^\circ\text{C}$; IR (CHCl_3) 1609 cm^{-1} ; ^1H NMR (300 MHz) δ 1.35 (s, 12H), 2.59 (s, 4H), 3.71 (s, 4H), 3.78 (s, 6H), 6.81 and 6.24 (d, $J = 9.92$ Hz, 4H each); HRMS (FAB), m/z 433.2109 calcd for $\text{C}_{24}\text{H}_{35}\text{O}_2\text{S}_2\text{N}$, $M^+ + 1$, 434.2198. Anal. Calcd for $\text{C}_{24}\text{H}_{35}\text{O}_2\text{S}_2\text{N}$: C, 66.51; H, 8.06. Found: C, 66.12; H, 8.62.

Preparation of *N,N*-Bis[(2-(4'-methoxybenzylthio)-2-methylpropyl)]methylamine (5**).** Sodium cyanoborohydride (500 mg, 8 mmol) was added to a solution of **4** (2 g, 5 mmol) and HCHO (2 mL, 37% aqueous) in CH_3CN . This was stirred for 15 min, and then glacial HOAc was added dropwise until the solution tested neutral on wet pH papers. The mixture was stirred for 45 min, the solvents were removed, and 2 N KOH (10 mL) was added to the residue. The resulting mixture was extracted with ether (3×10 mL). The ether extracts were combined, washed with 0.2 N KOH (10 mL), and extracted with 1 N HCl (2×20 mL). The acid extracts were combined, neutralized with solid KOH, and re-extracted with ether (3×10 mL). Ether layers were combined and concentrated *in vacuo* to obtain a viscous oil, **5**, which was purified on silica (EtOAc): yield 73%; oil; IR (CHCl_3) 1604 cm^{-1} ; ^1H NMR (300 MHz) δ 1.38 (s, 12H), 2.56 (s, 3H), 2.66 (s, 4H), 3.74 (s, 4H), 3.77 (s, 6H), 6.83 and 7.25 (d, $J = 8.67$ Hz, 4H each); HRMS (FAB), m/z 447.2265 calcd for $\text{C}_{25}\text{H}_{37}\text{O}_2\text{S}_2\text{N}$, $M^+ + 1$, 448.2361.

Preparation of *N,N*-Bis[(2-mercapto)-2-methylpropyl)]methylamine (6**).** Amine **5** (2.6 g, 6 mmol) and anisole (1.5 mL) in TFA (45 mL) were cooled to 0°C , and $\text{Hg}(\text{OAc})_2$ (1.91 g, 6 mmol) was added. This mixture was stirred at 0°C for 15 min and concentrated *in vacuo* at room temperature. The residue was dried under a high vacuum for 30 min, and dry ether (50 mL) was added.

The resulting solid was collected by suction filtration and redissolved in EtOH (100 mL). H₂S gas was then bubbled through the EtOH solution for 15 min, and the black precipitate that formed was filtered through a thick pad of Celite. The filtrate was concentrated *in vacuo* to obtain a colorless oil that was dried under a high vacuum for 30 min. HCl (1 N, 20 mL) and ether (20 mL) were added to the above oil, and the resulting mixture was vigorously stirred for 15 min and transferred to a separating funnel. The aqueous layer was separated and basicified with concentrated NH₄OH, and the resulting colorless product was extracted with CH₂Cl₂ (2 × 20 mL). The CH₂Cl₂ layer was dried (Na₂SO₄) and concentrated *in vacuo* to obtain a viscous oil, **6**, which was stored under a blanket of argon: yield 66%; oil; IR (CHCl₃) 1604 cm⁻¹; ¹H NMR (300 MHz) δ 1.3 (s, 12H), 2.17 (br s, 2H), 2.5 (s, 3H), 2.63 (s, 4H); HRMS (EI), *m/z* 207.1115 calcd for C₉H₂₁NS₂, M⁺ - 2, 205.0955.

Preparation of *N,N*-Bis(2'-mercaptoethyl)-2-methylpropylamine (9). Ethylene sulfide (2 g, 22 mmol), isobutylamine (1.4 mL, 14 mmol), and toluene (5 mL) were heated at 110 °C in a sealed tube for 8 h. Upon cooling, the solution was filtered to remove colorless solid formed during the reaction, and the filtrate obtained was concentrated *in vacuo*. The product, **9**, was obtained by fractional distillation: yield 23%; bp 78–80 °C (4 mmHg); IR (neat) 2964, 2799, 2551 cm⁻¹; ¹H NMR (300 MHz) δ 0.82 (d, *J* = 9 Hz, 6H), 1.64 (br s, 3H), 2.03 (d, *J* = 9 Hz, 2H), 2.54 (m, 8H); HRMS (EI), *m/z* 193.0959 calcd for C₈H₁₉NS₂, M⁺ + 1, 194.1043.

Preparation of *N,N*-Bis(2'-mercaptoethyl)-2-aminoethyl-4-morpholine (11). Ethylene sulfide (5 g, 38 mmol), 4-(2-aminoethyl)morpholine (7.6 mL, 76 mmol), and toluene (30 mL) were heated at 110 °C in a sealed tube overnight. Upon cooling, the solution was filtered to remove colorless solid formed during the reaction, and the filtrate obtained was concentrated *in vacuo*. The product was purified by flash column chromatography on silica (5% methanol/ethyl acetate) to obtain the title compound, **11**, with some impurity. Compound **11** (300 mg) was purified one more time using TLC preparative plates (5% methanol/ethyl acetate) to obtain 160 mg (24%) of **11**: IR (neat) 2958–2807 (s, CH), 2543 (weak, SH) and 1455, 1293, 1114 cm⁻¹; ¹H NMR (300 MHz) δ 1.95 (s, 2H), 2.43–2.45 (m, 6H), 2.65–2.75 (m, 10H), 3.68 (m, 4H). Anal. Calcd for C₁₀H₂₄OS₂N₂Cl₂: C, 37.15; H, 7.48; N, 8.66. Found: C, 37.42; H, 7.33; N, 7.29.

General Procedure for the Preparation of Trityltropane Compounds (15–18). Tritylthiol (Ph₃CSH) (1 g, 3.6 mmol) in THF (5 mL) was added over 5 min to a pentane-washed NaH (60% dispersion, 145 mg, 3.6 mmol) in THF (5 mL) at 0 °C, and the resulting mixture was stirred at room temperature for 10 min. It was then cooled to 0 °C and treated with 1,2-dibromoethane (3.6 mmol) or 1,3-dibromopropane (3.6 mmol). After stirring at room temperature for 30 min, the reaction mixture was concentrated and partitioned between EtOAc (20 mL) and water (10 mL). The EtOAc layer was dried over Na₂SO₄ and concentrated *in vacuo* to obtain a yellow oil that solidified on standing.

1,4-Dioxane (25 mL), Na₂CO₃ (1.15 g, 10.8 mmol), KI (598 mg, 3.6 mmol), and nortropine (**13** or **14**) (2.64 mmol) were sequentially added to the bromo compound obtained above (1 g, 3.6 mmol), and the contents were heated at reflux for 18 h. The reaction mixture was concentrated and partitioned between CH₂Cl₂ (50 mL) and water (20 mL). The CH₂Cl₂ layer was separated, dried over Na₂SO₄, and concentrated *in vacuo* to obtain a brownish yellow oil that was purified by flash chromatography on silica (10% EtOAc/hexane).

***N*-[2'-(Triphenylmethylmercapto)ethyl]-3β-(4''-fluorophenyl)tropane 2β-Carboxylic Acid Methyl Ester (15):** yield 51%; mp 49–51 °C; IR (CHCl₃) 1743 cm⁻¹; ¹H NMR (300 MHz) δ 1.43 (m, 3H), 1.92 (m, 2H), 2.17 (m, 1H), 2.28 (m, 2H), 2.45 and 2.62 (m, each 1H), 2.93 (m, 2H), 3.3 and 3.5 (m, each 1H), 3.43 (s, 3H), 7.3 and 7.5 (m, 19 H); HRMS (FAB), *m/z* 565.2450 calcd for C₃₆H₃₆O₂NSF, M⁺ + 1, 566.2263. Anal. Calcd for C₃₆H₃₆O₂NSF: C, 76.46; H, 6.37. Found: C, 76.27; H, 6.07.

***N*-[2'-(Triphenylmethylmercapto)ethyl]-3β-(4''-chlorophenyl)tropane 2β-Carboxylic Acid Methyl Ester (16):** yield (1.2 g) 57.4%; mp 120–122 °C; IR (CHCl₃) 1739 cm⁻¹; ¹H NMR (300 MHz) δ 1.47 (m, 3H), 1.92 (m, 2H), 2.17 (m, 1H), 2.28 (m, 2H), 2.45 and 2.62 (m, each 1H), 2.93 (m, 2H), 3.3 and 3.5 (m, each 1H), 3.43 (s, 3H), 7.3 and 7.5 (m, 19H); HRMS (EI), *m/z* 581.2155 calcd for C₃₆H₃₆O₂NSCl, M⁺ - Tr, 338.0983; FAB 581.2155 calcd for C₃₆H₃₆O₂NSCl, M⁺ + 1, 582.2233. Anal. Calcd for C₃₆H₃₆O₂NSCl: C, 74.35; H, 6.19. Found: C, 74.01; H, 6.53.

***N*-[2'-(Triphenylmethylmercapto)propyl]-3β-(4''-fluorophenyl)tropane 2β-Carboxylic Acid Methyl Ester (17):** yield 45.9%; mp 72–74 °C; IR (CHCl₃) 1743 cm⁻¹; ¹H NMR (300 MHz) δ 1.5 (m, 5H), 2.12 (m, 6H), 2.55 (dt, *J*₁ = 2.88, *J*₂ = 12.27, 1H), 2.88 (t, *J* = 4.2, 1H), 2.95 (td, *J*₁ = 4.83, *J*₂ = 12.27, 1H) 3.32 and 3.59 (m, each 1H), 3.43 (s, 3H), 6.9–7.4 (m, 19 H); HRMS (FAB), *m/z* 579.2607 calcd for C₃₇H₃₈O₂NSF, M⁺ + 1, 579.2693. Anal. Calcd for C₃₇H₃₈O₂NSF: C, 76.55; H, 6.55. Found: C, 76.10; H, 6.74.

***N*-[2'-(Triphenylmethylmercapto)propyl]-3β-(4''-chlorophenyl)tropane 2β-Carboxylic Acid Methyl Ester (18):** yield 41%; foam; IR (CHCl₃) 1743 cm⁻¹; ¹H NMR (300 MHz) δ 1.5 (m, 5H), 2.12 (m, 6H), 2.55 (dt, *J*₁ = 2.88, *J*₂ = 12.27, 1H), 2.88 (t, *J* = 4.2, 1H), 2.95 (td, *J*₁ = 4.83, *J*₂ = 12.27, 1H) 3.32 and 3.59 (m, each 1H), 3.43 (s, 3H), 6.9–7.4 (m, 19 H); HRMS (FAB), *m/z* 595.2311 calcd for C₃₇H₃₈O₂NSCl, M⁺ + 1, 596.2316. Anal. Calcd for C₃₇H₃₈O₂NSCl: C, 74.62; H, 6.38. Found: C, 74.23; H, 6.74.

General Procedure for the Preparation of Mercaptotropane Compounds (19–22). The appropriate trityltropane compound (**15**–**18**) (1 mmol) was dissolved in CF₃COOH (6.5 mL) and anisole (0.2 mL) at 0 °C, and Hg(OAc)₂ (382 mg, 1.2 mmol) was added. The resulting mixture was stirred at 0 °C for 30 min. The reaction mixture was then concentrated *in vacuo* to obtain a brownish red oil that was dried under high vacuum for 1 h. Anhydrous ether (50 mL) was added to the above oil, and the mixture was kept under sonication for 15 min. The resulting mixture was magnetically stirred for an additional 30 min. The colorless precipitate that formed was collected by suction filtration, dried under a high vacuum for 15 min, and redissolved in EtOH (50 mL). H₂S gas was bubbled through the EtOH solution for 15 min, and the black precipitate that formed was filtered through a thick pad of Celite. The filtrate was concentrated *in vacuo* to obtain a colorless oil that was dried under a high vacuum for 30 min. HCl (1 N, 20 mL) and ether (20 mL) were added to this oil, and the resulting mixture was vigorously stirred for 15 min and transferred to a separating funnel. The aqueous layer was separated and basicified with concentrated NH₄OH, and the resulting colorless product was extracted with CH₂Cl₂ (2 × 20 mL). The CH₂Cl₂ layer was dried (Na₂SO₄) and concentrated *in vacuo*.

***N*-[2'-(Mercapto)ethyl]-3β-(4''-fluorophenyl)tropane 2β-Carboxylic Acid Methyl Ester (19):** yield 68%; 46–48 °C; IR (CHCl₃) 1743 cm⁻¹; ¹H NMR (300

MHz) δ 1.43–1.92 (m, 6H), 2.17 (m, 1H), 2.28 (m, 2H), 2.45 and 2.62 (m, each 1H), 2.93 (m, 2H), 3.3 and 3.5 (m, each 1H), 3.43 (s, 3H), 7.3 (m, 4H); HRMS (FAB), m/z 323.1355 calcd for $C_{17}H_{22}O_2NSF$, $M^+ + 1$, 324.1355.

N-[2'-(Mercapto)ethyl]-3 β -(4''-chlorophenyl)tropane 2 β -Carboxylic Acid Methyl Ester (20): yield 77.5%; mp 69–71 °C; IR (CHCl₃) 1743 cm⁻¹; ¹H NMR (300 MHz) δ 1.7 (m, 4H), 2.05 (m, 3H), 2.17 (m, 3H), 2.60 (dt, $J_1 = 2.97$, $J_2 = 12.39$, 1H), 2.94 (m, 2H), 3.39 and 3.64 (m, each 1H), 3.52 (s, 3H), 7.22 (m, 4 H); HRMS (FAB), m/z 339.1060 calcd for $C_{17}H_{22}O_2NSCl$, $M^+ + 1$, 340.1141.

N-[2'-(Mercapto)propyl]-3 β -(4''-fluorophenyl)tropane 2 β -Carboxylic Acid Methyl Ester (21): yield 71%; waxy solid; IR (CHCl₃) 1741 cm⁻¹; ¹H NMR (300 MHz) δ 1.49 and 2.09 (m, each 6H), 2.53 (dt, $J_1 = 2.88$, $J_2 = 12.27$, 1H), 2.86 (t, $J = 4.2$, 1H), 2.95 (td, $J_1 = 4.83$, $J_2 = 12.27$, 1H), 3.32 and 3.59 (m, each 1H), 3.43 (s, 3H), 6.9 and 7.1 (m, each 2H); HRMS (FAB), m/z 337.1512 calcd for $C_{18}H_{24}O_2NSF$, $M^+ + 1$, 338.1579.

N-[2'-(Mercapto)propyl]-3 β -(4''-chlorophenyl)tropane 2 β -Carboxylic Acid Methyl Ester (22): yield 66%; waxy solid; IR (CHCl₃) 1741 cm⁻¹; δ 1.7 (m, 6H), 2.05 (m, 3H), 2.17 (m, 3H), 2.60 (dt, $J_1 = 2.97$, $J_2 = 12.39$, 1H), 2.94 (m, 2H), 3.39 and 3.64 (m, each 1H), 3.52 (s, 3H), 7.22 (m, 4H); HRMS (FAB), m/z 353.1216 calcd for $C_{18}H_{24}O_2NSCl$, $M^+ + 1$, 354.1211.

Labeling with Tc-99m, Described for Ligand Mixture of 20 and 7 (25). A lyophilized sample of a mixture of **20** (3 μ mol) and **7** (2 μ mol) was dissolved in 200 μ L of CH₃CN, and 100 μ L of 1N HCl and 1 mL of tin glucoheptonate were successively added. [^{99m}Tc]Pertechnetate (1 mL; ranging from 1 to 90 mCi) saline solution was then added to the mixture. The reaction was allowed to stand at room temperature for 30 min. After the complex was extracted from the aqueous reaction medium with ethyl acetate (2 $\times$ 1.5 mL) and the organic solution was dried over Na₂SO₄, ethyl acetate was removed with a flow of N₂. The residue was dissolved in 200 μ L of ethyl acetate and purified by HPLC on a PRP-1 column (4.1 $\times$ 250 mm) with CH₃CN/DMGA (dimethyl glutarate) buffer (5 mM, pH 7; 8:2) as the eluent and a flow rate of 1 mL/min. The retention time of **25** was 16.6 min (radiochemical yield 40%, radiochemical purity >95%). All of the complexes displayed stability at 4 and 24 h after preparation; little change in radiochemical purity was observed. Identical labeling procedures were used for the preparation of the labeled complexes **24–34**, with radiochemical yields of 46, 40, 36, 46, 20, 10, 33, 22, 10, 5, and 21%, respectively (radiochemical purities were all >95%).

Biodistribution in Rats. Male Sprague-Dawley rats (225–300 g) that were allowed free access to food and water were used for *in vivo* biodistribution studies (29, 30). While the rats were under ether anesthesia, 0.2 mL of a saline solution containing **24–34** (50–100 μ Ci) was injected directly into the femoral vein, and the rats were sacrificed by cardiac excision at various time points postinjection. The organs of interest were removed and weighed, and the radioactivity was counted with an automatic gamma counter (Packard 5000). The percentage dose per organ was calculated by a comparison of the tissue counts to suitably diluted aliquots of the injected material. Total activities of blood and muscle were calculated under the assumption that they were 7 and 40% of the total body weight, respectively.

Regional brain distribution in rats was obtained after an injection of **24–34**. Samples from different brain regions (cortex, striatum, hippocampus, and cerebellum) were dissected, weighed, and counted, and the percentage

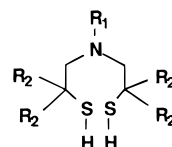
dose per gram of sample was calculated by comparing the sample counts with the count of the diluted initial dose. The uptake ratio of each region was obtained by dividing the percentage dose per gram of that region by that of the cerebellum. For blocking studies, rats were injected with either β -CIT or haloperidol (iv, 1 mg/kg) 5 min prior to injection of **25**. The rats were dissected and brain tissue samples counted as described above.

Partition Coefficients (PC). The PC was measured by mixing the Tc-99m compound with 3 g each of 1-octanol and buffer (pH 7.0 or 7.4, 0.1 M phosphate) in a test tube. The test tube was vortexed for 3 min at room temperature and then centrifuged for 5 min. Two weighed samples (0.5 g each) from the 1-octanol and buffer layers were counted in a well counter. The PC was determined by calculating the ratio of counts per minute per gram of octanol to that of buffer. Samples from the octanol layer were repartitioned until consistent PC values were obtained. The measurement was repeated three times.

In Vitro Autoradiography. Male Sprague-Dawley rats (200–250 g) were sacrificed by decapitation, and the brains were removed, placed in OTC embedding medium (Miles Laboratory, Elkhart, IN), and frozen in powdered dry ice. After equilibration to –15 °C, 20 μ m coronal sections were sliced on a cryostat (Hacker Instruments, Fairfield, NJ), thaw-mounted onto gelatin-coated slides, desiccated at 4 °C for 3 h, and kept at –70 °C until use. Prior to the experiment, the slides were dried at room temperature and preincubated for 30 min in buffer containing 50 mM Tris-HCl (pH 7.4) and 120 mM NaCl. The slides were then incubated for 2 h in preincubation buffer containing **25** (385 000 cpm/200 μ L) in a coplin jar. After incubation, the slides were washed for 1 h in cold Tris buffer with one change of buffer, then dipped in ice-cold water to remove buffer salts, and allowed to dry at room temperature. Nonspecific binding was determined in the presence of 1 μ M IPT. These slides were simultaneously exposed to DuPont X-ray film in an autoradiographic cassette for 18 h. The exposed film was developed with a Kodak automatic film processor.

RESULTS

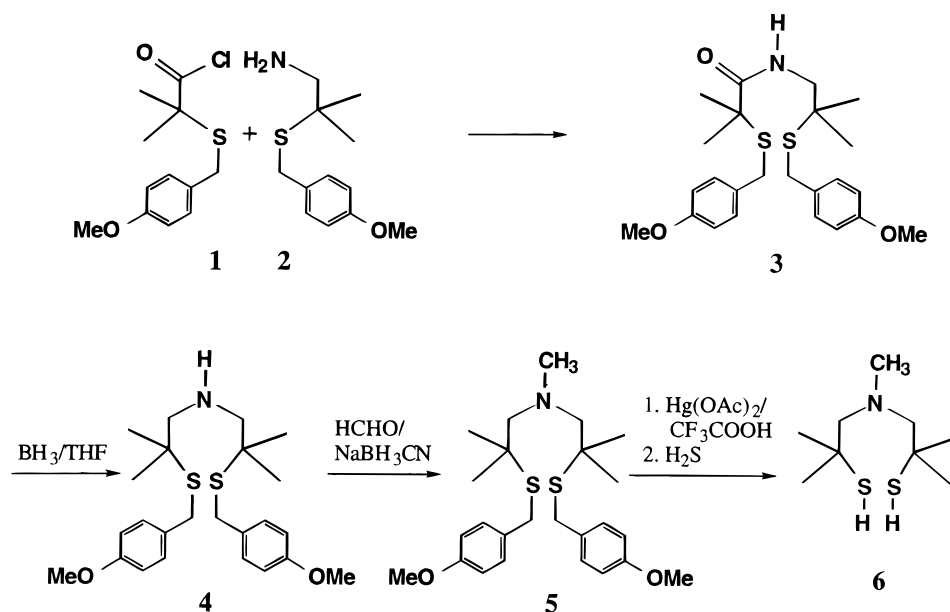
The aminobis(ethanethiol) ligand with *gem*-dimethyl groups, **6**, was synthesized according to a previously reported procedure (31), as shown in Scheme 1. The aminobis(ethanethiol) ligand without *gem*-dimethyl groups, **7**, was synthesized according to the literature (32). The



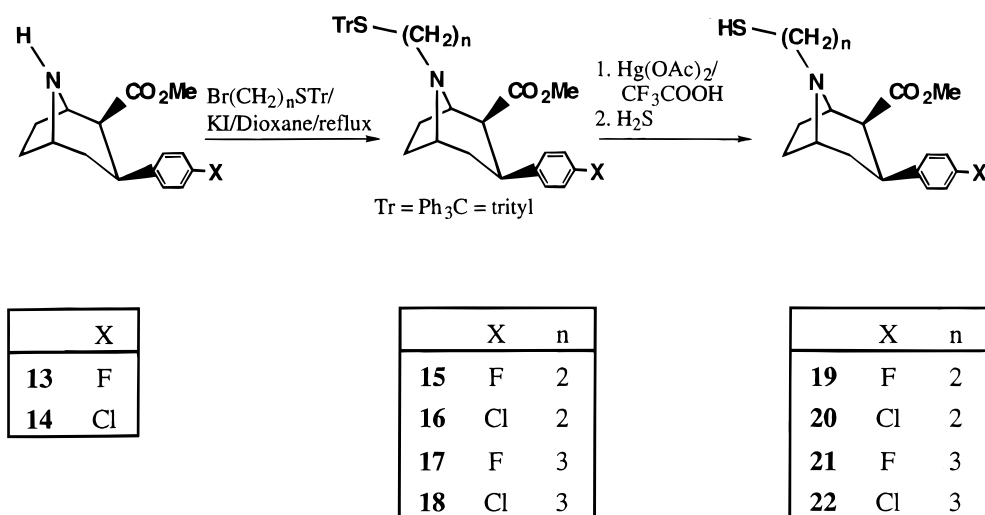
	R ₁	R ₂
6	CH ₃	CH ₃
7	CH ₃	H
8	Et	H
9	i-Bu	H
10		H
11		H
12		H

N-ethyl-substituted aminobis(ethanethiol), **8**, was prepared according to the procedure employed for the synthesis of **7**, using *N*-ethylbis(chloroethylamine) as the starting material. The other *N*-substituted bis(ethanethiols), **9–12**, were prepared by bisalkylation of benzyl-, isobu-

Scheme 1



Scheme 2



tyl-, morpholinoethyl- and *N,N*-bis(ethylamino)ethylamine with ethylene sulfide, respectively (33).

The syntheses of thiol tropane ligands **19–22** are shown in Scheme 2. Demethylated tropane derivatives **13** and **14** were prepared from cocaine in four steps, as previously reported (34). *N*-Alkylation was achieved by reacting them with *S*-trityl-protected 2-bromoethanethiol and 3-bromopropanethiol (35), and the resulting alkylated products, **15–18**, were successfully deprotected with $\text{Hg}(\text{OAc})_2/\text{H}_2\text{S}$ to produce free thiols **19–22**. Although the bisalkylation of amines with ethylene sulfide generally produced the expected bis(ethanethiol)s in poor yields, it provided a very short route for obtaining the required dithiols quickly for the initial studies. No attempts were made to improve the yields of these reactions. The vacuum distillation yielded fairly pure dithiols but further reduced the yields. The dithiols seem to have only moderate stability and tend to produce a white solid, presumably disulfides, over time. However, the monothiols, **15–18**, seem to have fairly good stability when stored at a low temperature under N_2 . As is evident from the X-ray crystallographic structure of Re complex **23**, none of the reaction conditions employed in the preparation of monothiols **15–18** and the Re complex

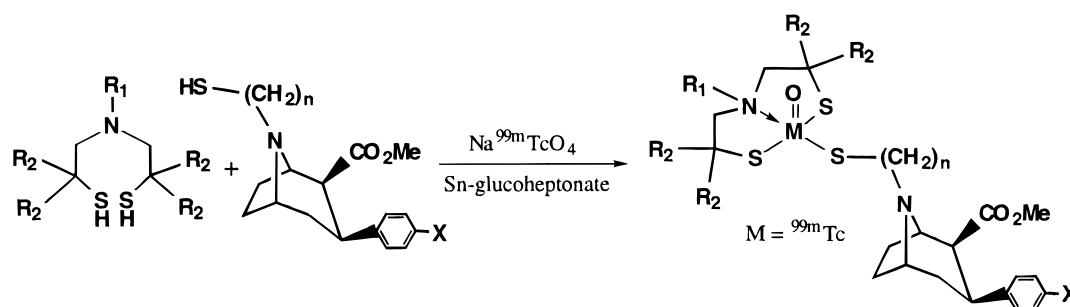
altered the stereochemistry at the C-2 position of the tropane ring.

Labeling with $^{99\text{m}}\text{Tc}$ was carried out by reacting the appropriate ligands in a molar ratio of 1:1.5 [aminobis(ethanethiol):tropanethiol ligand] with sodium [$^{99\text{m}}\text{Tc}$]-pertechnetate, in the presence of tin(II) glucoheptonate as a reducing agent at room temperature, with yields of about 40% (Scheme 3).

Byproducts were labeled complexes in which either the tridentate ligand aminobis(ethylthiol) or the monothiol is complexed to a TcO core to form the neutral binuclear complex $\text{Tc}_2\text{O}_2[\text{RN}(\text{CH}_2\text{CH}_2\text{S})_2]_3$ (36) and ionic complexes (37), which were not further investigated.

The labeled compounds were purified by HPLC, and final radiochemical purity of >95% was obtained. All of the $^{99\text{m}}\text{Tc}$ -labeled complexes **24–34** displayed good *in vitro* stability. The lipophilicities of **24–34** were measured by partition between 1-octanol and buffer (Table 1). The complexes with *gem*-dimethyl groups, **26** and **27**, displayed a much higher partition coefficient than the analogous complexes, **24** and **25**, without *gem*-dimethyl groups. In addition, the partition coefficients of the complexes containing the fluorine atom, **24**, **26**, and **28**, are always lower than those of the corresponding com-

Scheme 3



	R ₁	R ₂
6	CH ₃	CH ₃
7	CH ₃	H
8	Et	H
9	i-Bu	H
10		H
11		H
12		H

	X	n
19	F	2
20	Cl	2
21	F	3
22	Cl	3

	R ₁	R ₂	X	n
24	CH ₃	H	F	2
25	CH ₃	H	Cl	2
26	CH ₃	CH ₃	F	2
27	CH ₃	CH ₃	Cl	2
28	CH ₃	H	F	3
29	CH ₃	H	Cl	3
30	Et	H	Cl	2
31	i-Bu	H	Cl	2
32		H	Cl	2
33		H	Cl	2
34		H	Cl	2

Table 1. PC, Brain Uptake, and ST/CB Ratios of Various Tc-99m Complexes

complex	retention time (min)	PC	brain uptake (% dose)		ST/CB ratios (30 min)
			2 min	30 min	
26	17.6 ^a	1800	0.08 ± 0.03	0.05 ± 0.002	1.0 (n = 3)
24	11.6 ^a	97	0.09 ± 0.01	0.063 ± 0.003	1.93 (n = 3)
27	27.4 ^a	2000	0.051 ± 0.009	0.046 ± 0.002	1.0 (n = 3)
25	16.6 ^a	307	0.100 ± 0.068	0.07 ± 0.003	2.2 (n = 12)
28	14.5 ^a	99	0.082 ± 0.014	0.045 ± 0.008	1.71 (n = 3)
29	12.5 ^b	125	0.12 ± 0.03	0.063 ± 0.013	1.72 (n = 3)
30	10.8 ^b	369	0.097 ± 0.003	0.058 ± 0.006	1.97 (n = 3)
31	14.8 ^b	881	0.080 ± 0.009	0.051 ± 0.006	0.97 (n = 3)
32	23.6 ^b	146	0.048 ± 0.021	0.046 ± 0.020	0.72 (n = 3)
33	9.8 ^b	86	0.062 ± 0.008	0.046 ± 0.009	0.47 (n = 3)
34	14.5 ^b	92	0.11 ± 0.02	0.061 ± 0.011	0.53 (n = 6)

^a Retention times of the complexes were measured by HPLC on a PRP-1 column (250 × 4.1 mm) eluted with acetonitrile/5 mM dimethylglutaric acid buffer, pH 7 (80:20), flow rate = 1 mL/min.

^b Retention times of the complexes were measured by HPLC on a PRP-1 column (250 × 4.1 mm) eluted with acetonitrile/5 mM dimethylglutaric acid buffer, pH 7 (90:10), flow rate = 1 mL/min.

plexes with the chlorine atom. In the series of alkyl-substituted NS₂ ligand complexes, **25**, **30**, and **31**, the PC follows an expected trend; the PC increases with the size of alkyl substituent (the PCs for *N*-methyl, *N*-ethyl, and *N*-isobutyl are 307, 369, and 881, respectively). Having a heteroatom in the substitution group at the NS₂ part (**33**, **34**) did not follow any general trend in PC.

Co-injections of Tc-99m-**25** and Re-**23** into HPLC under various elution conditions appeared to confirm that they

have similar retention times. It is likely that the Tc-99m complexes have the same chemical structures as those of the corresponding Re complexes. Compounds **25** and **23** behaved similarly under identical HPLC conditions (co-injection), suggesting that **23** is indeed a good surrogate for **25**. The retention time of **25** is similar to that of **23**; on a C₁₈ column (Partisil 10-ODS-3, 250 × 4.6 mm) with MeOH/NH₄HCO₃ (0.1 M, pH 7, ratio 8:2, flow rate = 1 mL/min) as the eluent, the retention times were 14.9 and 15.3 min for **25** and **23**, respectively (Figure 4). On a Chiralpak AD column (250 × 4.6 mm) with hexane/EtOH (1:1, flow rate = 1 mL/min) as the eluent, the retention times were 10.4 and 10.9 min for **25** and **23**, respectively. On a PRP-1 column (Hamilton, 250 × 4.1 mm) with CH₃CN/dimethyl glutarate buffer (5 mM, pH 7, ratio 9:1, flow rate = 1 mL/min) as the eluent, the retention times were 10.2 and 9.8 min for **25** and **23**, respectively. Whether the ^{99m}Tc complex has indeed the same chemical structure as Re complex **23** (methyl *anti* to the Re=O) cannot be determined definitively at this point. Under the applied conditions, only one of the two possible isomers (*anti* or *syn*) can be detected. If the other isomer is indeed present, the ratio must be >98:2 and the compounds must behave very similarly.

In vivo biodistribution studies of various Tc-99m-labeled complexes, **24**–**34**, were evaluated in male Sprague-Dawley rats. The brain uptakes of these complexes appeared to be similar and were all relatively low (0.1% dose/organ or less at 2 min postinjection) despite the

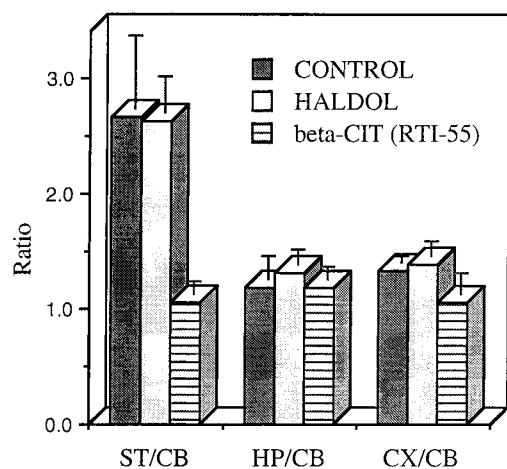


Figure 2. Ratios of regional brain uptakes at 60 min post iv injection of **25** in control rats and rats pretreated with haldol (1 mg/kg, iv) or β -CIT (1 mg/kg, iv) at 5 min prior to radiotracer injection. Values shown are means $\pm$ SD ($n = 3-4$, $p < 0.05$, Student t -test). ST, striatum; HP, hippocampus; CX, cortex; CB, cerebellum. The ST area, where dopamine transporters are located, displayed selective regional brain uptake, with the highest concentration ratio (ST/CB = 2.6). Pretreatment with β -CIT, which competes with dopamine transporter binding, showed blocking of the specific uptake of **25** in the ST area.

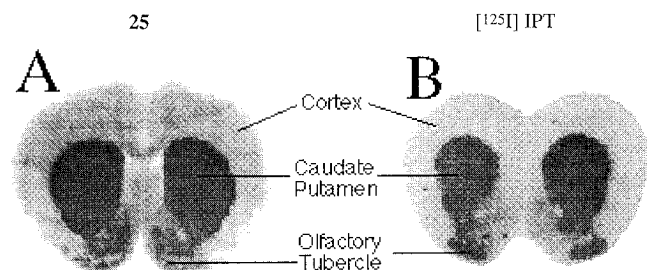


Figure 3. *In vitro* autoradiogram of **25** binding to dopamine transporters in a rat brain section demonstrated that it localized in caudate putamen and olfactory tubercle, areas known to have high dopamine transporter densities. A comparable section labeled with [^{125} I]IPT, a known iodinated dopamine transporter ligand (11), showed an almost identical regional labeling pattern. The coronal section of rat brain shown corresponds to Plate 17 in the atlas by Paxinos and Watson (38).

different lipophilicities measured by PC between 1-octanol and phosphate buffer (Table 1). Slow washout from the brain was observed for all of these complexes. Interestingly, only complexes without any *gem*-dimethyl groups displayed specific uptake in striatum, where dopamine transporters are highly concentrated. Furthermore, only complexes with smaller R groups on the NS₂ ligand showed any specific brain uptake; striatum to cerebellum ratios (ST/CB) were 1.93, 2.2, and 1.97 at 30 min postinjection for **24**, **25**, and **30**, respectively. Increasing the chain length in the tropane thiol ligand from two to three carbons (compounds **28** and **29**) did not change the brain uptake dramatically, but the ST/CB ratio was decreased (1.71 and 1.72, respectively). The cerebellum area, which does not contain dopamine transporters, was used as the background region for comparison.

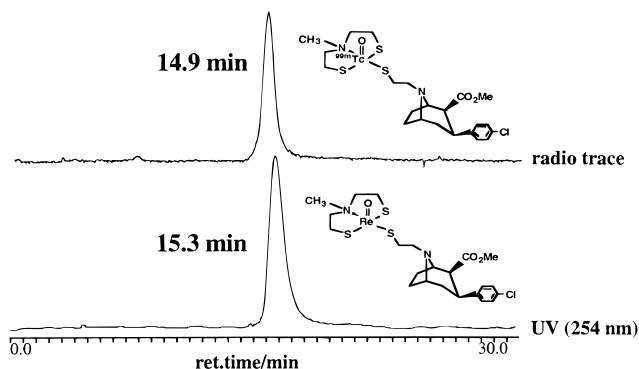


Figure 4. Comparison of HPLC traces of **23** (UV) and **25** (radio trace) on a C₁₈ column (Partisil 10-ODS-3, 250 $\times$ 4.6 mm) with MeOH/NH₄HCO₃ (0.1 M, pH 7, ratio 8:2, flow rate = 1 mL/min).

Table 2. Biodistribution of 25 in Rats (iv Injection; Percent Dose/Organ $\pm$ SD, $n = 3-6$)

organ	2 min	30 min	60 min	120 min
blood	4.72 $\pm$ 1.37	2.75 $\pm$ 0.62	2.83 $\pm$ 0.42	2.31 $\pm$ 0.27
heart	1.46 $\pm$ 0.37	0.30 $\pm$ 0.04	0.22 $\pm$ 0.23	0.12 $\pm$ 0.013
muscle	23.30 $\pm$ 3.82	16.70 $\pm$ 3.62	13.63 $\pm$ 0.99	4.33 $\pm$ 1.20
lung	6.41 $\pm$ 2.04	2.09 $\pm$ 0.47	1.26 $\pm$ 0.06	0.69 $\pm$ 0.061
kidney	5.67 $\pm$ 0.94	3.10 $\pm$ 0.68	2.87 $\pm$ 0.43	2.74 $\pm$ 0.096
spleen	0.36 $\pm$ 0.07	0.61 $\pm$ 0.10	0.50 $\pm$ 0.09	0.58 $\pm$ 0.009
liver	13.51 $\pm$ 0.36	29.6 $\pm$ 2.22	32.15 $\pm$ 3.00	35.37 $\pm$ 1.52
skin	2.94 $\pm$ 0.36	4.71 $\pm$ 1.27	4.57 $\pm$ 0.65	2.30 $\pm$ 0.082
brain	0.10 $\pm$ 0.02	0.06 $\pm$ 0.02	0.046 $\pm$ 0.002	0.025 $\pm$ 0.002

Biodistribution showed that **25** had the highest ST/CB ratio (2.2) at 30 min post iv injection. Therefore, **25** was studied further in rats. The study at 2, 30, 60, and 120 min post iv injection indicated that the complex followed the initial blood flow and localized in organs with high blood flow, such as muscle, liver, and kidney. The Tc-99m complex cleared from muscle, while liver accumulation increased with time (Table 2). Accumulation of radioactivity was highest in all brain regions at 2 min post iv injection and declined gradually over the 120 min period. The washout was slowest from the striatal region, followed by cortex and hippocampus. Maximum regional contrast ratio observed for ST/CB was 3.5 at 60 min postinjection (Table 3). Therefore, additional blocking studies in rats were performed at 60 min postinjection to further characterize the uptake of this Tc-99m compound. In this series of regional brain uptake studies in rats, the ST/CB ratio at 60 min postinjection was 2.6 (Figure 2). The specific uptake of **25** in striatum could be blocked by pretreating rats with a dose of β -CIT (1 mg/kg, iv), a dopamine transporter ligand (ST/CB = 1.0), but not with haldol (1 mg/kg, iv), an agent with a mixed pharmacological profile (binding to various CNS receptors but not to the dopamine transporter); no blocking effect was observed. The ST/CB ratio (2.6) was identical to that in control rats (Figure 2). The results suggested that uptake in the rat striatum was specifically related to the dopamine transporter. In addition, *in vitro* autoradiography of a rat brain coronal section incubated with **25** clearly demonstrated a regional distribution pattern of intense labeling in caudate putamen and olfactory tubercle, areas known to have a high density of dopamine transporters (Figure 3).

Table 3. Regional Brain Distribution of 25 in Rat Brain (Percent Dose/Gram)

region	2 min	30 min	60 min	120 min
cerebellum	0.066 $\pm$ 0.017	0.027 $\pm$ 0.006	0.018 $\pm$ 0.001	0.011 $\pm$ 0.002
striatum	0.054 $\pm$ 0.010	0.060 $\pm$ 0.011	0.063 $\pm$ 0.001	0.031 $\pm$ 0.003
hippocampus	0.046 $\pm$ 0.005	0.028 $\pm$ 0.008	0.025 $\pm$ 0.003	0.011 $\pm$ 0.000
cortex	0.060 $\pm$ 0.009	0.038 $\pm$ 0.007	0.026 $\pm$ 0.002	0.015 $\pm$ 0.003
ST/CB ratio	0.82	2.2	3.5	2.8

DISCUSSION

In this paper, a series of complexes with a TcO^{3+} center core, a tropane thiolate, and an aminobis(ethanethiolate) ligand (three plus one complexes) are reported as potential dopamine transporter imaging agents. To maintain the ability to penetrate the blood-brain barrier, it is essential to keep the neutral Tc complex core of these dopamine transporter ligands small. The addition of *gem*-dimethyl groups to a compound dramatically enhances lipophilicity and significantly reduces specific binding to the striatal regions of the brain, where dopamine transporters are located. Fine tuning of the size and lipophilicity of the technetium complexes appears to be critical for brain uptake and receptor binding. It is apparent that compound **25**, with a partition coefficient of 307, was the best among this series of three plus one mixed-ligand complexes. Specific uptake in the striatum can be selectively blocked with pretreatment of β -CIT, suggesting that the localization of **25** in rat brain is selective to dopamine transporter sites. To characterize further the chemical structure of the technetium complexes, ReO complex **23**, the corresponding complex to technetium complex **25**, was prepared and characterized (27). The nonradioactive rhenium is chemically close to technetium-99, but without the usual hazards associated with the handling of radioactive material. X-ray crystallography of complex **23** displayed an expected structure, with a square pyramidal geometry. The $\text{Re}=\text{O}$ group is in the apical position, and the *N*-methyl group is at the *anti* position to the $\text{Re}=\text{O}$ functionality.

On the basis of the data for this series of Tc-99m-labeled tropane derivatives, **24–34**, it is evident that there are stringent structural requirements for achieving both neutral lipophilicity and dopamine transporter binding affinity. Our examples clearly showed that substitution at the NS_2 moiety with *gem*-dimethyl groups completely destroys the specificity (Table 1), and this is also true for *N*-substitution groups larger than a butyl group and for additional amino groups (Table 1). Both of these observations are not readily predictable by simple estimation of partition coefficients or molecular weight or size. Apparently, the *in vivo* biodistribution and receptor binding of dopamine transporter place unique and highly selective requirements on this novel group of agents.

In vitro binding studies of Re complex **23** with rat striatal homogenates displayed excellent binding affinity ($K_i = 0.3$ nM, using $[^{125}\text{I}]\text{-IPT}$ as the ligand; $K_d = 0.2$ nM) (11). The major drawback of this series of agents is that the brain uptake (0.1% dose/organ in rat brain at 2 min post iv injection) is too low to be useful for human imaging study. New complexes that produce at least 0.5% dose/organ in rat brain must be obtained before any are further developed as dopamine transporter imaging agents. Nevertheless, this series of $^{99\text{m}}\text{Tc}$ -labeled mixed ligand complexes [aminobis(ethanethiol) and monothiol complexed to a TcO^{3+} center core] is the first example of technetium-labeled agents displaying specific regional uptake in rat brain that reflects receptor distribution.

In conclusion, this series of Tc-99m compounds demonstrates that the basic approaches of designing a selective Tc-99m-labeled imaging agent for CNS receptors may be feasible. It can be reasonably predicted that, with additional structural modifications, Tc-99m-labeled ligands derived from this series may be developed in the future for human use.

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LITERATURE CITED

- (1) Eckelman, W. C. (1991) The status of radiopharmaceutical research. *Nucl. Med. Biol.* 18, iii–vi.
- (2) Fowler, J. S., and Wolf, A. P. (1989) New directions in positron emission tomography. *Annu. Rep. Med. Chem.* 24, 277–286.
- (3) Fowler, J. S., Wolf, A. P., and Volkow, N. D. (1990) New directions in positron emission tomography. Part II. *Annu. Rep. Med. Chem.* 25, 261–268.
- (4) Jurisson, S., Berning, D., Jia, W., and Ma, D.-S. (1993) Coordination compounds in nuclear medicine. *Chem. Rev.* 93, 1137–1156.
- (5) Steigman, J., and Eckelman, W. C. (1992) *The Chemistry of Technetium in Medicine*; National Academy Press, Washington, DC.
- (6) Frost, J. J., Rosier, A. J., Reich, S. G., Smith, J. S., Ehlers, M. D., Snyder, S. H., Ravert, H. T., and Dannals, R. F. (1993) Positron emission tomographic imaging of the dopamine transporter with $[^{11}\text{C}]\text{-WIN 35,428}$ reveals marked declines in mild Parkinson's disease. *Ann. Neurol.* 34, 423–431.
- (7) Scheffel, U., Pöggö, S., Stathis, M., Boja, J. W., and Kuhar, M. J. (1991) *In vivo* labeling of cocaine binding sites on dopamine transporters with $[^3\text{H}]\text{WIN35,428}$. *J. Pharmacol. Exp. Ther.* 257, 954–958.
- (8) Shaya, E. K., Scheffel, U., Dannals, R. F., Ricaurte, G. A., Carroll, F. I., Wagner, H. N., Jr., Kuhar, M. J., and Wong, D. F. (1992) *In vivo* imaging of dopamine reuptake sites in the primate brain using single photon emission computed tomography (SPECT) and iodine-123 labeled RTI-55. *Synapse* 10, 169–172.
- (9) Innis, R. B. (1994) Single photon emission tomography imaging of dopamine terminal innervation: a potential clinical tool in Parkinson's disease [editorial; review]. *Eur. J. Nucl. Med.* 21, 1–5.
- (10) Innis, R. B., Seibyl, J. P., Scanley, B. E., Laruelle, M. A., Abi-Dargham, A., Wallace, E., Baldwin, R. M., Zea-Ponce, Y., Zoghbi, S. S., Wang, S., et al. (1993) Single photon emission computed tomographic imaging demonstrates loss of striatal dopamine transporters in Parkinson's disease. *Proc. Natl. Acad. Sci. U.S.A.* 90, 11965–11969.
- (11) Kung, M.-P., Essman, W. D., Frederick, D., Meegalla, S. K., Goodman, M. M., Mu, M., Lucki, I., and Kung, H. F. (1995) IPT: a novel iodinated ligand for the CNS dopamine transporter. *Synapse* 20, 316–324.
- (12) Malison, R. T., Vessotskie, J. M., Kung, M.-P., McElgin, W. T., Romaniello, G., Kim, H.-J., Goodman, M. M., and Kung, H. F. (1995) Striatal dopamine transporter imaging in non-human primates with iodine-123-IPT SPECT. *J. Nucl. Med.* 36, 2290–2297.
- (13) Mozley, P. D., Stubbs, J. B., Kim, H.-J., McElgin, W. T., Kung, M.-P., Meegalla, S. K., and Kung, H. F. (1996) Dosimetry of an iodine-123-labeled tropane to image dopamine transporters. *J. Nucl. Med.* 37, 151–159.
- (14) Neumeyer, J. L., Wang, S., Gao, Y., Milius, R. A., Kula, N. S., Campbell, A., Baldessarini, R. J., Zea-Ponce, Y., Baldwin, R. M., and Innis, R. B. (1994) *N*- ω -Fluoroalkyl analogs of (1*R*)-2*β*-carbomethoxy-3*β*-(4-iodophenyl)-tropane (β -CIT): radiotracers for positron emission tomography and single photon emission computed tomography imaging of dopamine transporters. *J. Med. Chem.* 37, 1558–1561.
- (15) Jurisson, S. S., Pirro, J., DiRocco, R. J., Rosenspire, K. C., Jagoda, E., Nanjappan, P., Eckelman, W. C., Nowotnik, D. P., and Nunn, A. D. (1995) Boronic acid adducts of technetium dioxime (BATO) complexes derived from quinuclidine benziolate (QNB) boronic acid stereoisomers: syntheses and studies of their binding to the muscarinic acetylcholine receptor. *Nucl. Med. Biol.* 22, 269–281.
- (16) Lever, S. Z., Baidoo, K. E., Mahmood, A., Matsumura, K., Scheffel, U. A., and Wagner, H. N., Jr. (1994) Novel technetium ligands with affinity for the muscarinic cholinergic receptor. *Nucl. Med. Biol.* 21, 157–164.
- (17) Del Rosario, R. B., Jung, Y.-W., Baidoo, K. E., Lever, S. Z., and Wieland, D. M. (1994) Synthesis and *in vivo* evalua-

- tion of a [$^{99m/99}\text{Tc}$]-DADT-benzovesamicol: a potential marker for cholinergic neurons. *Nucl. Med. Biol.* 21, 197–203.
- (18) Chi, D. Y., and Katzenellenbogen, J. A. (1993) Selective formation of heterodimeric bis-bidentate aminothiol-oxometal complexes of rhenium(V). *J. Am. Chem. Soc.* 115, 7045–7046.
- (19) Chi, D. Y., O'Neil, J. P., Anderson, C. J., Welch, M. J., and Katzenellenbogen, J. A. (1994) Homodimeric and heterodimeric bis(amino thiol) oxometal complexes with rhenium(V) and technetium(V): control of heterodimeric complex formation and an approach to metal complexes that mimic steroid hormones. *J. Med. Chem.* 37, 928–937.
- (20) DiZio, J. P., Fiaschi, R., Davison, A., Jones, A. G., and Katzenellenbogen, J. A. (1991) Progesterone-rhenium complexes: metal-labeled steroids with high receptor binding affinity, potential receptor-directed agents for diagnostic imaging or therapy. *Bioconjugate Chem.* 2, 353–366.
- (21) DiZio, J. P., Anderson, C. J., Davison, A., Ehrhardt, G. J., Carlson, K. E., Welch, M. J., and Katzenellenbogen, J. A. (1992) Technetium- and rhenium-labeled progestins: synthesis, receptor binding and *in vivo* distribution of an 11 beta-substituted progesterone labeled with technetium-99 and rhenium-186. *J. Nucl. Med.* 33, 558–569.
- (22) O'Neil, J. P., Wilson, S. R., and Katzenellenbogen, J. A. (1994) Preparation and structural characterization of monoamine-monoamide bis(thiol) oxo complexes of technetium(V) and rhenium(V). *Inorg. Chem.* 33, 319–323.
- (23) O'Neil, J. P., Carlson, K. E., Anderson, C. J., Welch, M. J., and Katzenellenbogen, J. A. (1994) Progesterone radiopharmaceuticals labeled with technetium and rhenium: synthesis, binding affinity, and *in vivo* distribution of a new progesterone N_2S_2 -metal conjugate. *Bioconjugate Chem.* 5, 182–193.
- (24) Mastrostamatis, S. G., Papadopoulos, M. S., Pirmettis, I. C., Paschali, E., Varvarigou, A. D., Stassinopoulou, C. I., Raptopoulou, C. P., Terzis, A., and Chiotellis, E. (1994) Tridentate ligands containing the SNS donor atom set as a novel backbone for the development of technetium brain imaging agents. *J. Med. Chem.* 37, 3212–3218.
- (25) Spies, H., Fietz, T., Glaser, M., Pietzsch, H.-J., and Johannsen, B. (1995) The “n+1” concept in the synthesis strategy of novel technetium and rhenium tracers. *Technetium and Rhenium in Chemistry and Nuclear Medicine* (M. Nicolini, G. Bandoli, and U. Mazzi, Eds.) Vol. 4, pp 243–246, S. G. Editoriale, Padova, Italy.
- (26) Spies, H., Abram, U., Uhlemann, E., and Ludwig, E. (1985) Synthesis and characterization of a new technetium complex tris(monothiodibenzoyl-methanato)technetium(III). *Inorg. Chim. Acta* 109, L3–L4.
- (27) Meegalla, S. K., Plössl, K., Kung, M.-P., Stevenson, D. A., Liable-Sands, L. M., Rheingold, A. L., and Kung, H. F. (1995) The first example of a Tc-99m complex as a dopamine transporter imaging agent. *J. Am. Chem. Soc.* 117, 11037–11038.
- (28) Mirza, S. A., Pressler, M. A., Kumar, M., Day, R. O., and Maroney, M. J. (1993) Oxidation of nickel thiolate ligands by dioxygen. *Inorg. Chem.* 32, 977–987.
- (29) Kung, H. F., Molnar, M., Billings, J. J., Wicks, R., and Blau, M. (1984) Synthesis and biodistribution of neutral lipid-soluble Tc-99m complexes which cross the blood-brain barrier. *J. Nucl. Med.* 25, 326–332.
- (30) Kung, H. F., Yu, C. C., Billings, J. J., Molnar, M., and Blau, M. (1985) Synthesis of new bis-aminoethanethiol (BAT) derivatives: possible ligands for Tc-99m brain imaging agents. *J. Med. Chem.* 28, 1280–1284.
- (31) Ohmomo, Y., Francesconi, L. C., Kung, M.-P., and Kung, H. F. (1992) New conformationally restricted [^{99m}Tc] N_2S_2 complexes as myocardial perfusion imaging agents. *J. Med. Chem.* 35, 157–162.
- (32) Kolb, U., Beuter, M., and Dräger, M. (1994) Heterocyclic systems containing Tin(IV). 10. Control of three-center interactions $\text{X}\cdots\text{Sn}\cdots\text{Hal}$ in stannocanes by halide type. *Inorg. Chem.* 33, 4522–4530.
- (33) Corbin, J. L., Miller, K. H., Pariyadath, N., Wherland, S., Bruce, A. E., and Stiefel, E. I. (1984) Preparation and properties of tripodal and linear tetradentate N,S-donor ligands and their complexes containing the MoO_2^{2+} core. *Inorg. Chim. Acta* 90, 41–51.
- (34) Meltzer, P. C., Liang, A. Y., Brownell, A.-L., Elmaleh, D. R., and Madres, B. K. (1993) Substituted 3-phenylpropane analogs of cocaine: synthesis, inhibition of binding at cocaine recognition sites, and positron emission tomography imaging. *J. Med. Chem.* 36, 855–862.
- (35) Dhar, T. G. M., Borden, L. A., Tyagarajan, S., Smith, K. E., Branchek, T. A., Weinshank, R. L., and Gluchowski, C. (1994) Design, synthesis and evaluation of substituted triaryl-nipecotic acid derivatives as GABA uptake inhibitors: identification of a ligand with moderate affinity and selectivity for the cloned human GABA transporter GAT-3. *J. Med. Chem.* 37, 2334–2342.
- (36) Ravert, H. T., Burns, H. D., Heindel, N. D., Kramer, A. V., and Epps, L. A. (1983) Synthesis of neutral, lipid soluble technetium complexes of bis(2-mercaptoethyl)amines. *J. Nucl. Med.* 24, P10.
- (37) Spies, H., Pietzsch, H.-J., Syhre, R., and Hoffmann, S. (1990) Lipophilic technetium complexes. VIII. Preparation and animal studies of oxotechnetium(V) complexes with tridentate/monodentate ligand coordination. *Isotopenpraxis* 26, 159–162.
- (38) Paxinos, G., and Watson, C. (1986) *The Rat Brain In Stereotaxic Coordinates*, Academic Press, New York.

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Biochemical Characterization of an L-Asparaginase Bioconjugate

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In this work is characterized a bioconjugate of L-asparaginase, obtained by linkage of palmitic acid chains to the native enzyme in the presence of substrate as a protein protective molecule. Comparisons between isoelectric points, hydrophobicity, pH, and temperature profiles for the bioconjugate and the native enzyme were performed. A shift of pI from 5.03 to 4.58 was observed after conjugation. The modified enzyme evidences a 10-fold increase of the hydrophobicity. A small shift from 7.5 to 7 of the pH for maximal catalytic activity and a 5 °C increase of temperature for maximal activity were observed with conjugation. Stability studies in human serum and on storage evidence similar behaviors for both bioconjugate and native enzyme. The retention of catalytic activity of the bioconjugate is dependent on the presence of micelles. The bioconjugate evidenced 65% retention of activity when catalytic activity was assayed without a surfactant and 98–100% retention of activity when catalytic activity was assayed in the presence of surfactant micelles. The kinetic characteristics of the bioconjugate and of the native enzyme, in micelles of different hydrophobicities, were compared. The Michaelis constant of native enzyme is 0.030 mM, independent of the surfactant, and the Michaelis constant of the bioconjugate varies with the surfactant, from 0.036 to 0.046 mM.

INTRODUCTION

There is increasing interest in the search for mechanisms to optimize the therapeutic action of enzymes to improve their function and properties (Konrad, 1989). One of the tools to achieve this goal is the construction of bioconjugates, obtained by linkage of different molecules to enzymes (Borman, 1989). This approach, requiring random covalent coupling, may result in modification of the enzyme active site or in conformational alterations of the macromolecule, with a consequent alteration of properties. So, characterization of the bioconjugates needs to be performed. In this paper we report the physicochemical and kinetic characterization of a bioconjugate of L-asparaginase (L-asparagine amidohydrolase, EC 3.5.1.1), which is an enzyme with antitumor activity against human acute lymphoblastic leukemia (Asselin et al., 1989; Capizzi and Handschumacher, 1982). The bioconjugate (Ac-L-asparaginase) was obtained as described in previous work (Martins et al., 1990), by covalent coupling of palmitic chains to the native enzyme using the conjugation method developed by Torchilin et al. (1980) but including substrate in the reactional medium. Ac-L-asparaginase was developed with the aim of supplying the enzyme surface with hydrophobic chains able to promote the association of the active enzyme with the bilayer of liposomes while preserving the other enzyme characteristics. The association of Ac-L-asparaginase with liposomes was evidenced in previous work in which the liposomal form of the bioconjugate was characterized (Jorge et al., 1994). The physicochemical characteristics of the Ac-L-asparaginase, such as hydrophobicity and charge, could be relevant for the optimization of the liposomal form, as both parameters can help the rational design of a bilayer (Boggs, 1987) to improve the incorporation of a protein. The interaction between proteins and liposomes, other than the covalent bond, can be mediated by electrostatic and/or hydrophobic interactions between the membrane and

the protein, such as the ones occurring with membrane proteins (Jain and Zakim, 1987), but the relative importance of this interaction is difficult to assess (Low, 1987). The electrophoretic mobility and the isoelectric point of the bioconjugate and native enzyme were evaluated by Doppler laser anemometry and compared with gel isoelectric focusing quantification. Due to the resolution of this techniques, some information on the homogeneity of the sample can be obtained (McNeil-Watson, 1988; Dwyer, 1993). The catalytic performances of a bioconjugate are relevant for its usefulness, mainly initial activity and stability and also kinetic constants and pH and temperature profiles. The preservation of catalytic properties of a bioconjugate may be dependent on the use of an appropriate stabilizer of its protein structure. Reported stabilizers include some solvents (Timasheff and Arakawa, 1990) and naturally occurring or synthetic micellar systems able to affect the rates of many chemical reactions either *in vivo* or *in vitro* (Fendler and Fendler, 1975). High affinity to L-asparagine, and consequently low Michaelis constant (K_m), at physiological pH, are required for the antitumor activity of L-asparaginase (Wirston and Yellin, 1973). With the aim of identifying microenvironment parameters able to maximize the activity of Ac-L-asparaginase, micelles of different hydrophobicities were used. The effect of micellization on enzymatic reactions of the bioconjugate is considered.

EXPERIMENTAL PROCEDURES

Materials. Asparaginase Elspar, L-asparagine amidohydrolase (EC 3.5.1.1), type EC-2 from *Escherichia coli*, was obtained from Merck Sharp & Dohme. The other enzymes or proteins used were from Sigma. 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) was from Sigma. For gel isoelectric focusing were used PhastGel IEF, precast homogeneous polyacrylamide gels, containing Pharmaly as carrier ampholyte, and PhastGel Blue R 350 for Coomassie Blue stain, all from Pharmacia. All other reagents were of analytical grade.

The reactional medium for modification of the enzyme was sonicated in a water bath sonicator from Sonorex,

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Model RK 156. The separations by dialysis were performed using membranes with 12 000 molecular weight cutoff. The absorption spectrophotometry results were obtained in a spectrophotometer from Shimadzu, Model UV-160A. The fluorescence spectroscopy results were obtained in a spectrofluorometer from Hitachi, Model F 3000. The gel isoelectric focusing quantifications were performed in a Phast System from Pharmacia. The Doppler laser light scattering studies were performed in equipment from Malvern, Model Zeta-Sizer-III.

Methods. *Preparation of Ac-L-asparaginase.* The bioconjugate of L-asparaginase was prepared according to the method of Torchilin et al. (1980) with some modifications (Martins et al., 1990). In brief, palmitoyl chloride was added to a 50 mM carbonate buffer, pH 9.4, containing 1% sodium cholate and 8 mM L-asparagine. The mixture was sonicated for 90 s and immediately added to the enzyme and incubated, with gentle stirring, at room temperature. The modified enzyme was recovered from the reactional medium using separation procedures to remove the other compounds of the reactional medium. The palmitic acid was removed by centrifugation (20000g for 30 min) and the modified enzyme recovered in the supernatant. The remaining compounds [surfactant, salts, substrate (L-asparagine), and products from its conversion] were removed by dialysis against water.

Native enzyme, used as a reference for all of the experiments, was solubilized in 50 mM carbonate buffer, pH 9.4, containing 1% sodium cholate and 8 mM L-asparagine and the solution dialyzed against water. With this, a similar presence of the different compounds, in the concentrations that remain after dialysis, can be assured.

The degree of modification was quantified by a fluorometric assay (Böhlen et al., 1973). Briefly, after the recovery of modified and native enzyme from dialysis, the unblocked ϵ -NH₂ groups of either acylated or native enzyme were bound to fluorescamine according to the method of Böhlen et al. (1973). The intensity of fluorescence emission of bound fluorescamine at 475 nm was measured for excitation at 390 nm. The degree of modification is $[1 - (\text{emission per microgram of modified enzyme/emission per microgram of native enzyme})] \times 100$. Since fluorescamine can react with trace amines present in the reagents and solvents, a reagent blank was run routinely.

For this work were prepared batches of Ac-L-asparaginase with a 30% degree of modification and a 100% retention of activity and of L-asparaginase treated as previously described. Amounts of bioconjugate and native enzyme were lyophilized in individual containers.

Assay of Catalytic Activity of the Enzyme. Two procedures were used to quantify the initial rate of conversion of substrate (L-asparagine): the quantification of ammonia, according to the procedure of Chaney and Marbach (1962), and a coupled enzyme system to directly follow the reaction rate as described by Abuchowski et al. (1979). Briefly, in the quantification according to Chaney and Marbach (1962), the reaction was initiated by the addition of the native enzyme or the bioconjugate to a solution of L-asparagine (10 mM) in phosphate buffer (50 mM), pH 7.5, at 37 °C. The enzyme concentration in this mixture was 130 nM. The ammonia was quantified in samples (150 μ L) of the reaction medium. To each sample was added 1 mL of a solution containing phenol (10 g L⁻¹) and sodium nitroprusside (0.05 g L⁻¹) plus 1 mL of a solution containing sodium hydroxide (5 g L⁻¹) and sodium hypochloride (0.42 g L⁻¹). The color development was produced after 3 min at 60 °C and the

absorbance quantified at 625 nm. A calibration curve with standard solutions of 0.02–0.16 μ M ammonium chloride was used.

In the coupled enzyme system assay the reaction rate of conversion of L-asparagine, at 37 °C, was directly followed by the decrease of absorbance at 340 nm (decrease of NADH concentration) of a solution containing 3 nM of enzyme (native enzyme or bioconjugate) and 10 mM of substrate (L-asparagine). The reaction was initiated by the addition of 10 μ L of an enzyme solution to 1000 μ L of a reaction mixture with the following composition: 20 mL of glycerol, 11 mg of NADH, 116 units of L-malate dehydrogenase, 180 units of L-glutamic oxaloacetic transaminase, 11 mg of α -ketoglutaric acid, and 1.1 mmol of L-asparagine, made up to 100 mL with 0.1 M Tris-HCl, pH 8.35.

Unless specifically noted otherwise, the catalytic assays were performed including 1% cholic acid in reaction medium.

Determination of Isoelectric Point. (a) *Isoelectric Focusing.* Samples of Ac-L-asparaginase and L-asparaginase rehydrated in water solutions of CHAPS (1%) were applied in the isoelectric focusing gel, and the staining procedure was performed using Coomassie Blue; the concentrations and the other procedures of the electrophoresis followed the instructions of the system used.

(b) *Doppler Laser Anemometry.* The microelectrophoresis cell of the Doppler laser light scattering equipment was filled using equivalent preparations of bioconjugate or native enzyme, rehydrated to a concentration of 500 μ g mL⁻¹, in electrolytes with different pH values. As electrolytes were used buffers of constant and low ionic strength ($I = 0.01$). The compositions of each buffer were as follows: for pH 2.5, chloroacetic acid and potassium hydroxide; for pH 3.5, formic acid and potassium hydroxide; for pH 4.5, acetic acid and potassium hydroxide; for pH 5.5, succinic acid and potassium hydroxide; for pH 6.5, phosphoric acid and disodium hydrogen phosphate; for pH 7.5, phosphoric acid and disodium hydrogen phosphate; for pH 8.5, boric acid and sodium tetraborate. The electrode chambers were filled with electrolyte free of enzyme. The electrophoretic mobility was monitored at low ionic strength to promote electrostatic interactions for the bioconjugate and for the native enzyme as a function of the pH. The experiments were performed at 20 °C. The isoelectric point corresponds to the pH of null electrophoretic mobility.

Determination of Partition Coefficient. Water and octanol (2.5 mL of each) were mixed in a vortex until formation of an emulsion. The mixture was added to 0.25 mg of protein. The emulsion was stirred during 2 h. The preparation was centrifuged at 5000g during 5 min. The two phases formed after centrifugation were analyzed for protein content using a quantification by intrinsic emission of fluorescence of L-asparaginase or Ac-L-asparaginase. Equivalent preparations without protein were used as reference. The partition coefficient octanol/water was determined as the ratio between the protein present in octanol and water phases.

Stability Studies. (a) *Storage Stability of Free and Conjugated Enzyme.* Several equivalent preparations containing around 600 μ g of lyophilized Ac-L-asparaginase or L-asparaginase were stored in a refrigerator (6 °C). After different periods of storage, the preparations were rehydrated with 1 mL of a sodium cholate (1%) water solution and assayed for activity (Chaney and Marbach, 1962) and protein content (Lowry et al., 1951). The retention of activity was determined in relation to initial conditions, either for L-asparaginase or for Ac-L-asparaginase.

(b) *Stability in Human Serum of Free and Conjugated Enzyme.* Equivalent preparations containing 500 μg of L-asparaginase or 450 μg of Ac-L-asparaginase, plus 2 mL of human serum and 2 mL of phosphate saline buffer, were incubated at 37 °C. After different periods of time, a group of three preparations was analyzed for catalytic activity by the direct quantification of product previously described. The retention of activity was determined in relation to initial conditions, either for L-asparaginase or for Ac-L-asparaginase.

Determination of Temperature–Activity Profiles. Solutions of L-asparaginase or Ac-L-asparaginase (500 μg mL^{-1}) in water containing 1% of sodium cholate were assayed for catalytic activity by the quantification of ammonia previously described, at temperatures in the range from 25 to 85 °C at pH 7.5. The results were normalized in relation to the maximal activity for each form of the enzyme.

Determination of pH–Activity Profiles. Equivalent preparations, containing 500 μg of L-asparaginase or Ac-L-asparaginase, were rehydrated with 1 mL of buffer with different pH values (5.5–9.0), containing 1% of sodium cholate. In the pH range from 4 to 5.5 was used a 50 mM sodium acetate-acetic acid buffer, in the pH range from 6 to 8 was used a 50 mM phosphate buffer, and in the pH range from 8.5 to 10.5 was used a 50 mM carbonate buffer. Each native enzyme or bioconjugate solution was assayed for protein content (Lowry et al., 1951) and for catalytic activity, at 37 °C, in a reaction medium made of 10 mM L-asparagine in the buffer used for rehydration and using the direct quantification of product previously reported. The results were normalized in relation to the maximal catalytic activity of each form of the enzyme.

Determination of the Retention of Catalytic Activity. The retention of catalytic activity (RA) is the ratio

$$\text{RA (\%)} = \frac{\text{initial rate of reaction catalyzed by enzyme under study}}{\text{initial rate of reaction catalyzed by native enzyme}} \times 100$$

The “enzyme under study” in the above equation could be the bioconjugate in a micellar system, the bioconjugate in a nonmicellar system, or the native enzyme in a micellar system. The “native enzyme” is an equal amount of native enzyme without surfactant.

All of the quantifications were performed, in conditions of high substrate concentration $S \gg K_m$, by the coupled enzyme assay with 10 mM L-asparagine.

Determination of Kinetic Parameters. The initial reaction rate for L-asparaginase or Ac-L-asparaginase as function of the initial L-asparagine concentration in the range 0.05–2 mM was quantified by the coupled enzyme system previously described, using 1–40 μmol of L-asparagine in the reaction mixture.

The kinetic parameters of the bioconjugate and of the native enzyme were determined using the Hanes linearization method (Cornish-Bowden and Wharton, 1988).

All of the experiments and quantifications reported were performed in triplicate. The results represent a mean of three independent determinations.

RESULTS AND DISCUSSION

Effect of Conjugation on Enzyme Charge and Hydrophobicity. An alteration of charge was observed after conjugation according to the electrophoretic mobility results. A decrease of 0.45 of a pI unit was observed for the conjugated enzyme: the isoelectric point (pI) for L-asparaginase as determined by Doppler laser anemometry (Figure 1) is 5.03 ± 0.07 . This result is in accordance

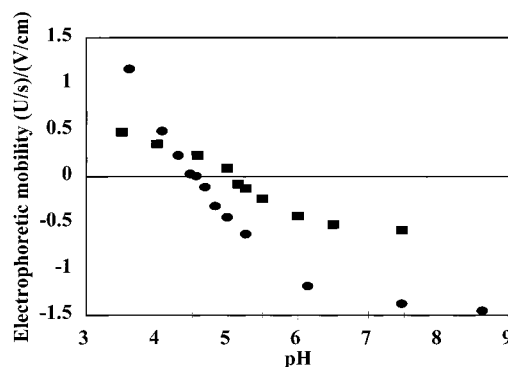


Figure 1. Electrophoretic mobility of Ac-L-asparaginase (●) and L-asparaginase (■) as function of pH.

Table 1. Octanol/Water Partition Coefficients of the Bioconjugate and the Native Enzyme

enzyme	partition coefficient
Ac-L-asparaginase	1.78
L-asparaginase	0.13

with the pI we obtain by isoelectric focusing, which is 5.03 ± 0.3 . Results reported in the literature for the pI of native *E. coli* L-asparaginase are in the range 4.6–5.35 (Wriston and Yellin, 1973) and 4.9–5.6 (Capizzi and Handschumacher, 1982). The pI of Ac-L-asparaginase determined by Doppler laser anemometry is 4.58 ± 0.05 (Figure 1), while by isoelectric focusing a discrepancy between the results of pI for the bioconjugate was observed (results not shown). As previously reported (Martins and Cruz, 1994), the Doppler laser anemometry technique evidences the advantage of quantification of the pI of hydrophobic macromolecules which have low mobility in a gel matrix. The decrease of pI was expected due to the blockage, by conjugation with palmitic chains, of 30% of the accessible $\epsilon\text{-NH}_2$ [$\text{p}K_a$ values of $\epsilon\text{-NH}_2$ are in the range 9.3–9.5 (Means and Feeney, 1971)]. This degree of modification, quantified by the fluorometric assay described under Experimental Procedures, is consistent with previous results (Martins et al., 1990). Figure 1 also evidences a deviation between the electrophoretic mobilities of both forms of the enzyme, which must be related to the different protonation of the two forms. This alteration of charge points to a different interaction of Ac-L-asparaginase with charged liposomal bilayers and could be an indicative factor to the selection of lipid compositions.

Table 1 shows the octanol/water partition coefficient for the native enzyme and the bioconjugate, quantified as a measure of hydrophobicity. To avoid perturbation of protein partitioning in the water/organic solvent systems by the presence of other compounds, the bioconjugate was separated from the reaction medium as described under Experimental Procedures. An increase of the affinity of the bioconjugate to octanol of more than 10-fold was observed without evidence for accumulation of Ac-L-asparaginase at the interface. The amount of bioconjugate solubilized in octanol is in accordance with results previously published (Martins et al., 1990). In these results a solubilization of bioconjugate in octanol, at concentrations as much as 0.46 μM , was correlated with the solutions turbidity. These results evidence a significant increase of the affinity of the bioconjugate to hydrophobic mediums, as expected after introduction of hydrophobic palmitoyl groups in the enzyme molecule.

Evaluation of the Homogeneity of the Bioconjugate. The unimodal zeta potential distribution of either Ac-L-asparaginase or L-asparaginase (Figure 2) can be correlated with a homogeneous charge distribution of

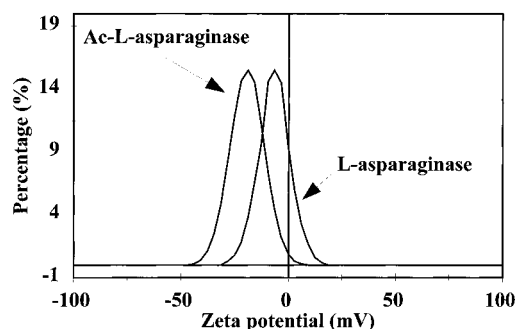


Figure 2. Zeta potential distribution of Ac-L-asparaginase and L-asparaginase at pH 6.5, quantified by microelectrophoresis in electrolyte solution. The concentration of native enzyme or bioconjugate was $500 \mu\text{g mL}^{-1}$.

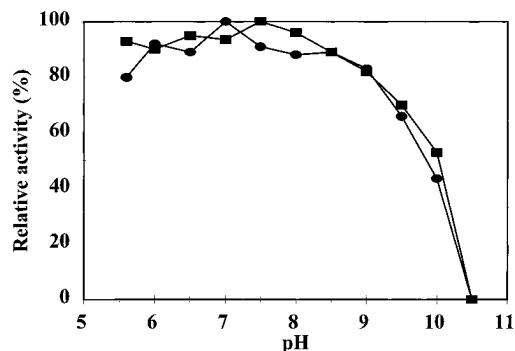


Figure 3. Effect of pH on the activity of Ac-L-asparaginase (●) and L-asparaginase (■).

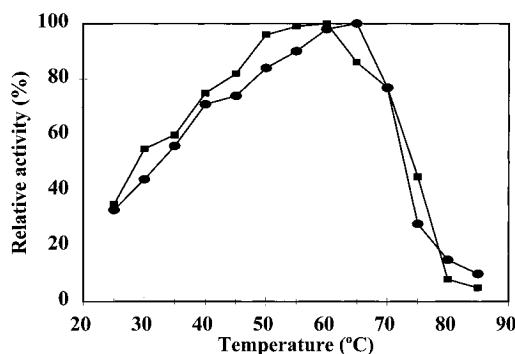


Figure 4. Effect of temperature on the activity of Ac-L-asparaginase (●) and L-asparaginase (■).

both samples (McNeil-Watson, 1988) and points to the absence of dissociation or aggregation either of native enzyme or of bioconjugate molecules for a concentration of $500 \mu\text{g mL}^{-1}$ at pH 6.5.

In this study we analyze the homogeneity of only those samples having electrophoretically similar populations. To perform a homogeneity evaluation, based on other characteristics, for example, the hydrophobic affinity, reversed phase chromatography could be used.

Effect of Conjugation on Catalytic Activity Dependence on pH and Temperature. Results of catalytic activity of the bioconjugate normalized in relation to the maximal catalytic activity are shown. Figure 3 evidences a similar catalytic activity for both bioconjugate and native enzyme over all of the pH range used. A similar pH dependence of the reaction catalyzed by the native enzyme in the pH range from 4 to 8 is reported in the literature (Ehrman, 1971).

Figure 4 shows a similar dependence of the catalytic activity on temperature both for bioconjugate and for

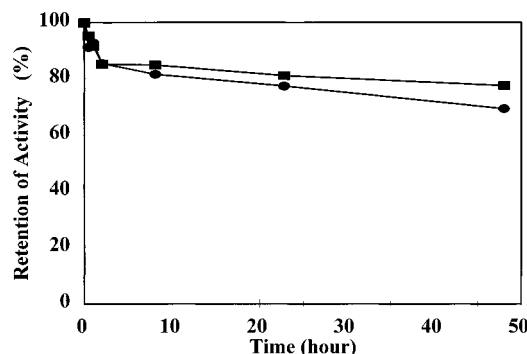


Figure 5. Stability of Ac-L-asparaginase (●) and L-asparaginase (■) in human serum at 37°C . Half-lives were calculated using the inverted decay model.

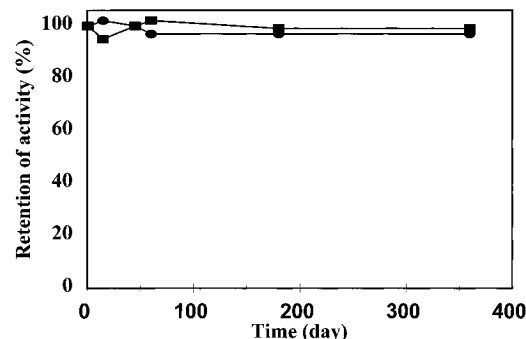


Figure 6. Stability of Ac-L-asparaginase (●) and L-asparaginase (■) on storage at 6°C in lyophilized form.

native enzyme; maximal activity was observed at 65 and 60°C for Ac-L-asparaginase and L-asparaginase, respectively.

Stability of Free and Conjugated Enzyme. A sharp decrease in the retention of activity of L-asparaginase and the conjugated form was observed in the first 2 h of incubation in human serum (Figure 5). This behavior is evidenced by the respective half-lives: 7.9 h for native enzyme and 6.9 h for bioconjugate, calculated using the inverted decay model. During the following 2–48 h the rate of activity variation is reduced.

No significant differences between the retention of activity for both the bioconjugate and the native enzyme after 360 days of storage at 6°C in lyophilized form were found (Figure 6).

Effect of Surfactant Micelles on Kinetic Characteristics of the Bioconjugate. The effect of micellar systems of natural and synthetic surfactants on the retention of catalytic activity and on the kinetic parameters (Michaelis constant, K_m , and maximal reaction rate, V) of the bioconjugate was evaluated and compared with the performances of native enzyme (Table 2). Surfactants with different charge and hydrophilic–lipophilic balance (HLB) were used: two anionic surfactants, cholic acid (HLB = 18) and deoxycholic acid (HLB = 16), and two nonionic surfactants, Brij 76 (HLB = 12) and Synperonic F-68 (HLB = 29). The results of retention of catalytic activity evidence a decrease of activity of bioconjugate in the absence of micellar medium. No decrease of catalytic activity of the bioconjugate was observed in micellar medium. This result points to the stabilization of the bioconjugate molecule by the presence of micelles independent of its charge or hydrophobicity.

A comparison between the Michaelis constants of L-asparaginase and Ac-L-asparaginase, in micellar medium (Table 2), evidences a slight decrease of the K_m of the bioconjugate. In contrast with a constant K_m for L-asparaginase, independent of the composition of mi-

Table 2. Effect of Surfactant Micelles on Kinetic Characteristics of Conjugated and Native Enzymes^a

enzyme	surfactant ^b	retention of act. (%)	K_m (M) ($\times 10^{-5}$)	V (M s ⁻¹)
Ac-L-asparaginase	cholic acid	>98	4.1	133
	deoxycholic acid	>98	nd ^c	nd
	Brij 76	>98	3.6	125
	Synperonic F-68	>96	4.6	125
L-asparaginase		65	nd	nd
	cholic acid	100	3.0	129
	deoxycholic acid	100	nd	nd
	Brij 76	100	3.1	125
	Synperonic F-68	100	3.1	118
		100	3.0	101

^a Linearization performed according to the Hanes method.^b Concentrations of surfactants: cholic acid, 1%; deoxycholic acid, 0.32%; Brij 76, 0.003%; Synperonic F-68, 0.053%. ^c nd, not done.

celles, a slight increase of this parameter was observed as function of the HLB of the surfactant. The maximal reaction (V) for the bioconjugate and the native enzyme also evidences slight differences. The effect of modification on these parameters can be discussed in terms of V/K_m , which is considered (Creighton, 1984) to be the most critical parameter in determining the specificity of an enzyme for a substrate. For the native enzyme in the absence of surfactant this ratio is 3.4×10^6 , while in the presence of micelles of surfactants it is as follows: cholic acid, 4.3×10^6 ; Brij 76, 4.0×10^6 ; Synperonic F-68, 3.9×10^6 . For the bioconjugate in micelles of cholic acid this ratio is 3.2×10^6 , in micelles of Brij 76 it is 3.5×10^6 , or in micelles of Synperonic F-68 it is 2.7×10^6 . The slight difference between the V/K_m of the bioconjugate and that of the native L-asparaginase, which can correspond to a small alteration of the affinity of the enzyme to substrate, can be due to hydrophobic interactions of the bioconjugate with micelles. Therefore, the localization of the bioconjugate and the native enzyme in a micellar medium may be different. If the bioconjugate becomes entrapped in the micelle, some conformational or microenvironmental alterations can occur and the accessibility of substrate to the bioconjugate can be perturbed, namely by steric hindrances. This may have consequences on the kinetic parameters of each enzyme form. These alterations, based on hydrophobic interactions, can depend on the surfactant hydrophilic-lipophilic characteristics.

The slight increase in alteration of affinity with conjugation which leads to a K_m for the bioconjugate in the range $(3.6-4.6) \times 10^{-5}$ M does not limit its clinical usefulness; the K_m range for clinically useful L-asparaginase is $(1-5) \times 10^{-5}$ M (Capizzi and Cheng, 1981).

CONCLUSIONS

Consistent with the purpose of the construction of a bioconjugate with affinity to the matrix lipid of liposomes, a significant increase in the hydrophobicity of the enzyme was observed after conjugation. Also, an alteration of surface charge was evidenced by the electrophoretic mobility curves. Considering these results, both hydrophobic and electrostatic interactions can explain the association between Ac-L-asparaginase and the bilayers of liposomes previously reported (Martins et al., 1990; Jorge, 1994). The solubilization of Ac-L-asparaginase in octanol, up to a concentration of $0.46 \mu\text{M}$, points to the potentiality of this bioconjugation to promote the solubilization of hydrophilic enzymes in organic solvents. The promotion of solubilization of hydrophilic enzymes in nonpolar organic solvents, by the development of specific methods of dissolution, was reported by Mozhaev et al. (1991). A minimized perturbation must be achieved for

the structure of this bioconjugate, considering the results of the effect of the chemical modification on the pH and temperature activity profiles, on the storage stability, on the stability in human serum, on the catalytic activity, and on the substrate affinity. The high retention of catalytic properties of this bioconjugate overcame the limitations reported in the literature (Claassen and Rooijen, 1983) to the modification of L-asparaginase by the palmitoyl chloride method. This must be the result of the following:

(1) the mild conditions of this conjugation process (Torchilin et al., 1980);

(2) the protective effect of substrate during conjugation (Martins et al., 1990); and

(3) the stabilizing effect of the hydrophobic portions of a naturally occurring or synthetic micellar systems.

In conclusion, the preservation of catalytic properties following conjugation of acyl chains to the native enzyme, as reported, evidences the potential of Ac-L-asparaginase as a candidate to substitute for the native enzyme in processes requiring a hydrophobic interaction with a microenvironment.

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LITERATURE CITED

- Abuchowski, A., Es, T. E., Palczuk, N. C., McCoy, J. R., and Davis, F. F. (1979) Treatment of L5178Y tumor-bearing BDF1 mice with a nonimmunogenic L-glutaminase-L-asparaginase. *Cancer Treatment Rep.* 63, 1127-1132.
- Asselin, B. L., Ryan, D., Frantz, N., Bernal, S. D., Leavitt, P., Sallan, S. E., and Cohen, H. J. (1989) *In vitro* and *in vivo* killing of acute lymphoblastic leukemia cells by L-asparaginase. *Cancer Res.* 49, 4363-4368.
- Boggs, J. M. (1987) Lipid intermolecular hydrogen bonding: influence on structural organization and membrane function. *Biochim. Biophys. Acta* 906, 353-404.
- Böhlen, P., Stein, S., Dairman, W., and Udenfriend, S. (1973) Fluorimetric assay of proteins in the nanogram range. *Arch. Biochem. Biophys.* 155, 213-220.
- Borman, S. (1989) Bioconjugate chemistry attracts growing interest. *Chem. Eng. News* 67 (May 8), 25-28.
- Capizzi, R. L., and Cheng, Y. C. (1981) Therapy of neoplasia with asparaginase. *Enzymes as Drugs* (J. C. Holcenberg, Ed.) pp 2-24, Wiley, New York.
- Capizzi, R. L., and Handschumacher, R. E. (1982) Asparaginase. *Cancer Medicine* (J. F. Holland and E. Frei III, Eds.) pp 920-933, Lea and Febiger, Philadelphia.
- Chaney A. L., and Marbach E. P. (1962) Modified reagents for determination of urea and ammonia. *Clin. Chem.* 8, 130-132.
- Claassen, E., and Rooijen, N. (1983) A comparative study on the effectiveness of various procedures for attachment of two proteins (L-asparaginase and horseradish peroxidase) to the surface of liposomes. *Prep. Biochem.* 13, 167-174.
- Cornish-Bowden, A., and Wharton, C. W. (1988) Simple enzyme kinetics. *Enzyme Kinetics* (D. Rickwood, Ed.) pp 1-18, IRL Press, Oxford, U.K.
- Creighton, T. E. (1984) *Proteins: Structure and Molecular Principles*, pp 405-409, W. H. Freeman, New York.
- Dwyer, D. L. (1993) Electrophoretic techniques of analysis and isolation. *Protein Biotechnology* (F. Franks, Ed.) pp 313-363, Humana Press, Totowa, NJ.
- Ehrman, M., Cedar, H., and Schwartz, J. H. (1971) L-Asparaginase of *Escherichia coli*: studies on the enzymatic mechanism of action. *J. Biol. Chem.* 246, 88-94.
- Fendler, J. H., and Fendler, E. J. (1975) Micellar effects on hydrophobic interactions and protein Structure. *Catalysis in*

- Micellar and Macromolecular Systems*, pp 305–312, Academic Press, New York.
- Jain, K. M., and Zakim, D. (1987) The spontaneous incorporation of proteins into preformed bilayers. *Biochim. Biophys. Acta* 906, 33–68.
- Jorge, J. C. S., Perez-Soler, R., Morais, J. G., and Cruz, M. E. M. (1994) Liposomal palmitoyl-L-asparaginase: characterization and biological activity. *Cancer Chemother. Pharmacol.* 34, 230–234.
- Konrad, M. (1989) Immunogenicity of proteins administered to humans for therapeutic purposes. *Trends Biotechnol.* 7, 175–179.
- Low, M. G. (1987) Biochemistry of the glycosyl-phosphatidylinositol membrane. *Bioch. J.* 244, 1–13.
- Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193, 265–275.
- Martins, M. B. F., and Cruz, M. E. M. (1994) Characterization of bioconjugates of L-asparaginase and Cu,Zn-superoxide dismutase. *Abstracts, Third European Symposium on Controlled Drug Delivery*, pp 188–191, University of Twente, Enschede, The Netherlands.
- Martins, M. B. F., Jorge, J. C. S., and Cruz, M. E. M. (1990). Acylation of L-asparaginase with total retention of enzymatic activity. *Biochimie* 72, 671–675.
- McNeil-Watson, F. K. (1988) A new instrument for particle size analysis and electrophoretic mobility measurement using photon correlation spectroscopy. *Particle Size Analysis* (P. J. Lloyd, Ed.) pp 89–99, Wiley, New York.
- Means, G. E., and Feeney, R. E. (1971) Proteins and their properties. *Chemical Modifications of Proteins*, pp 3–20, Holden-Day, San Francisco.
- Mozhaev, V. V., Poltevisky, K. G., Slepnev, V. I., Badun, G. A., and Levashov, A. V. (1991) Homogeneous solutions of hydrophilic enzymes in nonpolar organic solvents. New systems for fundamental studies and biocatalytic transformations. *FEBS* 292, 159–161.
- Nakahara, T., Higuchi, K., Hirata, H., and Ishikawa, K. (1988) Effect of N-acylation of lysine residue on lipoxygenase activity: modification for use in organic solvents. *Enzyme Microb. Technol.* 10, 474–478.
- Nickle, E. C., Solomon, R. D., Torchia, T. E., and Wriston, J. C. (1982) Chemical modifications of *Escherichia coli* L-asparaginase and their effect on plasma clearance rate and other properties. *Biochim. Biophys. Acta* 704, 345–352.
- Timasheff, S. N., and Arakawa, T. (1993) Stabilization of protein structure by solvents. *Protein Structure: A Practical Approach* (T. E. Creighton, Ed.) pp 331–345, IRL Press, Oxford U.K.
- Torchilin, V. P., Omel'Yanenko, V. G., Klivanov, A. L., Mikhailov, A. I., Gol'Danskii V. I., and Smirnov, V. N. (1980) Incorporation of hydrophilic protein modified with hydrophobic agent into liposome membrane. *Biochim. Biophys. Acta* 602, 511–521.
- Wriston, J. C., and Yellin, T. O. (1973) L-Asparaginase: a review. *Adv. Enzymol. Relat. Areas Mol. Biol.* 39, 185–248.

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Specific Activity of Polypyrrole Nanoparticulate Immunoreagents: Comparison of Surface Chemistry and Immobilization Options[†]

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Polypyrrole-based colloids with differing surface chemistries were compared with respect to the specific activity of immobilized antibody. Monoclonal antibody to the alpha subunit of human chorionic gonadotropin (hCG) was modified by incorporation of cystamine into the Fc-carbohydrate, followed by reduction with dithiothreitol resulting in the generation of 4.5 free thiols per IgG. The reduced IgG was added to clean, unmodified and surface-modified polypyrrole colloids. Functionalized colloids included carboxylate-modified polypyrrole, poly[pyrrole-co-1-(2-carboxyethyl) pyrrole]–silica composite, and amine forms of the carboxylated colloids. The amine-functionalized colloids were subsequently treated with sulfosuccinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate to provide thiol-reactive maleimide surface groups. Following the conjugation of IgG to the colloids, bound and soluble antibody activity was quantitated using a sequentially competitive immunoassay for hCG, based on an automated commercial hCG kit. The results indicated that all forms of polypyrrole retained the equivalence of between 12 and 33 μg of IgG activity/mg of colloidal solids, relative to the unmodified soluble IgG.

INTRODUCTION

Polypyrrole nanoparticles are a unique class of materials with applications in immunodiagnostics. The conductive properties of polypyrrole are well-known; however, it is its intense optical absorbance that provides opportunities for the development of visually read immunoassay formats (1, 2). The preparation of base polypyrrole latex in aqueous media has been described by Armes et al. (3, 4) and Bjorklund and Liedberg (5). Subsequently, Tarcha et al. (1) developed methods for the surface modification of the base polypyrrole providing functional groups for the covalent attachment of specific binding ligands. More recently, composite particles of ultrafine silica sols and polypyrrole, in which the silica provides charge stabilization, were developed by Maeda and Armes (6, 7). The intense dark coloration is present with these composite particles as well; however, the need for a steric stabilizer, such as poly(vinyl alcohol), is eliminated. These latter researchers further developed one-step syntheses for surface carboxylation of these composites employing the co-monomer 1-(2-carboxyethyl)pyrrole in the latex polymerizations (8).

Immunoreagents of the base polypyrrole and the polypyrrole–silica particles can be made by coating the particles with the specific binding ligand (antibody or macromolecular antigen) by adsorption. It is known that the specific activity of solid phase based immunoreagents and soluble immunoconjugates can be enhanced if the attachment is done through an intervening spacer molecule (9, 10). Additional specific activity can often be gained if the attachment to the specific binding member

is made to a region isolated from the binding site, such as the carbohydrate, located predominantly in the Fc region of IgGs. This was demonstrated by Hoffman and O'Shannessy (11) and Brillhart and Ngo (12) for the attachment of antibody to hydrazide surfaces via the carbohydrate region, which had been previously oxidized by periodate, producing aldehyde moieties.

This paper compares the specific activity of a monoclonal antibody to human chorionic gonadotropin (anti-hCG)¹ in solution to that immobilized on three particulate polypyrrole surfaces: (1) polypyrrole latex made by chemical oxidation of pyrrole in the presence of poly(vinyl alcohol), (2) a carboxylated derivative of (1), and (3) a carboxylated polypyrrole–silica nanocomposite. The antibody was passively coated onto all of the surfaces and covalently linked via the carbohydrate region to the functionalized polypyrrole surfaces with intervening spacer molecules.

EXPERIMENTAL PROCEDURES

Materials. All aqueous solutions were pressure-filtered through a Millipore 0.45 μm filter cartridge before use. The poly(vinyl alcohol) used was DuPont Elvanol HV, 99+% hydrolyzed, 125 000–186 000 molecular weight. Pyrrole was obtained from various sources, e.g. BASF, vacuum-distilled between 50 and 60 °C, and stored under nitrogen at –80 °C before use. High-purity *N*-methylpyrrolidone was obtained from Burdick and Jackson. Ferric chloride hexahydrate, bromoacetyl bromide, so-

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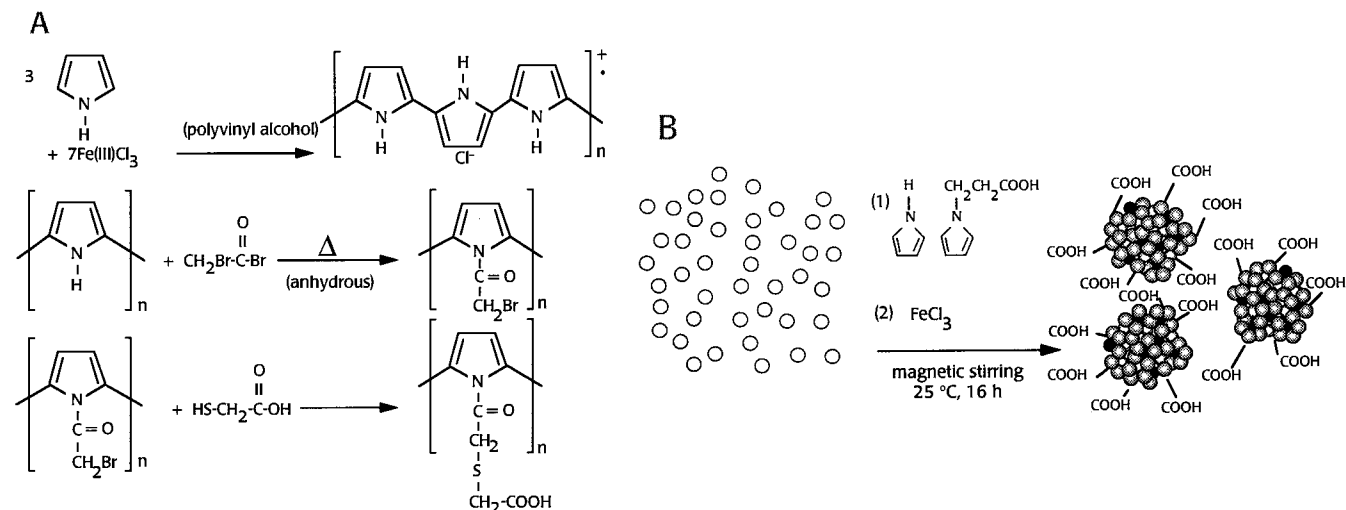
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[‡] Abbott Laboratories.

[§] University of Sussex.

[®] Abstract published in *Advance ACS Abstracts*, June 1, 1996.

¹ Abbreviations: hCG, human chorionic gonadotropin; EDA, ethylenediamine dihydrochloride; MES, 2-(*N*-morpholino)ethanesulfonic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; EDC, 1-ethyl-3-[(dimethylamino)propyl]carbodiimide hydrochloride; sulfo-SMCC, sulfosuccinimidyl 4-(*N*-maleimidomethyl)-cyclohexane-1-carboxylate; XPS/SIMS, X-ray photoelectron spectroscopy/secondary ion mass spectroscopy; NMP, *N*-methylpyrrolidone; TEA, triethanolamine; HEPES, *N*-(2-hydroxyethyl)piperazine-*N'*-ethanesulfonic acid; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); DTT, dithiothreitol; TEM, transmission electron microscopy.

Scheme 1. Synthesis of (A) Carboxylated Polypyrrole Latex and (B) Carboxylated Polypyrrole–Silica Composite Nanoparticles

dium periodate, sodium cyanoborohydride, 2,2,2-trifluoroethanol, 2,2,2-trifluoroethanethiol, 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), and mercaptoacetic acid were obtained from Aldrich Chemical Co., and all were used as received. Ethylenediamine dihydrochloride (EDA), 2-(*N*-morpholino)ethanesulfonic acid (MES), 3-(*N*-morpholino)propanesulfonic acid (MOPS), *N*-(2-hydroxyethyl)piperazine-*N'*-ethanesulfonic acid (HEPES), and cystamine dihydrochloride were obtained from Sigma. 1-Ethyl-3-[(dimethylamino)propyl]carbodiimide hydrochloride (EDC), dithiothreitol (DTT), and sulfosuccinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (sulfo-SM-CC) were from Pierce Chemical Co. and used as received. Colloidal silica, kindly donated by Nyalcol Products, had an average particle diameter of ca. 20 nm and was supplied as a 40 w/w % aqueous dispersion. Antibodies directed against human chorionic gonadotropin (anti-hCG) were murine monoclonal and were prepared by the Abbott Diagnostics Division according to standard methods. Diafiltration cartridges (0.1 μm cutoff), which could handle process volumes from 5 mL to 1 L, were obtained from Microgon Inc., Laguna Hills, CA. Regenerated cellulose dialysis membranes with a molecular weight cutoff of 12 000–14 000 were obtained from Spectrum Medical Industries. Stirred-cell ultrafiltration devices and membranes (YM10, nominal molecular weight cutoff of 10 000) were obtained from Amicon, Inc. Human chorionic gonadotropin (hCG) was obtained either from Abbott Laboratories or from Sigma. Assay kits for quantitative determination of hCG were obtained from Abbott Laboratories.

Particle Size and Distribution. Particle size distributions were obtained on a Brookhaven Instruments BI-DCP disk centrifuge photosedimentometer. The general theory and method of this technique have been described by Thomas and Fairhurst (13). For this work the samples were diluted into distilled water and injected onto the spinning disk which contained 4% sucrose as the spin fluid. All colloidal suspensions were sonicated for 30 s in a laboratory sonic bath prior to analysis to break up any loose aggregates. A density of 1.36 g cm^{-3} was assumed for the polypyrrole latex (14); however, the unmodified latex contains an adsorbed layer of stabilizer, and hence this value should be considered an upper limit. The particle density of the carboxylated polypyrrole–silica nanocomposite was determined to be 1.57 cm^{-3} using helium pycnometry (average of two measurements using a Micromeritics AccuPyc 1330 pycnometer). Trans-

mission electron microscopy (TEM) was done with a Phillips CM12 transmission electron microscope operating at 100 kV, using carbon-coated grids containing the dried suspensions.

X-ray Photoelectron Spectroscopy (XPS). The surface spectroscopy data were collected on the various particulates of this study using a Physical Electronics 5600 XPS/SIMS system using a monochromatic Al ($\text{Al K}\alpha = 1486.6 \text{ eV}$) X-ray source. When maleimide surfaces were analyzed, a fluorinated reactive marker, 2,2,2-trifluoroethanethiol, was used, since the maleimide did not have uniquely differentiable atoms from the underlying surface. 2,2,2-Trifluoroethanol was used as a control. Suspensions (5 mL) were combined with 20 μL of the thiol or the alcohol and were allowed to react for 1 h at room temperature. All suspensions were dialyzed against distilled water prior to preparation for XPS analysis. The particulate samples were dried from suspension on 1 cm^2 silver squares, which had been previously surface-roughened by immersion in 6 M nitric acid for 20 s and rinsed in distilled water.

Synthesis and Purification of Polypyrrole Latex. Polypyrrole latex was synthesized according to a procedure similar to that described by Armes et al. (3). A 2 L glass reactor filled with a Teflon paddle stirrer was used for the latex reaction. An 8.0 g sample of poly(vinyl alcohol) was dissolved in distilled water (500 mL) by heating at 95 $^{\circ}\text{C}$ with stirring for 1 h. After cooling to room temperature overnight, the solution was pressure-filtered through a 0.45 μm filter cartridge into the reactor. Ferric chloride hexahydrate (176.0 g) was dissolved in 500 mL of distilled water, pressure-filtered, and combined with the poly(vinyl alcohol) solution. The solution was stirred at 500 rpm, and distilled pyrrole (20 mL) was added all at once. The solution turned black after a few seconds. After 10 min, the stirring rate was reduced to 250 rpm, and the reaction was allowed to proceed for a total of 4 h. Purification was achieved by diafiltration (980 cm^2 , 0.1 μm cutoff cartridge), which involved 10–15 volume exchanges with distilled water. At this point the conductivity of the supernatant reached a plateau at 300 $\mu\text{S cm}^{-1}$, where distilled water had a conductivity of 1 $\mu\text{S cm}^{-1}$. The latex was stored at room temperature in distilled water until further use.

Synthesis and Purification of Carboxylated Polypyrrole Latex. This procedure is a modification of that reported by Tarcha et al. (1) and is shown in Scheme 1A. An aliquot of aqueous latex was solvent-exchanged into

N-methylpyrrolidone (NMP) and dried by azeotrope with toluene. A 95 g sample of the anhydrous latex at 13.5% solids (12.8 g of polymer, 0.191 equiv) was cooled to 10 °C, and 0.191 mol (38.6 g) of bromoacetyl bromide was added with stirring. The reaction was allowed to warm to room temperature over 1 h. Then it was heated using a glycerol bath at 80 °C for a further 4 h. An equal volume of 50% NMP in water (v/v %) was then added to the reaction, and the particles became flocculated. They were centrifuged at 12000*g* for 30 min, and the supernatant was decanted. The pellets were redispersed with the aid of a sonic probe into 5% sodium carbonate solution, containing 0.35% Brij 35, to a total volume of 220 mL.

A 145 mL aliquot of the bromoacetylated suspension (pH 9.2, 0.13 equiv of pyrrole units; conversion to bromoacetyl derivative was not measured) was reacted with 13.2 g (0.14 equiv) of mercaptoacetic acid at room temperature with stirring. Some foaming initially occurred due to the release of CO₂. The reaction mixture was stirred overnight.

The resulting surface-modified latex was purified by diafiltration using a smaller cartridge (600 cm², 0.1 μm cutoff). After 30 volume exchanges, the latex effluent had a conductivity of 400 μΩ⁻¹.

Synthesis and Purification of Carboxylated Polypyrrole–Silica Nanocomposite. The carboxylic acid-functionalized polypyrrole–silica particles were synthesized as follows (see Scheme 1B): 200 mL of aqueous silica sol was diluted to 500 mL with distilled, deionized water. To this stirred solution was added a 100 mL aqueous solution containing 5.0 mL of pyrrole and 10.0 g of 1-(2-carboxyethyl)pyrrole (1:1 molar ratio). This reaction mixture was stirred at room temperature for 3 h prior to the addition of an aqueous solution of ferric chloride hexahydrate (91 g in 400 mL). The reaction solution turned black, and stirring was continued for at least 5 h at room temperature.

The final black dispersion was centrifuged at 6000 rpm for 30 min using a Beckman J2-21 centrifuge, and the resulting black sediment was redispersed in de-ionized water using an ultrasonic bath. This centrifugation–redispersion cycle was repeated twice to completely remove the excess small silica particles and (in)organic byproducts from the larger carboxylated polypyrrole–silica composite particles. Diafiltration was found to be unsuitable for purifying these suspensions because excess silica particles of the dimensions used tended to clog the diafiltration membranes, rendering them ineffective.

Analysis of Carboxylic Acid Content. The polypyrrole particles, which had been prepurified by either centrifugation and washing or diafiltration, were ion-exchanged by shaking with a mixed bed of cation and anion exchange resins in the H⁺ and OH⁻ forms. The mixed bed was prepared from equal wet volumes of Dowex 1X4 (anion) and Dowex 50WX4 (cation), 20–50 mesh. The individual resins were purified prior to use according to the method of Vanderhoff et al. (15). After ion exchange, the solids content was measured gravimetrically and the latex was titrated conductimetrically using a standard barium hydroxide solution. The ion exchange process, redetermination of solids content, and titration were repeated until a constant specific titer was obtained.

Amination by Amidation of Surface Carboxylic Acid with EDA. A stock solution of 2 M ethylenediamine dihydrochloride/20 mM *N*-(morpholino)ethanesulfonic acid buffer (EDA/MES) was prepared by adding EDA (133 g) and MES (1.95 g) to 400 mL of distilled water. The pH was adjusted to 5.5 with dilute sodium

hydroxide. The volume was brought up to 500 mL with distilled water and stored at room temperature in an amber glass container.

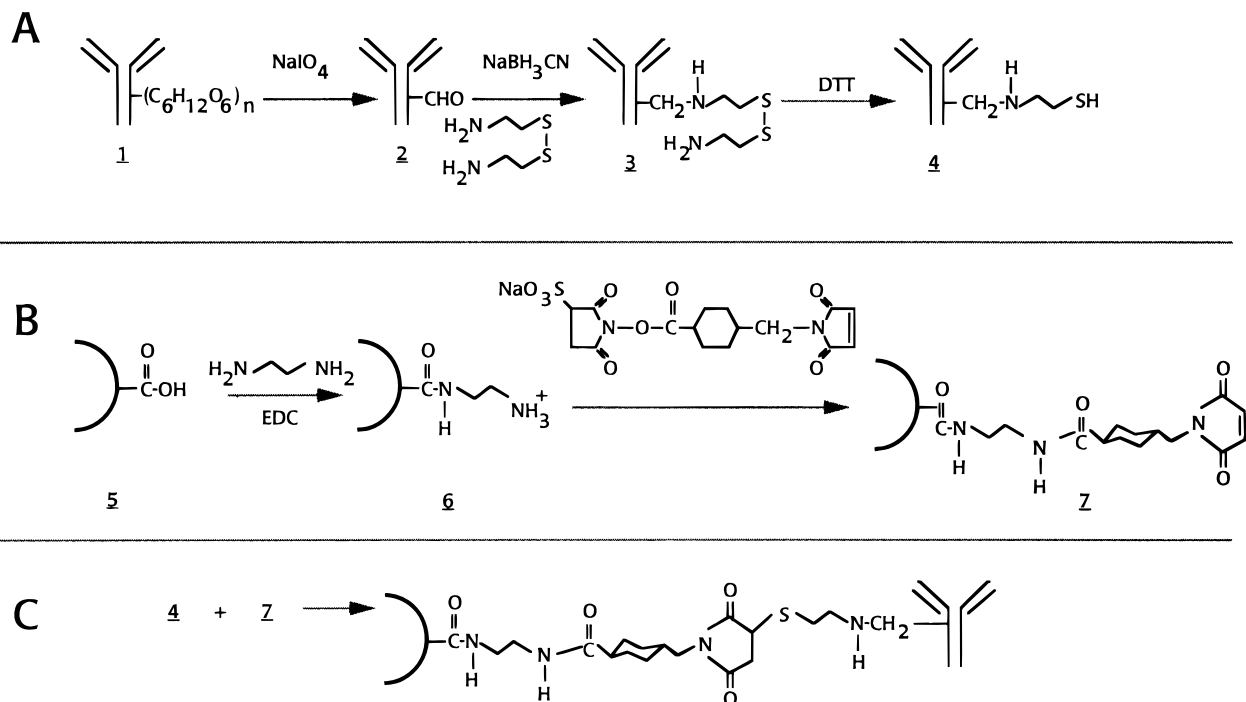
Carboxylated polypyrrole particles (10 mL, 2.0% solids) were added to a polypropylene tube followed by a 10 mL aliquot of EDA/MES buffer. The suspension was mixed end-over-end for 10 min. Then a freshly prepared aqueous solution of 0.52 M EDC (0.1 mL) was added followed by brief vortex mixing. The suspension was mixed end-over-end at room temperature. After 1 h, 0.6 mL of 4 M acetate buffer, pH 5.5, was added. The suspension was purified by dialysis against 2 L of deionized water, with 10 changes of dialysate. This is shown in Scheme 2B.

Preparation of Maleimido Surface-Functionalized Particles. Aminated latex (10.0 mL of 0.3–0.5% solids) was placed into a polypropylene tube to which 10.0 mL of a 4 mg/mL sulfo-SMCC solution in 0.1 M MOPS buffer, pH 7.0, was added. The suspension was mixed end-over-end at room temperature for 1 h. The latex was concentrated to 5 mL by tangential flow filtration using a MicroKross diafiltration cartridge (4 cm², 0.1 μm cutoff) followed by diafiltration with 50–75 mL of MOPS buffer. The maleimido-functionalized particles were used immediately after preparation to minimize the extent of hydrolysis of the maleimide.

Fc Modification of Monoclonal Anti-hCG. The introduction of thiol groups into the Fc carbohydrate region of monoclonal antibodies was performed similarly to the method described by Husain and Bieniarz (16, 17). Anti-hCG (17 mL at 10 mg/mL) was exchanged into triethanolamine (TEA) buffer (50 mM, 160 mM NaCl, pH 8.0) by dialysis overnight at 5 °C. In an amber glass vial, the antibody solution was combined with 1.1 mL of 200 mM sodium periodate in TEA buffer. The solution was incubated for 1 h at 5 °C with slow shaking on a tabletop rotary shaker. A Sephadex G-25 column, 2.5 × 40 cm, was equilibrated with 0.1 M sodium phosphate/0.1 M NaCl, pH 7.0. The oxidized antibody was eluted at a flow rate of 2.5 mL/min. Fractions with an absorbance greater than 0.3 at 280 nm were pooled to yield a final volume of 40 mL.

Cystamine was dissolved in 10 mM phosphate buffered saline, pH 7.2, at a concentration of 169 mg/mL. Sodium borohydride was dissolved in the same buffer at 20 mg/mL. The oxidized antibody was reacted with 9.9 mL of cystamine solution followed immediately with 2.5 mL of cyanoborohydride solution to reduce the Schiff base. The final mixture was incubated overnight at room temperature with slow shaking on a rotary shaker. It was then concentrated to 10 mL, using stirred cell ultrafiltration, and desalted using a Sephadex G-25 column under the same conditions stated above. The antibody fractions were combined, giving a final protein yield of 84%. Modified antibody that was not used immediately was rapidly frozen using liquid nitrogen and stored at –70 °C.

Reduction of the Disulfide. Fc-cystamine-modified antibody was reduced prior to conjugation to particles. A 40 mM solution of DTT was prepared by dissolving the DTT in 0.1 M sodium phosphate/0.1 M NaCl containing 2 mM EDTA. An aliquot of DTT solution (0.05 mL) was added to 1.0 mL of modified antibody, and the mixture was incubated for 0.25 h at room temperature. The solution was passed through a Sephadex G-25 column under conditions stated previously, and the antibody fractions were pooled. Reduced antibody was stored at 5 °C, protected from light, and was added to latex for conjugation within 24 h of desalting. Scheme 2A illustrate the antibody modification process.

Scheme 2. Fc Immobilization of Mouse IgG to Particles: (A) Activation of the Antibody; (B) Activation of the Particles; and (C) Coupling of the Antibody to the Particles

Determination of the Number of Thiol Groups per Fc-Thiolated Anti-hCG. The concentrations of IgG were determined by the bicinchoninic acid method (BCA method, Pierce, Rockford IL), using the protocol supplied by the manufacturer. The thiol content of the IgG was quantitated spectrophotometrically by reaction with DTNB (18). An aliquot of 0.25 mL of IgG containing sample was mixed with 0.75 mL of 0.1 M HEPES, pH 8.0. To this was added 0.1 mL of DTNB (4 mg/mL in 0.1 M HEPES, pH 8.0). After 15 min at room temperature, the absorbance at 412 nm was measured and the thiol concentration determined using an extinction coefficient of $13\,600\text{ M}^{-1}\text{ cm}^{-1}$.

Coupling of Thiolated Antibody to Maleimido Particles. Maleimide-functionalized particles (5.0 mL) were combined with 1.0 mL (3.0 mg) of Fc-thiolated antibody. The suspensions were mixed gently at room temperature overnight, followed by the addition of 10 mg/mL (1.0 mL) BSA in MOPS buffer. The particles were then concentrated and diafiltered, as before, using 25 mM MOPS, 0.5% BSA, and 0.1% Brij 35, pH 7.2, as the continuous phase. This is shown in Scheme 2C.

Adsorption of Anti-hCG Antibody to Unmodified, Carboxylated, and Aminated Particles. The polypyrrole dispersions (2.5 mL of 1.0% solids) were diluted with 2.5 mL of 0.1 M MOPS, pH 7.0, to which was added 1.0 mL (3.0 mg) of antibody. The suspension was gently mixed at room temperature overnight, followed by the addition of 1.0 mL of 10 mg/mL BSA in MOPS buffer. The dispersions were then concentrated and diafiltered as before, using 25 mM MOPS, 0.5% BSA, and 0.1% Brij 35, pH 7.2, as the continuous phase. The following combinations of particles and antibodies were processed: nonfunctionalized polypyrrole, carboxylated polypyrrole, carboxylated polypyrrole-silica nanocomposite, and the amino-modified carboxylated particles with non-reduced or reduced antibody which had been derivatized in the Fc region with cystamine.

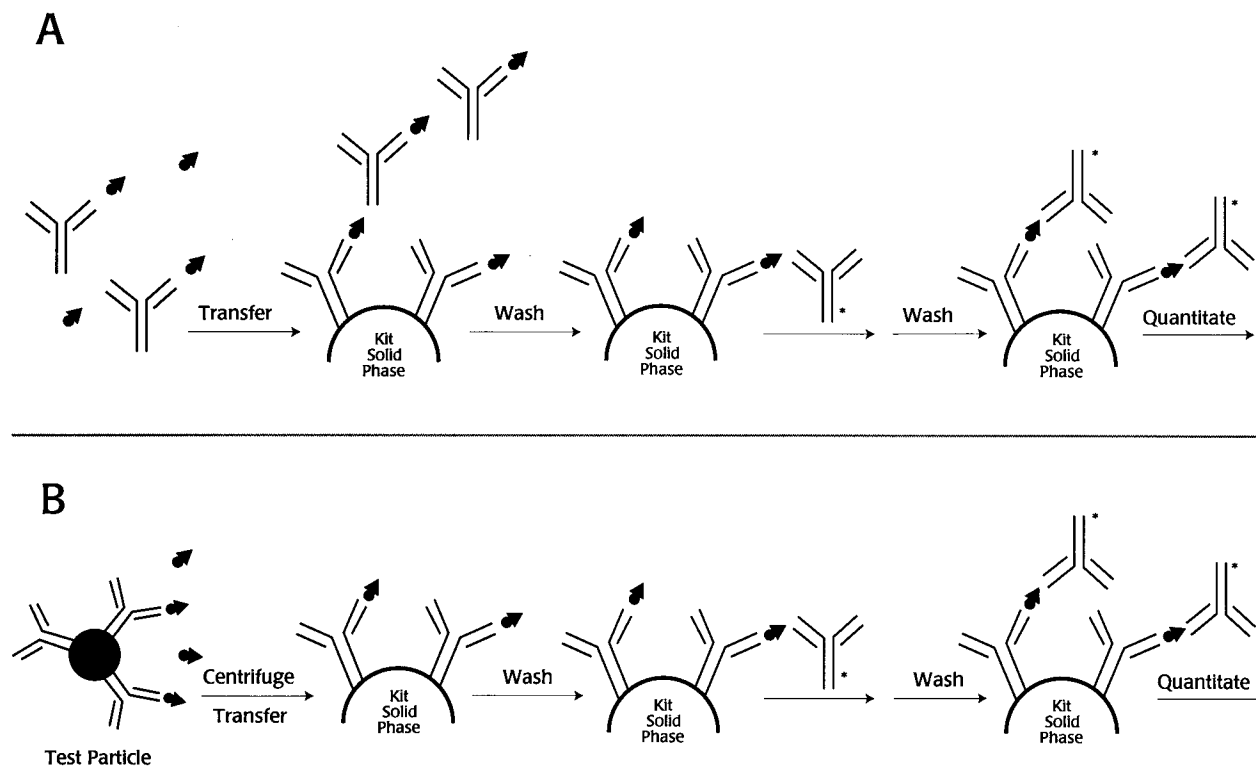
Inhibition Assays for the Measurement and Comparison of Antibody Activities. *General Principles* (Scheme 3). The relative immunoreactivities of native

and Fc-modified anti-hCG solutions and surface-immobilized anti-hCG were measured by sequential competitive analysis. The method is similar to that of Van Heyningen et al. (19), who compared the affinities of monoclonal antibodies. An IMx immunoanalyzer, manufactured by Abbott Laboratories, was used to perform the analyses, and the principle of the technique has been described previously (20). The assay method used here has a fixed concentration of antigen (hCG) and varies the concentration of monoclonal antibody added (Ab). The antigen concentration used should fall within the linear range of the assay kit, where more than 95% of the antigen will be complexed by the kit immunoreagents (Ab_{kit}). The hCG and Ab are premixed and allowed to complex. After equilibrium has been reached, the solution is introduced to the automated immunoassay, where it is added to the kit solid phase (Ab_{kit}). The solid phase is then washed to remove the free, soluble complex, and the remainder of the automated assay protocol is then completed.

The data may be analyzed by nonlinear regression, analogous to Michaelis-Menton kinetics:

$$[\text{hCG}]_{\text{bound}} = [B_{\text{max}}][\text{Ab}]/(\text{AB}_{50} + [\text{Ab}]) \quad (1)$$

In this form, $[\text{hCG}]_{\text{bound}}$ is the concentration of hCG bound by the monoclonal antibody (Ab). B_{max} is the asymptote for the maximum binding of antigen by the monoclonal antibody. This should approach, but may not equal, the total amount of antigen used in the assay. After the antibody-antigen complex has been added to the automated assay reagents, some of the complex will dissociate. The amount of dissociation is dependent on the rate of dissociation and the assay incubation period (t). The dissociated antigen may then partition between the monoclonal antibody and the kit solid phase. Using conditions in which the kit solid phase is in excess results in a B_{max} somewhat lower than the total level of antigen added. If the amount of soluble antibody added is increased further, it will eventually compete for all of the antigen.

Scheme 3. Sequential Competitive Assay Process for (A) Soluble Test Antibody and (B) Test Antibody Immobilized on a Solid Phase

The term AB_{50} identifies the level of antibody that inhibits the automated assay by 50%. This value is used to compare antibody preparations, under similar assay conditions. The AB_{50} is affected by the antibody's binding constant, purity, and level of denaturation and by the concentration of the antigen. The data may also be analyzed in terms of free antigen as a function of $[Ab]$:

$$[hCG]_{\text{free}} = [hCG]_{\text{total}} - \frac{[B_{\text{max}}][Ab]/(AB_{50} + [Ab])}{([hCG]_{\text{total}} - [B_{\text{max}}])} \quad (2)$$

Equation 2 describes the inhibition function of antibody on the assay. This equation has the general form of $[free] = [total] - [bound] - [offset]$, where $[offset] = [hCG]_{\text{total}} - B_{\text{max}}$. The offset is the difference between the calculated maximum bound analyte and the total analyte in the assay. As stated above, the offset is expected as a result of the level of dissociation of preformed antibody-antigen complex. Assay variation, incomplete competition by the soluble antibody, or other features may also contribute to the offset.

Comparison of the Specific Activity of the Nanoparticulate Immunoreagents. The particulates containing anti-hCG antibody immobilized by various means were subjected to activity testing by measuring their ability to remove hCG from the test hCG standard and hence inhibit the immunoassay signal. Antibody-coated particles were mixed with hCG, the suspension was centrifuged, and the supernatant was assayed for residual hCG using the automated immunoassay. The sequential competition assay for the particulate reagents is depicted in Scheme 3B. The assay is comparable to the assay used for the soluble antibody except that the initial complex is removed by centrifugation prior to analysis by the automated assay. This difference in protocol is necessary since antibody-coated particulates would act as additional solid phase in the automated assay. In addition, the intense absorbance of the polypyrrole particles interferes with the fluorometric, automated assay.

The assay results are generated with known particulate concentrations and are related directly to the inhibition curves generated for the soluble antibodies. This relation allowed the calculation of the amount of active antibody per latex particle in terms of active binding equivalents. From these values the effective surface area per active antibody can be calculated. The quantitation of immunoreagent activity according to this method gives relative comparisons of specific binding activity relative to the particle, but not true specific activity of the immobilized antibody. Also, it should be noted that the separation of antigen-(polypyrrole)particulate complex precludes the opportunity of dissociation present with the analysis of soluble antibody. This protocol difference may lead to an overestimation of the amount of immobilized antibody activity. In addition, the presence of antibody inactivated upon immobilization is not derived by this method. The preferred method to determine the percentage of active antibody on the particles in the final reagent would be a radiolabeling experiment. In view of the multisteps involved in the activation of the antibody for coupling, the development of a suitably tagged, and well-characterized, activated antibody was beyond the scope of this initial study.

Detailed Procedures: Preparation of hCG Stock Solution. Lyophilized hCG was dissolved in distilled water to prepare a 1000 IU/mL solution. An aliquot of 0.1 mL of the hCG solution was added to 200 mL of assay specimen diluent (Abbott hCG kit) to prepare a stock solution of 500 mIU/mL hCG. The actual concentration was determined experimentally on the IMx analyzer.

Evaluation of Soluble Anti-hCG Activity and Generation of a Standard Inhibition Curve. Dilutions of anti-hCG and Fc-cystamine-modified anti-hCG were prepared in 25 mM MOPS, 0.5% BSA, and 0.1% Brij 35, pH 7.2. The diluted antibody (200 μ L) was mixed with 800 μ L of 500 mIU/mL hCG and incubated at room temperature for 30 min. Aliquots of 200 μ L were transferred to the IMx analyzer for quantitation of hCG. Assay inhibition

curves were established as the detected [hCG] vs micrograms of test anti-hCG added.

Evaluation of Particle-Bound Anti-hCG Immunoactivity. Dilutions of anti-hCG bound to polypyrrole were prepared using 25 mM MOPS, 0.5% BSA, and 0.1% Brij 35, pH 7.2. The diluted particles (200 μ L) were mixed with 800 μ L of 500 mIU/mL hCG and incubated at room temperature for 30 min. The suspension was centrifuged at 12000*g* for 15 min to remove all particulates. Aliquots of supernatant (200 μ L) were transferred to the IMx analyzer for quantitation of hCG. Appropriate dilutions of the latex were made to obtain between 25 and 75% inhibition of measured hCG. Anti-hCG activity was determined as microgram anti-hCG equivalents from the standard inhibition curve generated for soluble antibody. The contribution by soluble anti-hCG in the particle suspensions was determined by centrifuging the preparation to remove the particulate solid phase, followed by sequential competitive analysis of the supernatant as described above.

RESULTS AND DISCUSSION

Particle Size and Distributions. It is apparent from the electron micrographs that the carboxylated polypyrrole latex has a similar particle size but a smoother and more spherical morphology compared to the carboxylated polypyrrole-silica composite particles (see Figure 1). The disk centrifuge data indicate that in aqueous solution the polypyrrole-silica composite particles have a higher degree of dispersion than the polypyrrole latex particles [the former has a polydispersity ratio (D_w/D_n) of 1.1 compared to 1.7 for the latter]. Presumably, the flocculation of the functionalized polypyrrole latex upon transfer from NMP to water is due in part to the removal of the poly(vinyl alcohol) steric stabilizer.

Carboxylic Acid Content. Conductimetric titration data indicated that the carboxylated polypyrrole latex and the carboxylated polypyrrole-silica composite particles had titers of 340 and 690 μ equiv of weak acid/g of solids, respectively. The specific surface area based on the particle size distribution was calculated automatically by the disk centrifuge program and gave values of 1.21×10^5 and 2.87×10^5 cm²/g for the carboxylated polypyrrole latex and polypyrrole-silica composite particles, respectively. From these values the calculated areas occupied by each carboxylic acid group were 35 and 48 $\text{\AA}^2$. The latter value does not take into account the fact that the polypyrrole-silica composite particles have a higher surface area than assumed due to the "raspberry-like" nature of the surface. Thus, the area per group may in fact be higher than 48 $\text{\AA}^2$. In addition, many of the surface acid groups present in aggregates may be accessible to titration and activation but not accessible for subsequent reaction with activated antibody due to steric reasons. This concern should apply to the more highly aggregated polypyrrole latex particles as well.

XPS Analysis of Particle Surface Compositions. The atomic surface compositions for unmodified polypyrrole latex, carboxylated polypyrrole latex, and carboxylated polypyrrole-silica composite particles are shown in Table 2 (samples a-m). The surface of the polypyrrole-silica composite particles is distinctly silica-rich as reported previously (21); however, a significant amount of polypyrrole is still present at the surface, as evidenced by the nitrogen 1s and carbon 1s signals (a). The sulfur signal observed in the carboxylated polypyrrole latex originates from the mercaptoacetic acid, which is used as a nucleophilic reactant with the bromoacetylated surface, thereby immobilizing pendant carboxylic acid groups. A low level (1%) of residual bromine was found

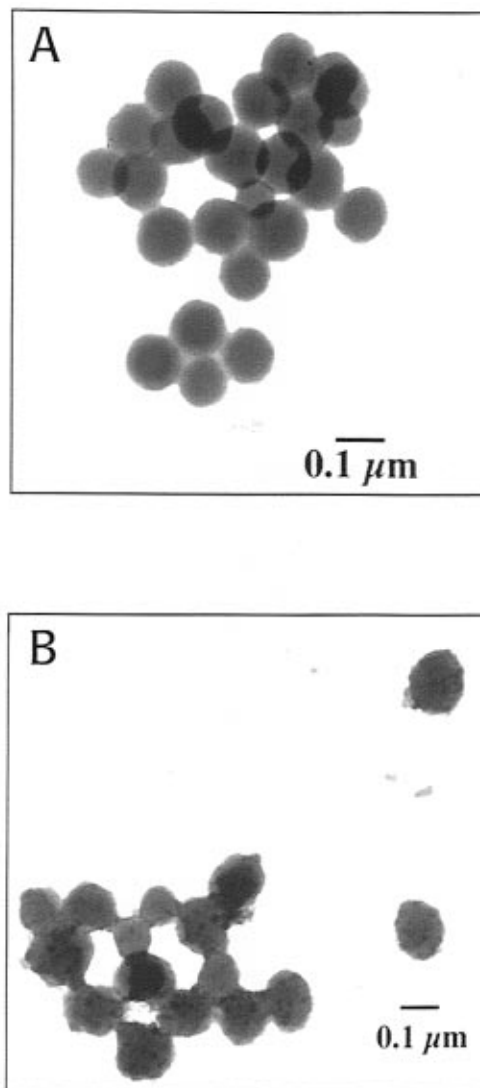


Figure 1. Particle size and morphology by electron microscopy of (A) carboxylated polypyrrole latex and (B) carboxylated polypyrrole-silica nanocomposite.

Table 1. Particle Size and Polydispersity (D_w/D_n) of Carboxylated Polypyrrole and Polypyrrole-Silica Nanocomposites before and after Antibody Coupling Determined by Disk Centrifuge Photosedimentometry

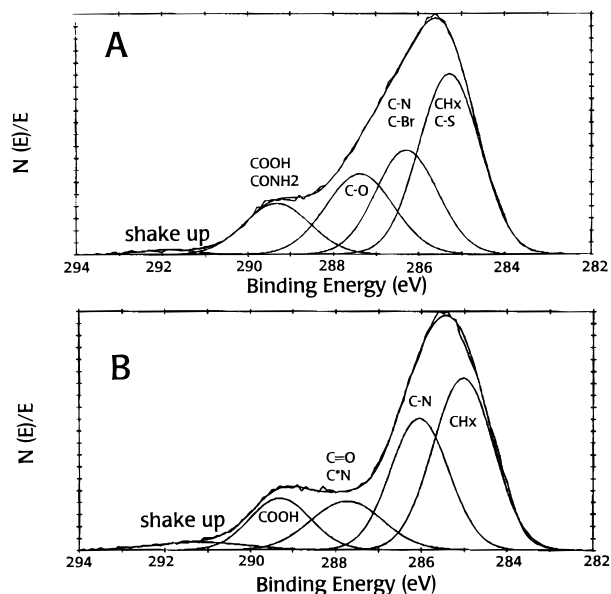
particle	D_n (nm)	D_w (nm)	D_w/D_n
carboxyl polypyrrole, no MAB	232	402	1.7
carboxyl polypyrrole-silica, no MAB	113	125	1.1
carboxyl polypyrrole, Fc-coupled MAB	301	484	1.6
carboxyl polypyrrole-silica, Fc-coupled MAB	131	156	1.2

on these samples after the reaction and cleaning (Scheme 1A) (g-l). The 17.6% oxygen content on the unmodified polypyrrole latex (m) is most likely due in part to the relatively high level of poly(vinyl alcohol) stabilizer which remains adsorbed even after cleaning. On the basis of the structure of polypyrrole and the measured nitrogen concentration of 9.2%, 46.0% of the carbon detected should be from unmodified polypyrrole. If it is assumed that the remaining 25.8% carbon comes from the poly(vinyl alcohol), this leaves 5% of the oxygen unaccounted for. This oxygen may be attributed to hydroxylated polypyrrole (22). Peak fitting of the carbon 1s signal for both carboxylated moieties and the corresponding peak assignments for these regions are shown (Figure 2). It is notable that the relative amounts of carboxyl carbon were similar in both cases. In addition, both spectra

Table 2. XPS Surface Analysis (Atomic Percent) of Particles

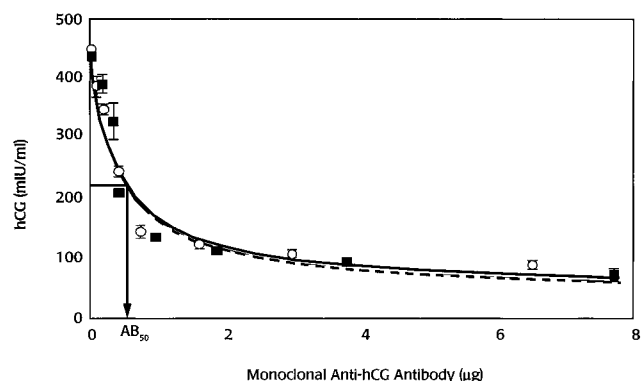
particle ^a	F	O	N	C	S	Si	Br
a	0.0	38.5	6.8	42.4	0.0	12.2	
b	0.0	23.8	11.3	60.4	0.0	4.5	
c	0.09	23.7	11.4	60.4	0.0	4.5	
d	0.0	20.7	11.7	65.7	0.0	1.8	
e	1.6	21.2	11.5	63.2	0.4	1.9	
f	1.7	20.5	10.7	64.3	0.5	1.7	
g	0.0	23.3	5.1	67.9	2.0	0.0	1.1
h	0.0	20.9	6.8	69.0	2.0	0.0	0.9
i	0.0	21.0	6.8	68.9	2.1	0.0	1.0
j	0.0	21.4	6.5	68.8	1.9	0.0	0.9
k	0.6	21.0	7.1	67.7	2.0	0.0	0.8
l	1.1	21.6	6.1	67.0	2.3	0.0	0.7
m	0.0	17.6	9.2	71.8	0.0	0.0	

^a Particle descriptions: a, carboxylated polypyrrole-silica nanocomposite; b, carboxylated polypyrrole-silica nanocomposite after reaction with ethylenediamine in the absence of the carbodiimide EDC as a control; c, carboxylated polypyrrole-silica nanocomposite after reaction with ethylenediamine and EDC; d, nanocomposite with a maleimido surface after reaction with 2,2,2-trifluoroethanol as a control; e, nanocomposite with an amino surface after reaction with 2,2,2-trifluoroethanethiol as a control; f, nanocomposite with a maleimido surface after reaction with 2,2,2-trifluoroethanethiol; g, carboxylated polypyrrole latex; h, carboxylated latex after reaction with ethylenediamine in the absence of the carbodiimide EDC as a control; i, carboxylated latex after reaction with ethylenediamine in the presence of EDC; j, latex with a maleimido surface after reaction with 2,2,2-trifluoroethanol as a control; k, latex with an amino surface after reaction with 2,2,2-trifluoroethanethiol as a control; l, latex with a maleimido surface after reaction with 2,2,2-trifluoroethanethiol; m, unmodified polypyrrole latex.

**Figure 2.** X-ray photoelectron spectroscopy of (A) carboxylated polypyrrole latex and (B) carboxylated polypyrrole-silica composite.

show a $\pi-\pi^*$ shake-up peak, which is characteristic of aromatic carbon species.

Subjecting the nanocomposite particles to the amination conditions both with and without the coupling promoter, EDC, resulted in decreased surface silica and oxygen concentrations and increased nitrogen and carbon (b and c vs a). The carboxylated latex also exhibited increases in nitrogen (h and i vs g). The relative increases in nitrogen (4.6 vs 1.7 atom %) for the carboxylated nanocomposite and latex, respectively, may be related to the measured carboxylic acid titers of 690 vs 340 $\mu\text{equiv/g}$. On the basis of controls run for both materials, in which the EDC was left out (b and g), the presence of

**Figure 3.** Sequential competitive assay comparing (○) native monoclonal anti-hCG antibody to (■) Fc-modified monoclonal anti-hCG. $n = 6$; error bars are ± 2 SD.

the promoter did not appear to affect the resulting surface composition. This suggests that another mechanism may be operating, such as ion exchange of the weak acid groups with protonated amine or a reaction with the polymer backbone (discussed later).

The next step in the functionalization scheme was the reaction of sulfo-SMCC with the aminated surface. The sulfo-SMCC should couple via the active ester moiety, leaving a free maleimide group. Since the sulfo-SMCC does not have any unique elements easily differentiable from the polypyrrole surface, the reactive marker 2,2,2-trifluoroethanethiol was used and 2,2,2-trifluoroethanol was the control. Samples d and j are maleimide surfaces on the nanocomposite and the latex, respectively. They were both reacted with 2,2,2-trifluoroethanol and, as expected, the surfaces contained no fluorinated groups as no evidence of fluorine was detected by XPS. Samples e and k were the aminated precursors and did not have maleimide surface groups. The samples were both reacted with 2,2,2-trifluoroethanethiol and were found to contain fluorinated surface groups. Samples f and l contained maleimide surface groups and were also reacted with the fluorinated thiol. A significant increase in fluorine was not seen for the nanocomposite (f) compared to the control (e), but for the maleimido latex (l), the fluorine level increased 2-fold over the control (k).

The interaction of nucleophiles such as thiols and amines with the polypyrrole chain is currently under study. Previously we reported (1) a significant reduction (80%) in the ability of the unmodified polypyrrole surface to adsorb protein after it had been exposed to ethanethiol. Gustafsson et al. (23) suggested mechanisms for the nucleophilic attack on the conjugated polypyrrole chain, where hydroxide and ammonia were presented as models. The incorporation of fluorinated thiol into the polypyrrole controls, which did not contain maleimide, and the nitrogen incorporation in samples c and h are rationalized in view of these papers.

Analysis of Thiol Content on the Modified Antibodies. Fc-cystamine-modified antibody was treated with 2 mM DTT to reduce the cystamine disulfides to thiols. Analysis of the thiol content of the product indicated that an average 4.5 thiols were present per antibody. This is comparable to what has been previously reported (16).

Specific Activity of Native Anti-hCG and Thiolated Anti-hCG Antibody. Equation 2 defines the inhibition curve as shown in Figure 3 for both native and chemically modified antibody. The data show that the ability to inhibit the signal from the standard hCG immunoassay is equivalent for both antibodies at a AB_{50} value of 0.5 μg . Hence, oxidation of the carbohydrate

Table 3. Specific Activity of Immobilized Anti-hCG Antibody

antibody and particle surface chemistry	antibody/particle ^a (w/w)	effective surface area/antibody ^b (nm ²)
Fc-coupled anti-hCG (cystamine reduced)		
polypyrrole—maleimide	0.0158	210
polypyrrole—silica—maleimide	0.0330	192
adsorbed anti-hCG (cystamine reduced)		
polypyrrole—amine	0.0133	255
polypyrrole—silica—amine	0.0275	231
polypyrrole carboxylic acid	0.0127	268
polypyrrole—silica—carboxylic acid	0.0176	361
polypyrrole	0.0323	159
adsorbed anti-hCG (cystamine not reduced)		
polypyrrole carboxylic acid	0.0192	177
polypyrrole—silica—carboxylic acid	0.0243	261
polypyrrole	0.0172	297

^a Antibody/particle weight ratio based on active binding equivalents from competitive assay. ^b based on active binding equivalents.

region of the IgG, with subsequent reductive amination with cystamine, had a minimal effect on the binding ability of that antibody. If the ligand binding ability of the modified antibody had been altered in a negative fashion by denaturation, steric hindrance, or alteration of the paratopes, a higher AB₅₀ would be expected relative to the value for the native antibody.

Chemical modification of the monoclonal antibody may be predicted to generate microheterogeneity in the equilibrium binding constant for the antibody preparation. This form of activity modification is not readily obtained from the sequential inhibition analysis. In Figure 3 the calculated maximum binding by soluble antibody ($B_{\max}$) resulted in 90% inhibition of the assay. The remaining [hCG] of 42 and 46 mIU/mL for native and modified antibodies, respectively, is the "offset" for eq 2. This residual level of hCG may be the result of a small amount of complex dissociation during the assay incubations. Additional assays using significantly higher levels of soluble antibody demonstrated complete inhibition (data not shown).

Comparison of the Specific Activity of the Nanoparticulate Immunoreagents. Comparison of the weight of active, covalently linked antibody per particle for the two carboxylated moieties with maleimide surface groups shows that at most a 2.6-fold greater amount of active antibody was present on the carboxylated polypyrrole—silica composite particles (Table 3). Compared to anti-hCG antibody immobilized on unmodified polypyrrole latex by passive adsorption, there is essentially no difference in active antibody between the two. We conclude that the modification of both the monoclonal anti-hCG antibody and the polypyrrole particles for covalent linkage by these methods provides no obvious advantage in terms of immobilized activity. On the other hand, different antibodies may behave differently and an advantage relative to long-term storage stability may be realized; however, that remains to be demonstrated.

It is interesting to note that polypyrrole surfaces derivatized with surface amine or carboxylic acid, in both latex or silica composite forms, have a good affinity for both the thiolated and disulfide-containing anti-hCG antibody. These antibody species were designated "reduced" and "not reduced", respectively, in Table 3. In fact, the relative performance of all particulate reagents differed by only a factor of 2. The measured levels of soluble antibody remaining in the particulate preparations were negligible, indicating that the immobilized antibody remained on the particles. These findings provide further evidence that covalent linkage may not justify the additional effort, unless the specific binding ligand of interest has only a weak affinity for the polypyrrole surface. In general, for IgGs examined to date this has not been seen to be the case.

CONCLUSIONS

Intrinsically colored nanoparticles of polypyrrole were prepared in functionalized form as composite particles with colloidal silica or a homopolymer latex which was subsequently modified. Anti-hCG was used as a model antibody, and the Fc region was functionalized for covalent attachment. Comparisons of various particle surface functionalities on both the nanocomposites and the latex revealed no obvious advantage for surface modification and covalent linkage compared to passive adsorption of the antibody. The "passive" adsorption may in fact be more than passive in that nucleophilic groups may bind to polypyrrole and its derivatives by an alternative mechanism. The minimal differences in immunoreactivity among the various surfaces studied, including native polypyrrole, as well as the surface chemical data on fluorinated thiols, suggest that nucleophilic groups on the protein may have a predominant interaction with the polypyrrole chains irrespective of the specific coupling chemistry employed.

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LITERATURE CITED

- (1) Tarcha, P. J., Misun, D., Finley, D., Wong, M., and Donovan, J. J. (1991) Synthesis, analysis, and immunodiagnostic applications of polypyrrole latex and its derivatives. *Polymer Latexes, Preparation, Characterization and Applications* (E. S. Daniels, E. D. Sudol, and M. S. El-Aasser, Eds.) ACS Symposium Series 492, pp 347–367, American Chemical Society, Washington, DC.
- (2) Tarcha, P. J., Donovan, J. J., and Wong, M. (1993) Indicator reagents, diagnostic assays and test kits employing organic polymer latex particles. U.S. Pat. 5,252,459.
- (3) Armes, S. P., Miller, J. F., and Vincent, B. J. (1987) Aqueous dispersions of electrically conducting monodisperse polypyrrole particles. *J. Colloid Interface Sci.* 118, 410–416.
- (4) Armes, S. P., and Vincent, B. (1987) Dispersions of electrically conducting polypyrrole particles in aqueous media. *J. Chem. Soc., Chem. Commun.*, 288–290.
- (5) Bjorklund, R. B., and Liedberg, B. J. (1986) Electrically conducting composites of colloidal polypyrrole and methylcellulose. *J. Chem. Soc., Chem. Commun.*, 1293–1295.
- (6) Maeda, S., and Armes, S. P. (1993) Preparation of novel polypyrrole-silica colloidal nanocomposites. *J. Colloid Interface Sci.* 159, 257–259.
- (7) Maeda, S., and Armes, S. P. (1994) Preparation and characterization of novel polypyrrole-silica colloidal nanocomposites. *J. Mater. Chem.* 4, 935–942.

- (8) Maeda, S., Corradi, R., and Armes, S. P. (1995) Synthesis and characterization of carboxylic acid-functionalized polypyrrole-silica microparticles. *Macromolecules* 28, 2905–2911.
- (9) Peterman, J. H., Tarcha, P. J., Chu, V. P., and Butler, J. E. (1988) The immunochemistry of sandwich-ELISAs IV. The antigen capture capacity of antibody covalently attached to bromoacetyl surface-functionalized polystyrene. *J. Immunol. Methods* 111, 271–275.
- (10) Bieniarz, C., Welch, C. J., Barnes, G., and Schlesinger, C. (1991) Covalent attachment of antibodies and antigens to solid phases using extended length heterobifunctional coupling agents, U.S. Pat. 5,002,883.
- (11) Hoffman, W. L., and O'Shannessy, D. J. (1988) Site-specific immobilization of antibodies by their oligosaccharide moieties to new hydrazide derivatized solid supports. *J. Immunol. Methods* 112, 113–120.
- (12) Brillhart, K. L., and Ngo, T. T. (1991) Use of microwell plates carrying hydrazide groups to enhance antibody immobilization in enzyme immunoassays. *J. Immunol. Methods* 144, 19–25.
- (13) Thomas, J. C., and Fairhurst, D. (1991) High resolution particle size analysis of coating materials. In *Surface Phenomenon and Fine Particles, Water-Based Coatings and Printing Technology* (M. K. Sharma and F. J. Micale, Eds.) pp 213–224, Plenum Press, New York.
- (14) Diaz, A. F., and Hall, B. (1983) Mechanical properties of electrochemically prepared polypyrrole films. *IBM J. Res. Dev.* 27, 342–347.
- (15) Vanderhoff, J. W., van den Hul, H. J., Tausk, R. J. M., and Overbeek, J. Th. G. (1970) In *Clean Surfaces: Their Preparation and Characterization for Interfacial Studies* (G. Goldfinger, Ed.) p 15, Dekker, New York.
- (16) Bieniarz, C., Husain, M., and Bond, H. E. (1993) Site-specific conjugation of immunoglobulins and detectable labels. U.S. Pat. 5,191,066.
- (17) Husain, M., and Bieniarz, C. (1994) Fc site-specific labeling of immunoglobulins with calf intestinal alkaline phosphatase. *Bioconjugate Chem.* 5, 482–490.
- (18) Ellman, G. L. (1959) Tissue sulphydryl groups. *Arch. Biochem. Biophys.* 82, 70–77.
- (19) Van Heyningen, V., Brock, D. J. H., and Van Heyningen, S. (1983) A simple method for ranking the affinities of monoclonal antibodies. *J. Immunol. Methods* 62, 147–153.
- (20) Fiore, M., Mitchell, J., Doan, T., Nelson, R., Winter, G., Grandone, C., Zeng, K., Haraden, R., Samith, J., Harris, K., Leszczynski, J., Berry, D., Safford, S., Barnes, G., Scholnick, A., and Ludington, K. (1988) The Abbott IMx automated benchtop immunochemistry analyzer system. *Clin. Chem.* 34, 1726–1732.
- (21) Maeda, S., Gill, M., and Armes, S. P. (1995) Surface characterization of conducting polymer-silica nanocomposites by X-ray photoelectron spectroscopy. *Langmuir* 11, 1899–1904.
- (22) Lei, J., and Martin, C. R. (1992) Infrared investigations of pristine polypyrrole—is the polymer called polypyrrole really poly(pyrrole-co-hydroxypyrrole)? *Synth. Met.* 48, 331–336.
- (23) Gustafsson, G., Lundstrom, I., Liedberg, B., Wu, C. R., Inganas, O., and Wennerstrom, O. (1989) The interaction between ammonia and poly(pyrrole). *Synth. Met.* 31, 163–179.

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Use of Morpholinonucleosides To Conjugate Oxidized DNA Bases to Proteins

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The universal method of Erlanger and Beiser was unsuccessful in conjugating 5-hydroxycytidine to proteins because of the instability of the base under the conditions used. A new strategy was developed to conjugate fragile modified nucleosides to proteins. This involves the use of morpholino derivatives of modified nucleosides. The validity of this method was demonstrated by the production of polyclonal antibodies specific for the DNA modification, 8-oxo-7,8-dihydro-2'-deoxyguanosine.

INTRODUCTION

Antibodies raised against modified bases are extremely useful tools for the detection and measurement of DNA adducts (Levine et al., 1966; Lewis et al., 1978; Müller et al., 1982; Wild, 1990; Shuker and Bartsch, 1994). The formation of antibodies to small molecules requires the binding of either the molecules or structurally related analogues to carrier proteins to elicit an immune response in mammals. Different methods of linking a DNA adduct to a protein are available. One approach is based on the use of 2'-deoxyribonucleosides. Selective oxidation of the nucleosugar in the presence of platinum catalyst yields a 5'-carboxylic acid (Beiser et al., 1968), which then is coupled to a carrier protein either via a mixed anhydride with ethyl chlorocarbonate (Boissonnas, 1951) or by means of a carbodiimide (Erlanger, 1973). An alternative method is based on the introduction of a carboxyl function on the 5'-hydroxyl group of an unmodified 2'-deoxyribonucleoside, via a succinic arm (Okabayashi et al., 1977). Deoxyribonucleoside 5'-monophosphates have also been successfully linked to proteins by using water-soluble carbodiimides as coupling agents (Halloran and Parker, 1966). Finally, a method described by Erlanger and Beiser (1964), originally developed for linking unmodified ribonucleosides to proteins, has become the most widely applied technique. This method involves production *in situ* of a 2',3'-dialdehyde of the nucleoside which reacts with side-chain amino groups on proteins and is stabilized by reduction. The reaction products have been poorly characterized but are probably morpholinonucleoside derivatives (Figure 1; Rayford et al., 1985). This method of Erlanger and Beiser was chosen to obtain antibodies specific for 5-hydroxycytosine, a DNA modification generated by an OH radical-mediated reaction or a one-electron oxidation (Wagner et al., 1990; Cadet, 1994).

EXPERIMENTAL PROCEDURES

Chemicals. Cytidine, sodium periodate, sodium borohydride, β -alanine, collidine, EDC,¹ and succinic anhydride were supplied by Aldrich Chemical Co. (Milwaukee, WI). Glycine, 6-aminocaproic acid, 8-bromoguanosine,

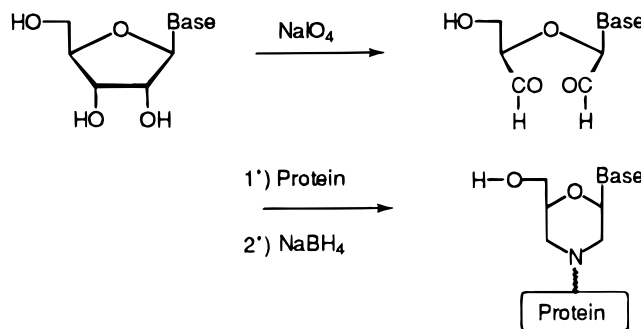


Figure 1. Principle of conjugation of 5-hydroxycytidine to BSA, utilizing the method of Erlanger and Beiser.

BSA, OVA, mBSA, TMB, H₂O₂, goat anti-mouse IgG peroxidase conjugate, and Tween 20 were purchased from Sigma Chemical Co. (St. Louis, MO). Ethylene glycol and formic acid were obtained from Merck (Darmstadt, Germany). Polystyrene 96-well plates were supplied by Dynatech (Polylabo, Strasbourg, France). PBS was made up as follows: 140 mM NaCl, 2.7 mM KCl, 1.5 mM KH₂PO₄, 10 mM Na₂HPO₄, pH 7.4. 5-OH-Cyd (1) and 5-OHd-Cyd (7) were synthesized as reported by Eaton and Hutchinson (1973). 8-OxoGuo was obtained from 8-bromoguanosine according to the method of Lin et al. (1985).

Liquid Chromatography. For analytical and semi-preparative HPLC separations, a gradient pump SpectraSystem P2000 (Spectra Physics, Fremont, CA) equipped with a Rheodyne 7125 (Berkeley, CA) injector loop was

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¹ Abbreviations: EDC, *N*'-[(3-dimethylamino)propyl]-*N*-ethylcarbodiimide hydrochloride; BSA, bovine serum albumin; OVA, ovalbumin; mBSA, methylated bovine serum albumin; TMB, 3,3',5,5'-tetramethylbenzidine; PBS, phosphate buffer saline; TEAA, triethylammonium acetate; TSP, 3-(trimethylsilyl)propionic acid; 5-OHdCyd (7), 5-hydroxy-2'-deoxycytidine; 5-OHCyd (1), 5-hydroxycytidine; 8-oxoGuo (10), 8-oxo-7,8-dihydroguanosine; 8-oxoGua, 8-oxo-7,8-dihydroguanine; 8-oxodGuo, 8-oxo-7,8-dihydro-2'-deoxyguanosine; CMCHM (4a), 4-(carboxymethyl)-2-(cytosin-1-yl)-6-(hydroxymethyl)morpholine; 5-OH-CMCHM (5a), 4-(carboxymethyl)-2-(5-hydroxycytosin-1-yl)-6-(hydroxymethyl)morpholine; CECHM (4b), 4-(2-carboxyethyl)-2-(cytosin-1-yl)-6-(hydroxymethyl)morpholine; 5-OH-CECHM (5b), 4-(2-carboxyethyl)-2-(5-hydroxycytosin-1-yl)-6-(hydroxymethyl)morpholine; CHCHM (4c), 4-(5-carboxypentyl)-2-(cytosin-1-yl)-6-(hydroxymethyl)morpholine; 5-OH-CHCHM (5c), 4-(5-carboxypentyl)-2-(5-hydroxycytosin-1-yl)-6-(hydroxymethyl)morpholine; CMGHM, 4-(carboxymethyl)-2-(guanosin-9-yl)-6-(hydroxymethyl)morpholine; 8-oxo-CMGHM (11), 4-(carboxymethyl)-2-(8-oxo-7,8-dihydroguanosin-9-yl)-6-(hydroxymethyl)morpholine; ELISA, enzyme-linked immunosorbant assay.

used. Compounds were detected by a SpectraSystem UV2000 set at 254 nm. Five different chromatographic systems were used: (system A) Lichrospher 100 RP-18 end-capped 5 μ m silica gel (125 $\times$ 4 mm) column (Merck) with 25 mM TEAA, pH 7 (100%) or 25 mM methanol/TEAA, pH 7 (1/99) as eluent at 1 mL/min; (system B) Nucleosil 10 C₁₈ 10 μ m (300 $\times$ 7.5 mm) column (Macherey-Nagel, Düren, Germany) with 25 mM methanol/TEAA, pH 7 (2/98, 6/94, or 10/90), as eluent at 2.5 mL/min; (system C) Delta Pak octadecylsilyl silica gel 15 μ m (300 $\times$ 19 mm) column (Waters Associates, Milford, MA); with methanol/TEAA (3/97) as eluent at 5 mL/min; (system D) Li-Chroprep RP-18 15–25 μ m (150 $\times$ 40 mm) silica gel column from Merck used in an axial compression system from Jobin-Yvon (Lonjumeau, France) with elution carried out with water only or methanol/H₂O with increasing amounts of methanol (10–20% in steps of 2%) delivered at 5 mL/min by a Modulprep pump (Jobin-Yvon) [compounds were detected with a SM 25 UV detector (Delsy-Nermag, Argenteuil, France)]; (system E) Hypersil-5-NH₂ 10 μ m (250 $\times$ 10 mm) column (Interchim, Montluçon, France) equipped with a Waters 6000 pump and a R401 differential refractometer (Waters) with acetonitrile/water (75/25) as eluent at a flow rate of 2.5 mL/min.

Spectroscopic Analysis. FAB mass spectra were recorded in the positive mode by using a ZAB2-SEQ spectrometer (Fisons-V.G., Manchester, U.K.) equipped with a LSIMS source. The molecules were dissolved in a glycerol matrix and desorbed by cesium ion bombardment (35 keV). The 400.13 and 250.13 MHz ¹H NMR spectra were recorded in the Fourier transform mode by using either a Bruker 400 or a Bruker 250 instrument, respectively (Bruker, Wissembourg, France). The 100.61 MHz ¹³C NMR spectra were obtained on the Bruker 400 instrument. Chemical shifts in 99.96% deuterium oxide are expressed in parts per million with respect to 3-(trimethylsilyl)propionic acid (TSP). Assignment of ¹H and ¹³C signals was achieved by specific homo- and heteronuclear decoupling experiments and two-dimensional ¹H–¹³C heteronuclear correlated NMR experiments (XH-CORR). According to nomenclature rules, the anomeric proton is labeled H2' in the morpholinonucleoside series.

Preparation of Protein–Nucleoside Conjugates.

Preparation of Immunizing and Coating Antigens According to the Erlanger and Beiser Method. BSA and OVA conjugates of periodate-treated 8-oxoGuo were prepared as described (Erlanger and Beiser, 1964). The amount of hapten bound to proteins was determined by measuring the absorbance of a standard solution of the protein and the modified protein at 270 and 300 nm. On average, 8.0 and 5.7 residues of 8-oxoGuo were linked to BSA and OVA, respectively.

Preparation of Immunizing Antigens Using Morpholinonucleoside Method. (a) **Synthesis of CMCHM (4a).** Cytidine (**3**) (2.43 g) was dissolved in water (35 mL), and NaIO₄ (2.35 g, 1.1 equiv) was added to the homogeneous solution. After 20 min of stirring at room temperature, the excess NaIO₄ was decomposed by addition of 112 μ L of ethylene glycol (0.2 equiv) followed by additional stirring for 5 min at room temperature. The reaction mixture was then added to glycine (3.75 g, 5 equiv in 20 mL of aqueous solution), and the pH was adjusted to 9–9.5 using potassium carbonate (5%). Stirring was continued for 45 min while the pH was maintained at 9–9.5. NaBH₄ (950 mg, 2.5 equiv) was then added and the reaction mixture set aside for 18 h. The pH was adjusted to about 4 using formic acid (25% in water), and stirring was continued for about 1 h. The purification of CMCHM was achieved by HPLC using system D (H₂O,

100%; yield 42%). The purity of the desired product was checked by using system A (TEAA, 100%): 400.13 MHz ¹H NMR (D₂O, TSP) δ 3.12 (pseudotriplet, 1H, H-5'), 3.21 (pseudotriplet, 1H, H-3'), 3.70 (m, 1H, H-5''), 3.79 (m, 1H, H-3''), 3.82 (m, 1H, H-7''), 3.84 (s, 2H, CH₂), 3.91 (m, 1H, H-7'), 4.35 (m, 1H, H-6'), 6.16 (d, 1H, H-5), 6.20 (dd, 1H, H-2'), 7.85 (d, 1H, H-6); 100.61 MHz ¹³C NMR (D₂O, TSP) δ 51.58 (C-5'), 52.69 (C-3'), 59.16 (CH₂), 61.15 (C-7'), 74.24 (C-6'), 78.33 (C-2'), 96.73 (C-5), 141.47 (C-6), 155.48 (C-2), 165.32 (C-4), 170.21 (COOH); FAB-MS, *m/z* [relative intensity], positive mode, 307 [10 (M + Na)⁺], 285 [32 (M + H)⁺], 174 [7 (morpholine moiety)⁺], 112 [47 (B + 2H)⁺].

(b) **Synthesis of CECHM (4b) and CHCHM (4c).** The method described above was used to prepare CECHM and CHCHM starting with either β -alanine (4.45 g) or 6-aminocaproic acid (6.55 g), respectively.

CECHM (yield 46%): 400.13 MHz ¹H NMR (D₂O, TSP) δ 2.74 (t, 2H, CH_{2 β}), 3.05 (pseudotriplet, 1H, H-5'), 3.14 (pseudotriplet, 1H, H-3'), 3.47 (t, 2H, CH_{2 α}), 3.63 (m, 1H, H-5''), 3.73 (m, 1H, H-3''), 3.83 (m, 1H, H-7''), 3.91 (m, 1H, H-7'), 4.28 (m, 1H, H-6'), 6.14 (dd, 1H, H-2'), 6.19 (d, 1H, H-5), 7.86 (d, 1H, H-6); 100.61 MHz ¹³C NMR (D₂O, TSP) δ 31.04 (CH_{2 β}), 50.93 (C-5'), 52.41 (C-3'), 54.52 (CH_{2 α}), 61.17 (C-7'), 74.45 (C-6'), 78.59 (C-2'), 96.77 (C-5), 141.49 (C-6), 155.69 (C-2), 165.51 (C-4), 177.60 (COOH); FAB-MS, *m/z* [relative intensity], positive mode, 321 [24 (M + Na)⁺], 299 [72 (M + H)⁺], 188 [74 (morpholine moiety)⁺], 112 [66 (B + 2H)⁺].

CHCHM (yield 52%): 400.13 MHz ¹H NMR (D₂O, TSP) δ 1.44 (m, 2H, CH_{2 γ}), 1.68 (m, 2H, CH_{2 δ}), 1.79 (m, 2H, CH_{2 β}), 2.28 (t, 2H, CH_{2 ϵ}), 2.84 (pseudotriplet, 1H, H-5'), 2.91 (pseudotriplet, 1H, H-3'), 3.09 (t, 2H, CH_{2 α}), 3.48 (m, 1H, H-5''), 3.59 (m, 1H, H-3''), 3.81 (m, 1H, H-7''), 3.88 (m, 1H, H-7'), 4.20 (m, 1H, H-6'), 6.07 (dd, 1H, H-2'), 6.16 (d, 1H, H-5), 7.84 (d, 1H, H-6); 100.61 MHz ¹³C NMR (D₂O, TSP) δ 23.36 (CH_{2 β}), 25.03 (CH_{2 δ}), 25.72 (CH_{2 γ}), 36.64 (CH_{2 ϵ}), 51.23 (C-5'), 52.64 (C-3'), 57.70 (CH_{2 α}), 61.39 (C-7'), 74.51 (C-6'), 78.69 (C-2'), 96.93 (C-5), 141.41 (C-6), 156.32 (C-2), 166.06 (C-4), 182.56 (COOH); FAB-MS, *m/z* [relative intensity], positive mode, 363 [91 (M + Na)⁺], 341 [38 (M + H)⁺], 230 [100 (morpholine moiety)⁺]; 112 [39 (B + 2H)⁺].

(c) **5-OH CMCHM (5a).** CMCHM (**4a**) (50 mg) was dissolved in 2.5 mL of H₂O, and the solution was cooled in an ice bath. Bromine (3 drops) was added dropwise until a pale yellow color persisted. Excess bromine was removed by bubbling air for 30 min. The solution was then allowed to come to room temperature, and collidine (100 μ L) was added. The resulting mixture was stirred for 2 h. The solvents were evaporated under reduced pressure, and collidine was coevaporated with ethanol. 5-OH CMCHM was purified by HPLC using system B (6% of methanol and repurification with 2% methanol; yield 80%): 400.13 MHz ¹H NMR (D₂O, TSP) δ 3.17 (pseudotriplet, 1H, H-5'), 3.28 (pseudotriplet, 1H, H-3'), 3.74 (m, 1H, H-5''), 3.80 (m, 1H, H-3''), 3.83 (m, 1H, H-7''), 3.88 (s, 2H, CH₂), 3.92 (m, 1H, H-7'), 4.36 (m, 1H, H-6'), 6.21 (dd, 1H, H-2'), 7.42 (s, 1H, H-6); 100.61 MHz ¹³C NMR (D₂O, TSP) δ 51.53 (C-5'), 52.41 (C-3'), 59.09 (CH₂), 61.07 (C-7'), 74.01 (C-6'), 78.21 (C-2'), 122.50 (C-6), 128.09 (C-5), 154.52 (C-2), 161.41 (C-4), 169.78 (COOH); FAB-MS, *m/z* [relative intensity], positive mode, 301 [92 (M + H)⁺], 174 [44 (morpholine moiety)⁺], 128 [100 (B + 2H)⁺]; UV (H₂O) λ_{max} = 290.2 nm (ϵ = 6640) λ_{min} = 262.5 nm (ϵ = 3520).

5-OH-CECHM (5b) and 5-OH-CHCHM (5c). Oxidation was achieved according to the previous protocol used for 5-OH CMCHM, starting from **4b** (0.1 g) and **4c** (0.17 g), respectively.

5-OH-CECHM: 400.13 MHz ^1H NMR (D_2O , TSP) δ 1.38 (t, 2H, $\text{CH}_{2\beta}$), 2.73 (t, 2H, $\text{CH}_{2\alpha}$), 3.00 (pseudotriplet, 1H, H-5'), 3.11 (pseudotriplet, 1H, H-3'), 3.58 (m, 1H, H-5''), 3.66 (m, 1H, H-3''), 3.85 (m, 1H, H-7'), 3.92 (m, 1H, H-7''), 4.26 (m, 1H, H-6'), 6.12 (dd, 1H, H-2'); 7.45 (s, 1H, H-6); 100.61 MHz ^{13}C NMR (D_2O , TSP) δ 31.16 ($\text{CH}_{2\beta}$), 51.12 (C-5'), 52.43 (C-3'), 54.73 ($\text{CH}_{2\alpha}$), 61.38 (C-7'), 74.54 (C-6'), 78.84 (C-2'), 122.98 (C-6), 128.21 (C-5), 154.93 (C-2), 161.80 (C-4), 177.70 (COOH); FAB-MS, m/z [relative intensity], positive mode, 315 [100 ($\text{M} + \text{H}$) $^+$], 188 [88 (morpholine moiety) $^+$], 128 [67 ($\text{B} + 2\text{H}$) $^+$]; UV (H_2O) λ_{max} = 290 nm, λ_{min} = 262.7 nm.

5-OH-CHCHM (yield 72%): 250.13 MHz ^1H NMR (D_2O , TSP) δ 1.46 (m, 2H, $\text{CH}_{2\gamma}$), 1.67 (m, 2H, $\text{CH}_{2\delta}$), 1.82 (m, 2H, $\text{CH}_{2\beta}$), 2.29 (t, 2H, $\text{CH}_{2\epsilon}$), 2.99 (pseudotriplet, 1H, H-5'), 3.08 (pseudotriplet, 1H, H-3'), 3.21 (t, 2H, $\text{CH}_{2\alpha}$), 3.60 (m, 1H, H-5''), 3.65 (m, 1H, H-3''), 3.81 (m, 1H, H-7''), 3.89 (m, 1H, H-7'), 4.23 (m, 1H, H-6'), 6.09 (dd, 1H, H-2'), 7.39 (s, 1H, H-6); 100.61 MHz ^{13}C NMR (D_2O , TSP) δ 23.27 ($\text{CH}_{2\beta}$), 25.06 ($\text{CH}_{2\delta}$), 25.60 ($\text{CH}_{2\gamma}$), 36.67 ($\text{CH}_{2\epsilon}$), 51.05 (C-5'), 52.39 (C-3'), 57.63 ($\text{CH}_{2\alpha}$), 61.20 (C-7'), 74.35 (C-6'), 78.58 (C-2'), 122.40 (C-6), 128.24 (C-5), 154.92 (C-2), 161.75 (C-4), 183.16 (COOH); FAB-MS, m/z [relative intensity], positive mode, 379 [11 ($\text{M} + \text{Na}$) $^+$], 357 [31 ($\text{M} + \text{H}$) $^+$], 230 [100 (morpholine moiety) $^+$], 128 [64 ($\text{B} + 2\text{H}$) $^+$]; UV (H_2O) λ_{max} = 290.2 nm, λ_{min} = 262.3 nm.

(d) *Preparation of 5-OH-CMCHM-Protein Conjugate [5-OH-CMCHM-mBSA, (6)].* 5-OH-CMCHM (30 mg dissolved in 2 mL of water) was added over a period of 3 h to a solution of mBSA (80 mg, 0.013 equiv) in 25 mL of water containing EDC (35 mg, 2 equiv). The reaction was then stirred at room temperature for an additional 20 h. Then, the reaction mixture was dialyzed against H_2O and finally lyophilized. The degree of hapten bound to the protein was determined by UV to be 7.8 residues/mol of protein.

(e) *Synthesis of 8-Oxo-CMGHM (11).* The protocol is similar to the previous one used for the formation of CMCHM. The reaction was carried out with 300 mg of 8-oxoGuo. However, there were some procedural differences. The solution, after addition of NaBH_4 , was only stirred for 2 h. Then, HCOOH was added to adjust the pH to 7, because the final compound was unstable at lower pH. The solution was stirred for another 30 min, with the pH maintained at 7.0. The desired 8-oxo-CMGHM was purified by HPLC using system C, and the purity was checked by HPLC (system A, 1% of methanol): 400.13 MHz ^1H NMR (D_2O , TSP) δ 3.01 (pseudotriplet, 1H, H-5'), 3.48 (m, 1H, H-5''), 3.60 (m, 1H, H-3''), 3.74 (s, 2H, CH_2), 3.81 (m, 1H, H-7''), 3.87 (m, 1H, H-7'), 4.01 (pseudotriplet, 1H, H-3'), 4.27 (m, 1H, H-6'), 5.91 (dd, 1H, H-2'); 100.61 MHz ^{13}C NMR (D_2O , TSP) δ 51.08 (C-3'), 51.83 (C-5'), 59.82 (CH_2), 61.39 (C-7'), 74.71 (C-6'), 76.33 (C-2'), 99.73 (C-5), 148.04 (C-8), 152.54 (C-4), 153.14 (C-2), 153.52 (C-6), 172.33 (COOH); FAB-MS, m/z [relative intensity], positive mode, 363 [15 ($\text{M} + \text{Na}$) $^+$], 341 [53 ($\text{M} + \text{H}$) $^+$], 174 [2 (morpholine moiety) $^+$], 168 [2 ($\text{B} + 2\text{H}$) $^+$]; UV (H_2O) λ_{max} = 294 nm, ϵ = 11 200, λ_{min} = 270.2 nm, ϵ = 5433.

(f) *Preparation of 8-Oxo-CMGHM-Protein Conjugate (12).* The protocol was the same as the one used for the preparation of 5-OH-CMCHM-mBSA, and 8-oxo-CMGHM (4 mg) was used for conjugation. The degree of hapten binding to protein was determined by UV: 7.6 residues of 8-oxoGuo were linked to mBSA.

(g) *Preparation of Immunizing and Coating Antigens with a Succinate Group: 5'- and 3'-Succinic Esters of 5-OHdCyd (8a and 8b).* 5-OHdCyd (7) (400 mg) was suspended in anhydrous pyridine (15 mL), dried by coevaporation (three times), and then dissolved in 15 mL

of anhydrous pyridine. Succinic anhydride (500 mg, 3 equiv) was then added quickly and the solution was stirred for 15 h at room temperature. The pyridine was evaporated under reduced pressure, and then the residue was coevaporated twice with ethanol and dissolved in water. The main fractions were isolated by HPLC (prepurification with system D with 10–20% methanol, followed by a purification with system B, 10% methanol). The first eluted compound corresponded to unreacted 5-OHdCyd. After this, three different compounds were obtained from the HPLC separation by following increasing order of elution: a compound substituted on the 3'-position of the sugar ring by succinic acid (**8b**), the isomer substituted on the 5-hydroxy function of the deoxyribose moiety (**8a**), and the disubstituted compound (**8c**). **8a** and **8b** were characterized by ^1H NMR measurements and FAB mass spectroscopy.

5'-Succinate (**8a**): 400.13 MHz ^1H NMR (D_2O , TSP) δ ppm 2.36 (m, 1H, H-2'), 2.54 (m, 1H, H-2''), 2.64 (m, 2H, CH_2), 2.76 (m, 2H, CH_2), 4.35 (m, 1H, H-4'), 4.40 (m, 1H, H-5'), 4.49 (m, 1H, H-5''), 4.61 (m, 1H, H-3'), 6.39 (pseudotriplet, 1H, H-1'), 7.42 (s, 1H, H-6); FAB-MS, m/z [relative intensity], positive mode, 344 [13 ($\text{M} + \text{H}$) $^+$], 207 [4 (sugar moiety) $^+$], 128 [21 ($\text{B} + 2\text{H}$) $^+$].

3'-Succinate (**8b**): 400.13 MHz ^1H NMR (D_2O , TSP) δ 2.45 (m, 1H, H-2'), 2.59 (m, 1H, H-2''), 2.67 (m, 2H, CH_2), 2.75 (m, 2H, CH_2), 3.90 (m, 1H, H-5'), 3.94 (m, 1H, H-5''), 4.31 (m, 1H, H-4'), 5.39 (m, 1H, H-3'), 6.39 (pseudotriplet, 1H, H-1'), 7.59 (s, 1H, H-6); FAB-MS, m/z [relative intensity], positive mode, 344 [85 ($\text{M} + \text{H}$) $^+$], 207 [13 (sugar moiety) $^+$], 128 [100 ($\text{B} + 2\text{H}$) $^+$]; UV (H_2O) λ_{max} = 290.8 nm, ϵ = 6590, λ_{min} = 262.8 nm, ϵ = 3480.

(h) *Preparation of the 5'- or 3'-Succinate-mBSA Conjugates (9a and 9b) and 3'-Succinate-OVA Conjugate.* The protocol is the same as that used for the preparation of 5-OH-CMCHM-mBSA. The reaction was carried out with 20.5 mg of **8a** and 5 mg (for mBSA) or 2 mg (for OVA) of **8b**. The degree of hapten binding to the protein was determined by UV: 3.6 residues of 5'-succinate and 9.3 residues of 3'-succinate were linked, respectively, to mBSA, whereas 4.1 residues of 3'-succinate were linked to OVA.

Degradation of 5-OHdCyd with Sodium Periodate and Sodium Borohydride. 5-OHdCyd (50 mg) was dissolved in water (5 mL) and treated with NaIO_4 (42 mg, 1.0 equiv) for 3.5 h at room temperature. The pH was adjusted to 9–9.5 using potassium carbonate (5%). NaBH_4 (10 mg, 2.5 equiv) was then added, and the solution was stirred for 60 h at room temperature. HPLC of the crude reaction mixture (system E) showed the presence of a large number of unresolved compounds; only the major component could be isolated and identified as a mixture of the α - and β -anomers of 1-(2-deoxy-D-erythro-pentofuranosyl)urea (20 mg).

Production and Characterization of Antibodies.

Two rabbits were immunized with 5-OHCyd-BSA (**1**) (1 mg in 1 mL of water) emulsified with Freund's complete adjuvant (1 mL) by sc injection at multiple sites on their shaved backs. The same procedure was used again 4 weeks later. Eight and 12 weeks after the beginning of the protocol, each rabbit received one im injection of the same amount of antigen in incomplete Freund's adjuvant. Two weeks later, both rabbits were bled from the carotid artery, yielding the maximum amount of serum (protocol conducted by the Elevage Scientifique des Dombes, Romans, France). Sera were assayed for anti-5-OHCyd activity by ELISA using a checkerboard procedure: 96-well polystyrene plates prepared by evaporating solutions of 5-OHCyd-OVA (0.5 ng to 1 mg/well in PBS) and sera were diluted with PBS over the range 1 in 10 to 1 in 10^6 .

The same protocol was used with the other antigens prepared: 5-OH-CMCHM-mBSA (**6**), 3'-succinate-mBSA (**9a**), 8-oxoGuo-BSA, and 8-oxo-CMGHM-mBSA (**12**).

The specificity of the antiserum was then determined using a competitive ELISA for 8-oxodGuo. Polystyrene 96-well plates were coated with 2 ng of 8-oxoGuo-OVA conjugate diluted in 40 μ L of PBS for the study of the antisera obtained after immunization of rabbits with 8-oxoGuo-BSA (5 ng of 8-oxoGuo-OVA/well for the 8-oxo-CMGHM-mBSA immunizing antigen) and dried at 37 °C overnight. Plates can be stored for up to 2 months at 4 °C under dry and light-protected conditions without detectable loss of performance. The plates were washed six times with PBS-Tween 20 (0.05%). Excess washing buffer was removed by tapping the plates onto paper towels.

Twenty-five microliters of standard solution or sample (10^{-1} to 10^7 fmol/well of inhibitor) was added to each well followed by 25 μ L of antiserum (diluted 1:10⁵ for 8-oxoGuo-BSA conjugate and 1:10⁴ for 8-oxo-CMGHM-mBSA in PBS). Controls included wells containing no inhibitor or antibody (background). After a 90 min incubation at room temperature with the antibody-antigen solution, the plates were washed six times in PBS-Tween 20.

A 50 μ L sample of second antibody, goat anti-mouse IgG peroxidase conjugate, was added to each well at 1:5000 dilution and incubated at room temperature for 90 min. This incubation was followed by six washings in a new PBS-Tween 20 bath and a final washing with distilled water. Substrate solution [50 μ L: 1 mg of TMB in 0.1 mL of DMSO and 2 μ L of H₂O₂ (30 wt %) in 10 mL of citrate buffer, pH 5.0] was added and incubated at room temperature for 45 min. After 10 min, the reaction was stopped with 50 μ L of 1 M HCl, and absorbances were read at 450 nm with a Labsystems Multiskan plate reader (Helsinki, Finland).

RESULTS AND DISCUSSION

Specificity of the Antibodies against 5-Hydroxycytosine. *Method of Erlanger and Beiser.* Our first approach was to prepare conjugate **2** according to the method of Erlanger and Beiser (1964) as depicted in Figure 1. Two rabbits were immunized and the antisera analyzed by ELISA tests. A checkerboard procedure established the conditions for optimal color development (with a limiting dilution of antiserum). This was found to be a 10 ng coating of antigen per well and a working antiserum dilution of 1:10³. The experiment showed that antibodies recognized the coating hapten 5-OHCyd linked to ovalbumin, via the method of Erlanger and Beiser. However, a competitive ELISA assay using 5-hydroxycytosine, 5-hydroxycytidine, or 5-hydroxy-2'-deoxycytidine as inhibitor showed that the antibodies did not cross-react with this modified cytosine residue.

This result was surprising since the technique of Erlanger and Beiser is the method of choice to prepare antibodies for modified DNA bases. One possible explanation is that the modified base was not stable during this chemical process. To ascertain this, a mock reaction on 5-hydroxy-2'-deoxycytidine (5-OHdCyd) was made in the absence of protein. Using the conditions described by Erlanger and Beiser, the complete degradation of the nucleoside was observed by HPLC. However, owing to the large amount of salts used for this reaction, it was not possible to isolate the different compounds formed. Therefore, the reaction was made with a reduced stoichiometry. When 5-OHdCyd was treated by 1 equiv of NaIO₄, the degradation of the nucleoside was complete,

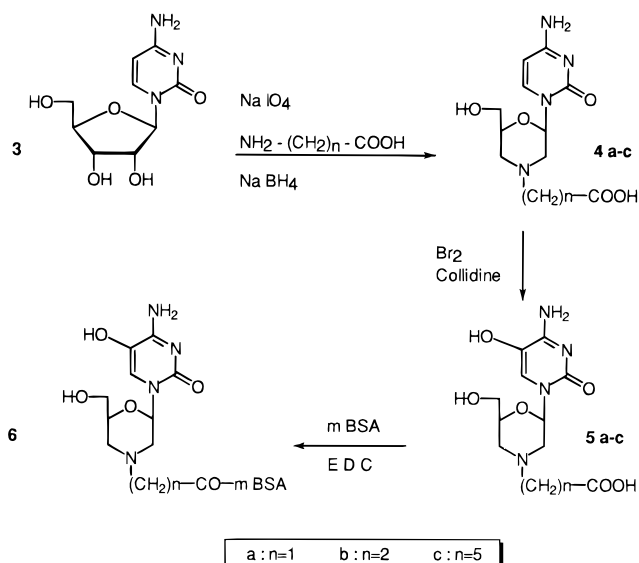


Figure 2. Preparation of the conjugate 5-hydroxycytidine linked to mBSA, via the morpholinonucleoside method.

as observed by HPLC and NMR (data not shown). The intermediates were not isolated but treated by 2.5 equiv of NaBH₄ during a longer period (60 h). NMR and HPLC analyses revealed the presence of more than 12 poorly resolved products. Preparative separation using an amino column gave a main fraction containing the α - and β -anomers of 1-(2-deoxy-D-erythro-pentofuranosyl)urea as indicated by ¹H and ¹³C NMR and FAB mass spectrometry (data not shown).

Therefore, it is apparent that the method of Erlanger and Beiser may not be suitable for some oxidized nucleosides. Another approach was sought for the conjugation of fragile modified nucleosides to a carrier protein.

Morpholinonucleosides. The approach consists in preparing a derivative of nucleoside, in which the sugar moiety is replaced by a morpholine cycle. The base is then modified and the compound is subsequently linked to a protein. This method seems to be well adapted since a morpholine pattern is involved, as in the Erlanger and Beiser conjugate, and this alternative avoids the contact between the fragile modified nucleoside and the drastic reagents, such as NaIO₄ and NaBH₄.

Morpholinonucleosides were prepared by condensation of cytidine 2',3'-dialdehyde with various amino acids. In a subsequent step, the base is modified as depicted in Figure 2 for the synthesis of conjugate **6**. The synthesis has been realized with three different amino acids having linear alkyl chains of different lengths. Compound **4a** was synthesized in 42% yield by the reaction with glycine ($n = 1$). When the length of the chain increased, the overall yields were similar. [Compound **4b** ($n = 2$) was synthesized in 42% yield and compound **4c** ($n = 5$) in 52% yield.] These yields are better than those reported by Rayford et al. (1985) for the synthesis of the corresponding adenine derivative. After the modification of the base, compounds **5a-c** were purified by reversed phase high-performance liquid chromatography.

The next step was the coupling of the modified morpholino derivatives to the protein. This involved the use of a coupling agent (EDC) and methylated BSA. In a first approach, **5a** was used to give conjugate **6a** because it was expected that with the shortest chain, antibodies should be more specific for the modified base.

The antisera obtained after the immunization of two rabbits with conjugate **6a** were analyzed using ELISA assays. A checkerboard procedure showed that no anti-

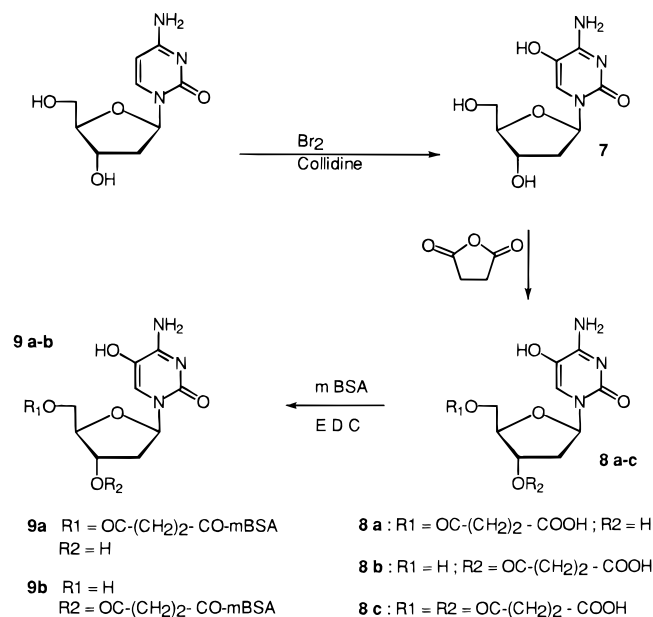


Figure 3. Condensation of 5-hydroxy-2'-deoxycytidine via a succinate arm.

bodies, directed against the antigen 5-OHCyd-OVA used as coating, were present. This was surprising since the structures of the conjugates obtained via the synthetic morpholinonucleosides and those prepared according to the Erlanger and Beiser procedure are very similar. Two possibilities seem likely: either 5-OHCyd does not induce any immune response in rabbits or the method we have developed is still not suitable for this kind of base. To study these alternatives, two different strategies were used. The first was to use the method of Okabayashi et al. (1977), and the second was to check the morpholinonucleosides with a DNA modification which is known to induce an immune response in rabbits.

Condensation of 5-OHCyd via a Succinate Group. The method of Okabayashi et al. (1977) consists in linking a succinic arm to the 3'- or 5'-position of a nucleoside via the formation of a trichloroethyl activated ester. We simplified this approach by using the reaction of succinic anhydride in the absence of any protecting group (Figure 3). The synthesis is not specific, but the reaction was improved to obtain a major compound (**8a**) substituted on the 5-hydroxyl function of the deoxyribose moiety, formed in 43% yield. Other compounds were also isolated: a compound substituted on the 3'-position of the sugar ring by succinic acid (**8b**, 16% yield) and another one disubstituted (**8c**, 20%). The position of the succinylation was determined using ^1H NMR. The substitution of a hydroxyl function of the sugar by an electronegative group leads to a deshielding of approximately 0.7 ppm of the corresponding sugar protons. UV analysis confirmed that the substitution has occurred on the sugar moiety since these products exhibit maximum and minimum absorption wavelengths similar to those of 5-hydroxy-2'-deoxycytidine. Minor compounds which are likely to involve substitution of the base were also present in the reaction mixture. They were observed by HPLC but were not isolated since they represented less than 20% of the overall mixture.

Under these conditions, the purification by HPLC was useful to obtain a homogeneous compound before **8a** and **8b** were bound to the protein. As the degree of modification of the protein was the highest for the 3'-succinate conjugate, two rabbits were immunized with antigen **9b**. ELISA tests showed, as for the morpholine conjugate,

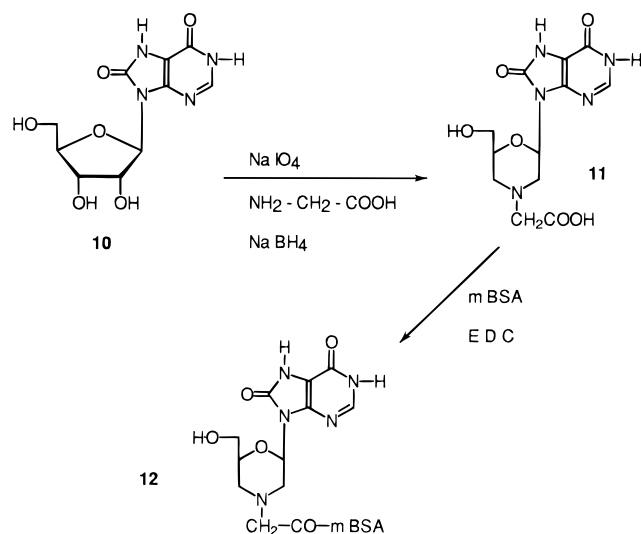


Figure 4. Preparation of the conjugate 8-oxoGua linked to mBSA, via the morpholinonucleoside method.

Table 1. Comparison of the Specificity for 8-OxoGua of Three Different Antibodies

inhibitor	concentration for 50% inhibition (pmol/well)		
	serum morpholino-nucleoside	serum Erlanger-Beiser	monoclonal antibodies (Park et al.)
8-oxodGua	7	10	0.5
8-oxoGua	7.5	40	0.9
8-oxoGua	10	40	3
2'-deoxyguanosine	>10.000	>10.000	3000
guanosine	>10.000	>10.000	8000
guanine	8000	>10.000	3000
8-mercaptopguanosine	200	70	200
8-bromoguanosine	8000	>10.000	3000
uric acid	7000		10.000
morpholinoguanosine	6000	500	700
Fapy	>10.000	>10.000	>10.000
8-oxodA	>10.000	>10.000	>10.000

that no antibodies from the antisera were able to recognize 5-hydroxycytosine.

Antibodies for 8-Oxo-7,8-dihydroguanine. 8-OxoGua was chosen because this modification has already been studied. Degan et al. (1991) have obtained polyclonal antibodies specific for 8-oxoGua, using the Erlanger and Beiser method, whereas Park et al. (1992) obtained a mAb specific for the same DNA modification.

A strategy different from that described above for 5-OHCyd was used to prepare 8-oxo-CMGHM. This compound was synthesized directly from the stable oxidized compound, 8-oxo-7,8-dihydroguanosine. Compound **11** was obtained with glycine in 44% yield (see Figure 4); 7.6 residues of nucleosides were linked to BSA. This rate is similar to the one obtained with the Erlanger and Beiser conjugate realized (8 residues of nucleosides by BSA). As above, two rabbits were immunized with conjugate **12**, and the antisera were analyzed by ELISA assays. A checkerboard procedure established conditions for optimal color development (with a limiting dilution of antiserum) which required 5 ng of coating antigen per well and a working antiserum dilution of 1:10⁴.

A competitive ELISA procedure was established to determine the specificity of the antibodies. 8-OxodGua, 8-oxoGua, and 8-oxoGua gave a characteristic inhibition curve over the range 0.1–10⁷ fmol/well. The specificity of the antibodies was determined using structurally related compounds as inhibitors (Table 1). 8-Mercaptopguanosine was about 10-fold less cross-reactive than

8-oxodGuo, whereas 8-bromoguanosine was about 1000-fold less cross-reactive. In addition, 2'-deoxyguanosine, guanosine, and guanine were, at least, 1000-fold less efficient in inhibiting antigen-antibody binding. This showed that the modification of the C8-position of the purine base of 8-oxodGuo was recognized by the antibodies. 8-Oxo-7,8-dihydroadenine also was not a good inhibitor. This showed the importance of the carbonyl function on the C6 position. Furthermore, the recognition of the C2-position was highlighted by the noninhibition of uric acid. Thus, the epitope recognized by the antibody is likely to be the upper part of the purine base (C2, C6, and C8) distal to the N9-position because the base was as well recognized as the nucleoside.

A comparison of the specificity of two antisera with one from a monoclonal antibody is presented in Table 1. An antiserum was obtained by the use of the morpholinonucleoside method and the other one according to the Erlanger and Beiser method. This showed that the polyclonal antibodies obtained by the morpholinonucleoside procedure and Erlanger and Beiser method present similar epitopes. The main differences concern the substitution of the N9-position. Indeed, with the Erlanger and Beiser serum, 8-oxoGuo and 8-oxoGua were about 10-fold less cross-reactive than 8-oxodGuo, whereas there were no significant differences found for the morpholinonucleoside serum. The specificity of the morpholinonucleoside serum also corresponds to the specificity of the mAb. This result shows that it is possible to obtain antibodies specific for 8-oxoGuo using the morpholinonucleoside method.

CONCLUSION

We have developed a new strategy to prepare antigenic derivatives of unstable modified 2'-deoxynucleosides, via the synthesis of morpholinonucleosides. Attempts to raise antibodies against 5-hydroxy-2'-deoxycytidine were unsuccessful by any of the methods that were tried. This modified base was extensively degraded during the standard Erlanger and Beiser coupling procedure. Moreover, no satisfactory antibody response was obtained even when the unambiguously synthesized morpholino derivative of 5-OHCyd was bound to carrier protein. Similarly, negative results were obtained using a succinate linker arm. Overall, these studies suggest that it may be very difficult to raise antibodies against 5-OHCyd even when the problems of chemical instability are overcome. However, the morpholinonucleoside approach was shown to be useful to raise a polyclonal antibody against 8-oxodGuo. This had similar properties to poly- and monoclonal antibodies raised by other methods. The approach is an alternative to the method of Erlanger and Beiser. When the latter method is applicable, it remains the easiest one. However, when the nucleoside is not stable during the oxidation and reduction steps of the classical Erlanger and Beiser procedure, the morpholinonucleoside method may be applied. Another potential application of the morpholinonucleoside derivatives would be to covalently link them to solid supports for use in the affinity purification of specific antibodies from sera and hybridoma supernatants.

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LITERATURE CITED

- Beiser, S. M., Tanenbaum, S. W., and Erlanger, B. F. (1968) Purine- and pyrimidine-protein conjugates. *Methods Enzymol.* 12, 889-893.
- Boissonnas, R. A. (1951) Une nouvelle méthode de synthèse peptidique (A novel method for the synthesis of peptides). *Helv. Chim. Acta* 34, 874-879.
- Cadet, J. (1994) DNA damage caused by oxidation, deamination, ultraviolet radiation and photoexcited psoralen. DNA adducts: identification and biological significance. *IARC Scientific Publication* 125 (K. Hemminki, A. Dipple, D. G. E. Shuker, F. F. Kadlubar, D. Segerbäck, and H. Bartsch, Eds.) pp 245-276, IARC, Lyon.
- Degan, P., Shigenaga, M. K., Park, E. M., Alperin, P. E., and Ames, B. N. (1991) Immunoaffinity isolation of urinary 8-hydroxyguanine and 8-hydroxy-2'-deoxyguanosine in DNA by polyclonal antibodies. *Carcinogenesis* 12, 865-871.
- Eaton, M. A. W., and Hutchinson, D. W. (1973) Poly(5-hydroxycytidylic acid). *Biochim. Biophys. Acta* 319, 281-287.
- Erlanger, B. F. (1973) Principles and methods for the preparation of drug-protein conjugates for immunological studies. *Pharmacol. Rev.* 25, 271-280.
- Erlanger, B. F., and Beiser, S. M. (1964) Antibodies specific for ribonucleosides and ribonucleotides and their reaction with DNA. *Proc. Natl. Acad. Sci. U.S.A.* 52, 68-74.
- Halloran, M. J., and Parker, C. W. (1966) The preparation of nucleotide-protein conjugates: carbodiimides as coupling agents. *J. Immunol.* 96, 373-378.
- Levine, L., Seeman, E. S., Hammer-schlag, E., and Van Vunakis, H. V. (1966) Antibodies to photoproducts of deoxyribonucleic acids irradiated with ultraviolet light. *Science* 153, 1666-1667.
- Lewis, H. L., Muhleman, D. R., and Ward, J. F. (1978) Serologic assay of DNA base damage. 5-hydroxymethyldeoxyuridine, a radiation product of thymidine. *Radiat. Res.* 75, 305-316.
- Lin, T. S., Cheng, J. C., Ishiguro, K., and Sartorelli, A. C. (1985) 8-substituted guanosine and 2'-deoxyguanosine derivatives as potential inducers of the differentiation of friend Erythroleukemia cells. *J. Med. Chem.* 28, 1194-1198.
- Müller, R., Adamkiewicz, J., and Rajewsky, M. F. (1982) Immunological detection and quantification of carcinogen-modified DNA components. *IARC Scientific Publication* 39, pp 463-479, IARC, Lyon.
- Okabayashi, T., Mihara, S., Repke, D. B., and Moffatt, J. G. (1977) A radioimmunoassay for 1-β-D-arabinofuranosylcytosine. *Cancer Res.* 37, 619-624.
- Park, E. U., Shigenaga, M. K., Degan, P., Korn, T. S., Kitzler, J. W., Wehr, C. M., Kolachana, P., and Ames, B. N. (1992) Assay of excised oxidative DNA lesions: isolation of 8-oxoguanine and its nucleoside derivatives from biological fluids with a monoclonal antibody column. *Proc. Natl. Acad. Sci. U.S.A.* 89, 3375-3379.
- Rayford, R., Anthony, D. D., O'Neill, R. E., and Merrick, W. C. (1985) Reductive alkylation with oxidized nucleotides. *J. Biol. Chem.* 260 (29), 15708-15713.
- Shuker, D. E. G., and Bartsch, H. (1994) Detection of human exposure to carcinogens by measurement of alkyl-DNA adducts using immunoaffinity cleanup with gas chromatography - mass spectrometry and other methods of quantitation. *Mutat. Res.* 313, 263-268.
- Wagner, J. R., van Lier, J. E., Decarroz, C., Berger, M., and Cadet, J. (1990) Photodynamic methods for oxyradical induced type DNA damage. *Methods in Enzymology, Oxygen Radicals in Biological Systems* (L. Packer and A. N. Glazer, Eds.) Part B, Vol. 186, pp 502-511, Academic, New York.
- Wild, C. P. (1990) Antibodies to DNA alkylation adducts as analytical tools in chemical carcinogenesis. *Mutat. Res.* 233, 219-233.

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Cell Targeting of a Pore-Forming Toxin, CytA δ -Endotoxin from *Bacillus thuringiensis* Subspecies *israelensis*, by Conjugating CytA with Anti-Thy 1 Monoclonal Antibodies and Insulin

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The cytolytic protein toxin CytA was linked to two monoclonal antibodies (mAb) directed against the mouse or the rat Thy 1 antigen. The purified CytA–mAb conjugates were not toxic to either target or nontarget cells. The conjugates did bind specifically to target cells since they agglutinated the target cells but not nontarget cells. When the conjugates were treated with dithiothreitol, the released CytA was toxic to all cells tested. These results suggested that the attachment of CytA to a molecule such as the mAb prevented it from forming a pore. Another conjugate was made by linking CytA to insulin. The purified insulin–CytA conjugate bound to and intoxicated cells bearing a high number of insulin receptors. Furthermore, the conjugate was far less toxic to cells expressing a low number of insulin receptors and not toxic to a known CytA target cell line from *Aedes aegypti*. However, CytA released from the conjugate by reduction was toxic to all cells tested. These results suggested that the cytotoxicity exhibited by CytA in the conjugate form against cells bearing insulin receptors was mediated through insulin and that, in the conjugate form, CytA no longer shows its broad *in vitro* cytolytic activity. The difference in toxicity between CytA–mAb conjugates and insulin–CytA conjugate is discussed in relation to size of the ligands, the number, distribution, and mobility of the target molecules, and intracellular trafficking.

INTRODUCTION

An immunotoxin (IT¹) is a chimeric protein consisting of a toxin or its subunit and a tumor-associated ligand linked either chemically or genetically. Such reagents are specifically designed to kill tumor cells, virally infected cells, or subsets of normal cells [reviewed by Blakey et al. (1988), Uhr (1991), Vitetta and Thorpe (1991), Vitetta et al. (1993), Pastan et al. (1992), and Brinkmann and Pastan (1994)]. The targeting ligands are usually cell-reactive monoclonal antibodies, hormones, or growth factors, all of which recognize and interact with specific receptors on the target cells. The most widely used toxins include plant toxins such as ricin, abrin, ribosomal inactivating proteins (Lord et al., 1991), and bacterial toxins such as diphtheria toxin and *Pseudomonas* exotoxin (Olsnes et al., 1991). These toxins are highly potent and kill cells by inhibiting protein synthesis. Recently, the first report has appeared concerning the conjugation of a cytolytic toxin (equinatoxin

II) to the cell-targeting ligand transferrin (Pederzoli et al., 1995).

Clinical trials of immunotoxins indicate that ITs can be effective antitumor agents, particularly against hematological malignancies (Grossbard et al., 1992; Vitetta et al., 1993; Uckun, 1993; Brinkmann and Pastan, 1994). However, the clinical effectiveness of these agents has been limited in solid tumors. Tumor heterogeneity, the failure of the ITs to penetrate solid tumors, and toxicity to normal cells are the major problems still to be overcome (Byers and Baldwin, 1991).

The limitations of ITs in current clinical use make the search for a new generation of ITs highly desirable. A major practical problem with the current ITs is that the toxic moiety must be translocated into the cytoplasm to kill the cell. This problem has been suggested to be the rate-limiting step in achieving a high selective killing of target cells. Furthermore, the emergence of toxin-resistant tumor cell mutants (Thorpe et al., 1987a), particularly cells deficient in the internalization process, clearly suggests the need for ITs with a different mode of action. One possible approach to overcome these limitations is to use membrane-acting toxins which kill cells by damaging the cell membrane (Drobniewski et al., 1988; Drobniewski, 1989). This strategy has potential advantages. (a) ITs made with membrane-acting toxins can be more effective in reaching the core of solid tumors by lysing cells that preclude accessibility of ITs, and (b) they can be used to enhance the effectiveness of ITs made with toxins that act in the cytosol by facilitating their access therein.

Among membrane-acting toxins (Alouf, 1976), the δ -endotoxins produced by the bacterium *Bacillus thuringiensis* (Bt) have potential for the design of a new generation of ITs. These are highly specific insecticidal proteins active against larvae of Lepidoptera, Coleoptera, and Diptera [reviewed by Hofte and Whiteley (1989) and Aronson (1993)]. The δ -endotoxins have been divided

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¹ Abbreviations: IT, immunotoxin; Bt, *Bacillus thuringiensis*; Bti, Bt subspecies *israelensis*; subsp., subspecies; IgG, immunoglobulin; FCS, fetal calf serum; DMEM, Dulbecco's Modified Eagle's medium; CCY, casein/casein/yeast extract broth; DTT, dithiothreitol; PMSF, phenylmethanesulfonyl fluoride; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*-2-ethanesulfonic acid; SPDP, *N*-succinimidyl-3-(2-pyridyldithio) propionate; SMPT, 4-(succinimidylloxycarbonyl)- α -methyl- α -(2-pyridyldithio)toluene; PBS, phosphate-buffered saline; FPLC, fast protein liquid chromatography; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; BSA, bovine serum albumin; LC₅₀, concentration of toxin causing 50% mortality.

into two families: Cry toxins and Cyt toxins (Hofte and Whiteley, 1989). These two classes have no amino acid sequence similarity, but they share common biochemical properties. They are produced as protoxins which upon solubilization at high alkaline pH are activated by proteolytic processing from both the N- and C-termini. The activated toxin acts by a two-step mechanism whereby the toxin first binds to a high-affinity protein receptor in the midgut epithelium (Van Rie et al., 1990) and then inserts into the membrane to create "pore" with a 1–2 nm diameter, leading to colloid-osmotic lysis (Knowles and Ellar, 1987; Knowles and Dow, 1993).

In addition to their specific activity against Diptera *in vivo*, the Cyt toxins when solubilized and proteolytically activated *in vitro* are cytolytic to a variety of invertebrate and vertebrate cells and lethal to animals when injected subcutaneously (Thomas and Ellar, 1983; Chilcott and Ellar, 1988; Chilcott et al., 1990; Drobniewski and Ellar, 1989; Knowles et al., 1992). The present work was designed to explore the potential use of this class of δ -endotoxin, represented by CytA, in immunotoxin targeting. CytA is a 27 kDa protein produced by *Bacillus thuringiensis* subsp. *israelensis* (Bti). The *cytA* gene has been cloned, sequenced, and expressed in *Bacillus subtilis* and acrySTALLIFEROUS Bti IPS/11 cured of its endogenous plasmids (Ward et al., 1986; Crickmore et al., 1990). Biochemical studies of CytA coupled with *in vitro* hemolytic assays against rat erythrocytes and cytolytic assays against *Aedes aegypti* cells revealed that CytA processed at both termini by proteinase K was the most toxic form of CytA (Al-yahyaee and Ellar, 1995).

In this model system, we have chemically linked the 22.5 kDa proteinase K-activated CytA (hereafter referred to simply as CytA unless otherwise specified) to two anti-Thy 1 monoclonal antibodies and insulin. The purified conjugates were assayed against target and nontarget cells.

EXPERIMENTAL PROCEDURES

Cell Lines. The mosquito *A. aegypti* cell line used and its growth and maintenance conditions have been described previously (Knowles and Ellar, 1986).

The Thy 1.2 positive EL-4 lymphoma cell line and the hybridoma cell line YBM 29.2 secreting the rat IgG2c monoclonal antibody which is specific for the mouse Thy 1 antigen (Cobbold et al., 1983) were obtained from Professor H. Waldmann (Department of Pathology, University of Cambridge). The hybridoma cell line MRC-OX7 secreting the mouse IgG1 monoclonal antibody which recognizes the Thy 1.1 antigen on the thymocytes of rat and AKR mouse (Mason and Williams, 1980) and the Thy 1.1-expressing mouse AKR-A/2 lymphoma cell line (Thorpe et al., 1987b) were obtained from Professor P. E. Thorpe (Department of Microbiology and Pharmacology, Southwestern Medical Center, Dallas, TX). All cell lines above were maintained in RPMI 1640 tissue culture media (Sigma) supplemented with 10% heat-inactivated fetal calf serum (Gibco), 1 mM pyruvate, and the antibiotic and antimycotic solution (Sigma) containing a final concentration of 100 units of penicillin, 100 μ g of streptomycin, and 0.25 μ g of amphotericin B per milliliter.

The insulin receptor-expressing cell lines, mouse Swiss 3T3 (Todaro and Green, 1963) and mouse NIH 3T3 N1R3.5 (Whittaker et al., 1987), were obtained from Dr. J. Metcalfe (Department of Biochemistry, University of Cambridge) and from Professor K. Siddle (Department of Clinical Biochemistry, University of Cambridge), respectively. Both cell lines were maintained in DMEM tissue culture medium (Sigma) supplemented with 20%

heat-inactivated fetal calf serum (FCS) (Gibco), 1 mM pyruvate, and the antibiotic and antimycotic solution (Sigma) containing a final concentration of 100 units of penicillin, 100 μ g of streptomycin, and 0.25 μ g of amphotericin B per milliliter. All mammalian cell lines were cultured at 37 °C in a humidified incubator gassed with 5% CO₂ in air and were passaged every 2–3 days.

Production and Purification of CytA Inclusion Bodies. Cloned CytA inclusion bodies were produced in Bti IPS 78/11 (a plasmid-cured mutant of Bti lacking δ -endotoxin genes) (Ward et al., 1984; Crickmore et al., 1990). For synchronous sporulation, Bti IPS 78/11 cells were grown in casein/casein/yeast extract (CCY) (Stewart et al., 1981) as described by Koni and Ellar (1994). Once the sporulating Bti IPS 78/11 cells had lysed to release over 90% of the inclusion bodies and spores as observed by phase contrast microscopy, the inclusion bodies were purified by a discontinuous sucrose density gradient as described by Thomas and Ellar (1983).

Solubilization and Activation of CytA Inclusion Bodies. CytA inclusion bodies were solubilized at 5–8 mg/mL in 50 mM Na₂CO₃/10 mM DTT (pH 10.3) at 37 °C for 1 h. Solubilized CytA was separated from insoluble material by centrifugation at 12000g for 15 min. Soluble CytA was then activated by treating it with 10% (w/w) insoluble proteinase K (proteinase K attached to agarose beads) (Sigma). In this activation regime and prior to incubation with soluble CytA, the insoluble proteinase K was washed with the solubilization buffer and centrifuged until no soluble protein in the supernatant was detected at A₂₈₀. The incubation was carried out at 37 °C overnight with continuous gentle mixing. Following activation, insoluble proteinase K was removed from activated CytA by centrifugation at low speed and filtration through a 0.20 μ m membrane filter (Sartorius). Any residual proteinase K activity was inhibited by adding 5 mM PMSF to the activated CytA before storage at –20 °C.

Production and Purification of Monoclonal Antibodies. Hybridoma cell lines producing monoclonal antibodies were grown in RPMI 1640 tissue culture medium containing 25 mM HEPES (Sigma), supplemented with 10% heat-inactivated fetal calf serum, 1 mM glutamine, 1 mM pyruvate, and antibiotic and antimycotic solution containing a final concentration of 100 units of penicillin, 100 μ g of streptomycin, and 0.25 μ g of amphotericin B per milliliter. The monoclonal antibodies were purified by ammonium sulfate precipitation followed by Protein A-Sepharose column chromatography (Pharmacia) as described by Harlow and Lane (1988).

Determination of Protein Concentrations. Protein concentrations were determined by A₂₈₀ measurement on the basis of the method of Cantor and Schimmel (1980). An absorbance of 1.0 is equivalent to 1.1, 0.9, and 0.7 mg/mL proteinase K-activated CytA, insulin, and monoclonal antibodies, respectively. For calculations, approximate molecular masses of 22.5, 6.0, and 150 kDa were used for CytA activated with proteinase K, insulin, and monoclonal antibodies, respectively.

Derivatization of CytA with SPDP or SMPT. Depending on the degree of derivatization required, CytA at 10 mg/mL in either borate saline buffer (0.05 M borate and 0.3 M NaCl at pH 9.0) or phosphate saline buffer (pH 7.4) (PBS) was reacted with a 1.5–2-fold molar excess of SPDP (Boehringer Mannheim) or SMPT (Pierce) for 30–60 min at room temperature. The derivatized CytA was purified on a Sephadex G25-F column equilibrated and eluted with a nitrogen-flushed phosphate–EDTA buffer [0.1 M phosphate (pH 7.3), 0.1 M NaCl, 1 mM EDTA, and 0.02% w/v sodium azide]. The degree of

derivatization was determined by the method of Carlsson et al. (1978). CytA carrying free sulfhydryl group(s) was prepared by reacting the derivatized CytA with an equal amount of DTT for 60 min at room temperature. CytA was then desalted on a Sephadex G25-F column equilibrated and eluted with nitrogen-flushed phosphate-EDTA buffer (pH 7.3).

Preparation of Conjugates. The rat IgG2c anti-Thy 1 containing on average two free sulfhydryl groups per molecule was prepared by the method of Cumber et al. (1985) using SPDP. The CytA-anti-Thy 1 rat IgG2c conjugate was prepared by mixing the emergent IgG2c peak from the Sephadex G25-F column immediately with a 5-fold molar excess of SPDP-derivatized CytA which had an average of 1.3 2-pyridyldithio groups per CytA molecule. The reaction mixture was then concentrated 5-fold by ultrafiltration using a PM10 membrane and left stirring in the Amicon ultrafiltration cell under nitrogen gas at room temperature for 48 h.

The mouse CytA-MRC-OX7 conjugate was prepared by the method of Thorpe et al. (1987b) using SMPT (Pierce). Conjugates were prepared by mixing a 5-fold molar excess of CytA derivatized with SMPT, containing on average one free sulfhydryl group immediately after elution from the Sephadex G25-F column, with the activated MRC-OX7 [i.e. carrying the α -methyl- α -(2-pyridyldithio)toluoyl groups] in an Amicon ultrafiltration cell fitted with a PM10 membrane (Amicon). The protein mixture was then concentrated approximately 5-fold and left stirring at room temperature under nitrogen for 72 h.

The insulin-CytA conjugate was prepared as follows. (a) In order to link insulin through lysine 29 of the B chain, the N-termini of bovine insulin (Sigma) were blocked by citraconylation prior to derivatization by the method of Roth et al. (1981) with the following modifications. Bovine insulin (Sigma) was dissolved at 5 mg/mL in either 10 mM NaHCO_3 (pH 6.9) containing 2 mM EDTA or 10 mM phosphate (pH 6.9) containing 5 mM EDTA. Citraconylation was carried out by reacting 20 mg of insulin with 5 μL of citraconic anhydride (Aldrich Chemicals) for 30 min at room temperature. The drop in pH was adjusted by adding approximately 20 μL of 1 N NaOH. Following citraconylation the N-terminally blocked insulin was first dialyzed at 4 °C against 5 L of NH_4HCO_3 (pH 8.0) for 12 h, followed by 10 L of borate saline buffer (pH 9.0) for another 12 h. (b) The blocked insulin in borate saline buffer (pH 9) was reacted with a 20-fold molar excess of SPDP (Boehringer Mannheim) or SMPT (Pierce) at room temperature for 2 h. Following derivatization, the reaction mixture was dialyzed against PBS (pH 7.4) at 4 °C to remove the unreacted cross-linker and the leaving *N*-succinimidyl groups. This derivatization regime gave on average one 2-pyridyldithio group per insulin molecule. (c) To unblock the insulin N-termini, the derivatized insulin was then dialyzed sequentially against deionized water for 2 h at room temperature, 10 mM HCl for 6 h at room temperature, and PBS-EDTA (pH 7.4) buffer at 4 °C overnight. (d) The insulin-CytA conjugate was then prepared by mixing a 5-fold molar excess of derivatized insulin with CytA carrying on average one free sulfhydryl group per CytA molecule in an Amicon ultrafiltration cell fitted with a YM1 membrane (Amicon). The reaction mixture was then concentrated 10-fold and left stirring at room temperature under nitrogen for 48 h.

Purification of the Conjugates. The CytA-anti-Thy 1 rat IgG2c and the CytA-MRC-OX7 conjugates were purified by fast protein liquid chromatography (FPLC) on a Waters 650E-FPLC system (Millipore) using

a combination of size exclusion (Superose 12, Pharmacia) and ion exchange chromatography (Mono Q, Pharmacia). Initially, the conjugation reaction mixture was loaded onto the Superose 12 column equilibrated and eluted with PBS (pH 7.4) at a flow rate of 0.25 mL/min. Contaminating IgG was then removed by loading fractions containing the conjugate onto a Mono Q column equilibrated with PBS (pH 7.4) and eluted with a linear gradient of 500 mM NaCl in PBS (pH 7.4) at a flow rate of 1.0 mL/min. Samples of protein peaks were analyzed by SDS-PAGE to determine their composition.

The insulin-CytA conjugate was purified by the Tris-tricine native polyacrylamide gel electrophoresis system (Schagger and von Jagow, 1987). The gel composition and running conditions were the same as the Tris-tricine SDS-PAGE system (see below) except that SDS was eliminated from the gel and the running buffers, and the sample was not boiled prior to loading on the gel. Following electrophoresis, a strip was excised from the gel and protein bands were visualized by Coomassie Blue staining (30 min of staining, 30 min of destaining). Using an R_f value for the conjugate obtained from the stained gel strip, the conjugate band on the remainder of the gel was localized and subsequently excised. The protein was eluted from the gel in either 25 mM Tris/192 mM glycine or 50 mM NH_4HCO_3 (pH 8.1-8.2) by electroelution for 4-6 h at room temperature using a Bio-Rad Model 422 Electro-Eluter as described by the manufacturer.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE). SDS-PAGE was carried out using the discontinuous Tris-glycine buffer system (Laemmli, 1970) as modified by Thomas and Ellar (1983) or the discontinuous Tris-tricine buffer system (Schagger and von Jagow, 1987). Gradient gels were cast by mixing the appropriate concentrations of the acrylamide in a gradient mixer and pumping the mixed gradient using a peristaltic pump (Microperpex, LKB) set at maximum speed into a mini-gel apparatus (Hoeffer) through the bottom port. To avoid the fast polymerization of the 20% acrylamide gel, the gradient mixer was placed on an ice block which was in turn placed on the top of a stirrer.

N-Terminal Protein Sequencing. N-Terminal amino acid sequencing was carried out as described by Koni and Ellar (1994).

In Vitro Hemolysis Assays. Hemolysis assays were carried out as described by Thomas and Ellar (1983) except that a 5% (v/v) blood cell suspension in hemolysis buffer [50% (w/v) phosphate-buffered saline (PBS) (pH 7.4), 2.25% (w/v) glucose, and 0.1% (w/v) gelatin] was used. Rat blood was collected in Alsevers solution (0.1 M trisodium citrate and 40 mM glucose) and stored at 4 °C until use. The erythrocytes were usually washed immediately before use with 50% (w/v) PBS and 2.25% (w/v) glucose until all lysed cells were removed. The cells were then diluted in hemolysis buffer to make up the 5% (v/v) blood cell suspension. The end point of hemolysis was assessed after 3-12 h as the last well in which coloration of the liquid was still visible.

Insulin-CytA Conjugate Binding to NIH 3T3 HIR3.5 Cells. The NIH 3T3 HIR3.5 cells were plated out at 2×10^4 cells/mL in a 24-well plate, each well containing 1 mL of DMEM medium (Sigma) supplemented with 20% FCS. Two days after plating, the medium was removed and the adherent confluent cells were washed twice with 0.5 mL of ice-cold PBS. To each well was then added 150 μL of binding buffer (120 mM NaCl, 1.2 mM MgSO_4 , 15 mM sodium acetate, 5 mM KCl, 10 mM glucose, 1 mM EDTA, 10 mg/mL BSA, 1 mg/mL bacitracin, and 50 mM HEPES at pH 7.8 at 4 °C; Millazzo

et al., 1992). Following this, the insulin–CytA conjugate, the derivatized insulin, or the native insulin was added in 100 μ L of the binding buffer to make the final concentrations indicated in the graph (Figure 3). The [^{125} I]insulin in 50 μ L containing approximately 20 000 cpm (a kind gift from Professor K. Siddle, Department of Clinical Biochemistry, University of Cambridge) was subsequently added to each well. After 4 h of incubation at 4 °C with gentle shaking, the cells were washed twice with 0.5 mL of ice-cold PBS to remove the unbound tracer. Cells were then solubilized by incubating them in 200 μ L of 0.03% SDS at room temperature for 30 min. Radioactivity was counted in a γ counter and nonspecific binding determined in the presence of 1 μ M insulin.

In Vitro Cytotoxicity Assays. *A. aegypti*, Thy 1.2-positive EL-4, and Thy 1.1-positive AKR-A/2 cell lines all growing in suspension were resuspended at $1\text{--}2 \times 10^6$ cells/mL in tissue culture medium. The adherent mouse NIH 3T3 HIR3.5 and mouse Swiss 3T3 cells were removed from the tissue culture flask by trypsinization. Since trypsin is known to cleave the insulin receptor (Clark et al., 1991), the detached resuspended cells in DMEM medium supplemented with 20% FCS were first washed to remove trypsin and then incubated in fresh medium/20% FCS at 37 °C for 2 h in a 5% CO₂ incubator with the flasks in an upright position to minimize the number of cells adhering to the surface of the flask. During this incubation, the cells are likely to repair the cleaved insulin receptors (Dr. R. Farndale, personal communication). Following this incubation, the cells were resuspended gently by pipetting and spun at 900g for 5 min at 37 °C. The recovered pellet was then resuspended in fresh medium without FCS to make approximately $1\text{--}2 \times 10^6$ cells/mL. Aliquots (47.5 μ L) of the cells were then distributed to 0.5 mL Eppendorf tubes and the test samples added in 2.5 μ L of PBS to make the desired concentrations. Each sample was tested in duplicate. The cells were then incubated at room temperature for the desired time with occasional gentle tapping of the tubes before 10 μ L was withdrawn and mixed with 10 μ L of 0.1% (w/v) Trypan blue in PBS to determine cell viability. Controls were performed by incubating the cells with 2.5 μ L of PBS and treating them exactly the same as the tested samples. Cells were counted in a hemocytometer counting chamber.

RESULTS

Purification and Biochemical Characterization of the Conjugates. Analysis of the CytA–IgGs conjugation reaction mixture by 5 to 20% gradient SDS–PAGE under nonreducing conditions showed that the reaction mixture contained conjugates of 170 and 190 kDa corresponding to one and two CytA molecules per IgG, respectively, as well as higher molecular mass aggregates (a typical result is shown in the top panel of Figure 1, lane 9). The conjugates were separated from unreacted antibody and CytA by FPLC on a Superose 12 column (Pharmacia) equilibrated and eluted with PBS (pH 7.4). The elution profile obtained from this column showed four peaks (data not shown). When fractions from each peak were analyzed by SDS–PAGE under nonreducing conditions, it was found that peak II contained the conjugates but was also significantly contaminated with free IgG (top panel of Figure 1, lane 4; bottom panel of Figure 1, lanes 5 and 6). Therefore, fractions from peak II were pooled and repurified on a Mono Q column (Pharmacia) equilibrated with PBS (pH 7.4) and eluted with a linear gradient of 500 mM NaCl in PBS (pH 7.4). This column gave two peaks (data not shown), which, when analyzed by SDS–PAGE under nonreducing condi-

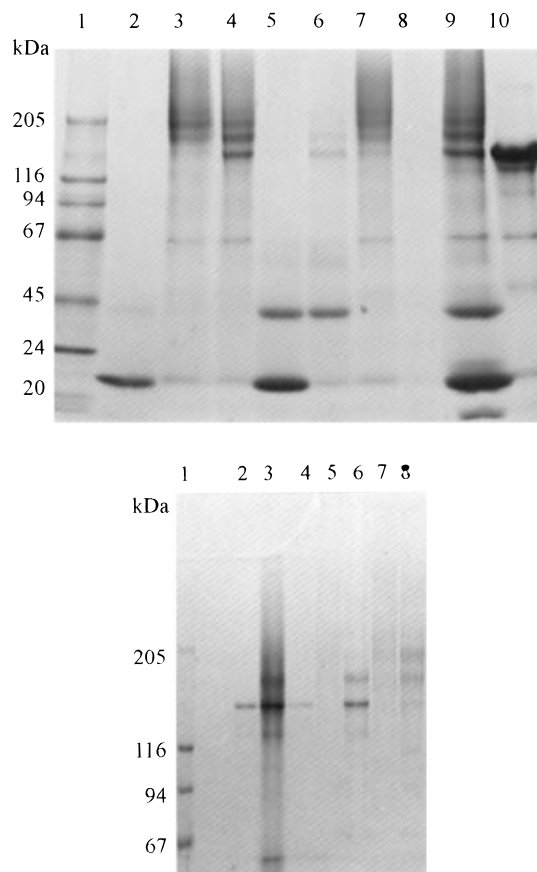


Figure 1. (Top) Coomassie Blue-stained SDS/5 to 20% gradient PAGE showing purification of the CytA–anti-Thy 1 rat IgG2c monoclonal antibody conjugate by Superose 12 and Mono Q columns (traces not shown). Twenty-five microliters of each fraction was mixed with 25 μ L of 2 $\times$ SDS sample buffer without DTT and boiled for 5 min before loading on the gel: lane 1, molecular mass markers; lane 2, CytA; lane 3, purified CytA–anti-Thy 1 rat IgG2c monoclonal antibody conjugate from the Mono Q column (trace not shown); lane 4, pooled fractions from peak II of the Superose 12 column (trace not shown); lanes 5–8, samples, respectively, from peaks IV–I of the Superose 12 column; lane 9, CytA–anti-Thy 1 rat monoclonal antibody conjugation reaction mixture; and lane 10, rat IgG2c monoclonal antibody. (Bottom) Coomassie Blue-stained SDS/7% PAGE showing purification of the CytA–MRC-OX7 conjugate by Superose 12 and Mono Q columns (traces not shown). Samples were treated as in the top panel before loading on the gel: lane 1, molecular mass markers; lanes 2 and 4, 10 and 5 μ g, respectively, of MRC-OX7 monoclonal antibody; lane 3, CytA–MRC-OX7 conjugation reaction mixture; lane 5, pooled fractions 20–23 from the Superose 12 column (trace not shown); lane 6, pooled fractions from peak I of the Mono Q run (trace not shown); and lanes 7 and 8, approximately 5 and 10 μ g, respectively, of the CytA–MRC-OX7 conjugate from peak II of the Mono Q run (trace not shown).

tions, showed that the pure conjugates were in peak II (top panel of Figure 1, lane 3; bottom panel of Figure 1, lanes 7 and 8). When the same samples in Figure 1 were analyzed on a 5 to 20% gradient SDS–PAGE under reducing conditions, the conjugates were reduced to their fundamental components, i.e. the heavy and the light chains of IgG as well as CytA (data not shown).

SDS–PAGE analysis of the insulin–CytA conjugation reaction mixture under nonreducing conditions showed several bands, three of which appeared to be insulin–CytA conjugates since they have higher molecular masses than CytA (Figure 2B, lane 4). Attempts were made to purify the insulin–CytA conjugate by gel filtration and ion exchange chromatography under various conditions, but unfortunately they were not successful (data not shown). Improved purification of the insulin–CytA

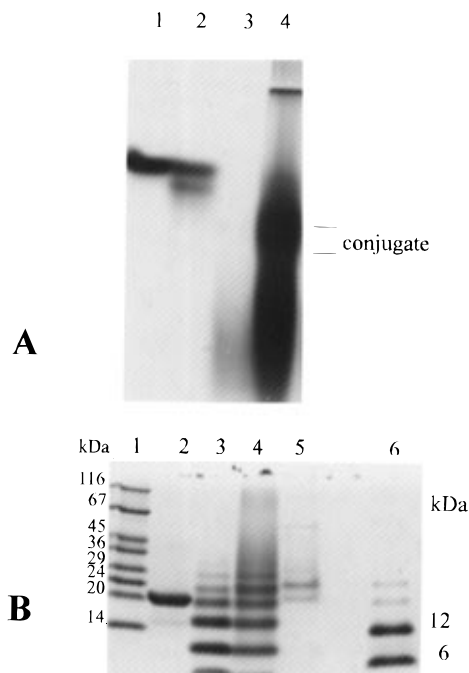


Figure 2. (A) Coomassie Blue-stained 10% native PAGE run with Tris-tricine buffer system showing the purification of the insulin-CytA conjugate. Each sample was mixed prior to loading with Tris-tricine sample buffer without SDS or DTT: lane 1, CytA; lane 2, derivatized CytA; lane 3, derivatized insulin; and lane 4, insulin-CytA conjugation reaction mixture. The cut insulin-CytA conjugate band is indicated with the two bars. (B) Coomassie Blue-stained SDS/10% PAGE run with the Tris-tricine buffer system showing the purity of the insulin-CytA conjugate eluted from the native gel (panel A). Approximately 20 μg of each sample was mixed with Tris-tricine SDS sample buffer without DTT. The samples were incubated at 40 $^{\circ}\text{C}$ for 30 min before loading on the gel: lane 1, molecular mass markers; lane 2, derivatized CytA; lane 3, derivatized insulin with SPDP (Boehringer Mannheim); lane 4, insulin-CytA conjugation reaction mixture; lane 5, the insulin-CytA conjugate eluted from the native gel (panel A); and lane 6, low-molecular mass markers [cytochrome c (12 kDa) and aprotinin (6 kDa)].

conjugate was obtained using 10% native polyacrylamide gel electrophoresed with either Tris-glycine or Tris-tricine systems. A typical result from a native Tris-tricine 10% PAGE showed that the insulin-CytA conjugate was migrating at an intermediate position between CytA and insulin (Figure 2A, lane 4). The fact that the conjugate migrated slower than CytA in this gel suggested that the separation was primarily driven by the net charge since the conjugate has a higher molecular mass than CytA. When the conjugate was eluted from such a gel and subsequently analyzed by SDS-PAGE under nonreducing conditions, it was found that the conjugate was resolved mainly into two bands, the lower of which corresponded in size to CytA and one of the derivatized insulin bands (Figure 2B, lane 5).

In order to identify these bands, they were N-terminally sequenced which showed that the lower bands had 38 pmol of CytA N-terminus and 10 pmol of each of the insulin N-termini. On the other hand, the higher band (running in Figure 2B as a 24 kDa band) gave 40 pmol each of the CytA and the insulin N-termini. This result suggested that the higher band was probably a 1:1 insulin-CytA conjugate, while the lower band might be a conjugate of CytA with one of the insulin chains and/or a contaminant of insulin plus CytA. If this lower band contained free CytA, it would be expected to lyse known CytA target cells such as *A. aegypti* cells. Assays against *A. aegypti* cells revealed that at 50 $\mu\text{g}/\text{mL}$ this conjugate

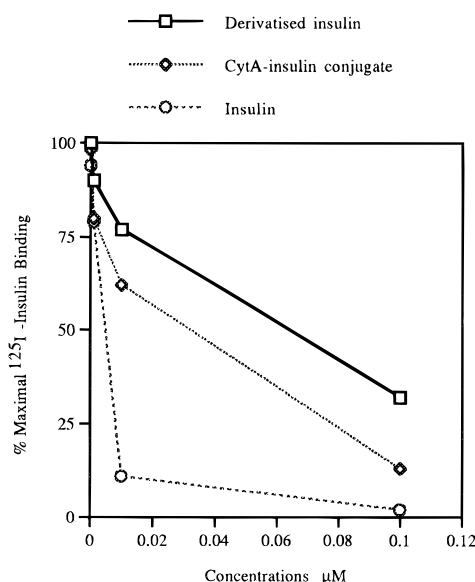


Figure 3. Competition binding study of native insulin, the SPDP-derivatized insulin, and the purified insulin-CytA conjugate on NIH 3T3 HIR3.5 cells carried out as described in Experimental Procedures. The displacement of [^{125}I]insulin binding by native insulin, the SPDP-derivatized insulin, and the insulin-CytA conjugate is plotted as a function of the concentrations indicated. Data points are the means of triplicate incubations of each sample from two independent experiments. Maximal percentage binding is expressed as a percentage of that in the absence of unlabeled insulin.

preparation was not toxic to the cells after 3 h of incubation (data not shown). However, when the conjugate incubated with *A. aegypti* cells for 3 h was subsequently treated with DTT, all the cells lysed after 15 min (control cells treated with DTT alone were unaffected). These data clearly demonstrated that the amount of free contaminating CytA in the conjugate preparation purified by the native gel was probably negligible, since *A. aegypti* cells are very susceptible to CytA, with an LC_{50} of $<1.0 \mu\text{g}/\text{mL}$ (S. A. S. Al-yahyaee, unpublished).

Binding of the Insulin-CytA Conjugate to NIH 3T3 HIR3.5 Cells. Binding studies were carried out as described in Experimental Procedures using the mouse cell line NIH 3T3 HIR3.5 that has been shown to express $>6.0 \times 10^6$ highly responsive insulin receptors per cell (Whittaker et al., 1987). Competitive binding studies using iodine-labeled insulin were performed and compared with those using insulin and the derivatized insulin. The results of such an experiment are shown as displacement curves in Figure 3. The concentrations of unlabeled insulin, the insulin CytA conjugate, and the derivatized insulin required for half-maximal displacement of [^{125}I]insulin were approximately 5, 35, and 65 nM, respectively. These data show that the insulin-CytA conjugate bound 7-fold less than native insulin. By contrast, a 1:1 insulin-ricin B chain conjugate made with the same cross-linker under the same conditions bound 20-fold less than native insulin (Roth et al., 1981). The reduction in binding of insulin in a conjugate form might have resulted from steric hindrance imposed by the ligand.

In Vitro Hemolytic Assays. To investigate the ability of CytA in the conjugate forms to lyse cells nonspecifically, the purified conjugates were assayed against rat erythrocytes and compared with CytA and derivatized CytA. None of the conjugates lysed erythrocytes at 200 $\mu\text{g}/\text{mL}$ (data not shown). A typical result is shown in Figure 4 (plate I) for the CytA-MRC-OX7 conjugate for which no hemolysis was detected at 125 $\mu\text{g}/$

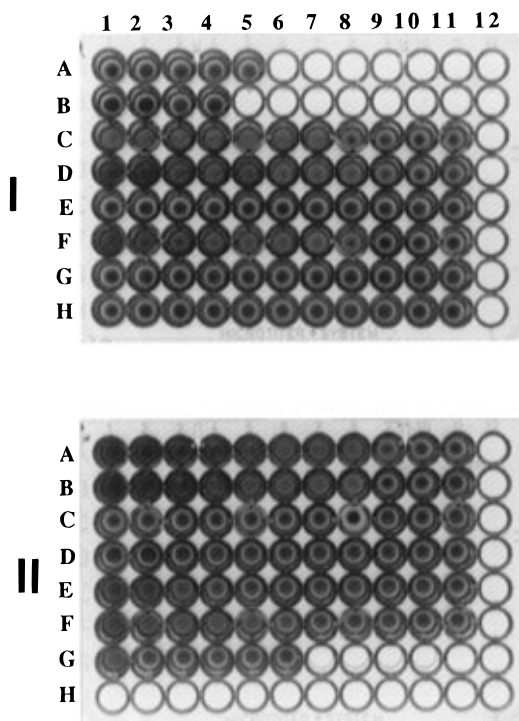


Figure 4. Hemolysis assay of the CytA-MRC-OX7 conjugate purified by Mono Q column chromatography. Fifty micrograms of each sample in 100 μ L of hemolysis buffer [50% PBS, 2.25% glucose, and 0.1% gelatin (pH 7.4)] was added to well number 1 in a 96-well microtiter plate, and a 2-fold serial dilution was subsequently constructed from left to right using hemolysis buffer. To each well was then added an equal volume of 5% rat red blood cells in the hemolysis buffer. Thus, the extreme left well contained a final sample concentration of 125 μ g/mL, and the extreme right well contained about 0.075 μ g/mL. The hemolysis assay was allowed to proceed at 20–22 $^{\circ}$ C overnight before photography. (Plate I) Row A, PBS (pH 7.4); row B, 50 mM Na_2CO_3 (pH 10.3); row C, CytA; row D, derivatized CytA; row E, MRC-OX7 monoclonal antibody; row F, CytA-MRC-OX7-CytA conjugation reaction mixture; row G, CytA-MRC-OX7 conjugate (Figure 1, bottom panel, lane 6); and row H, CytA-MRC-OX7 conjugate (Figure 1, bottom panel, lanes 7 and 8). (Plate II) Samples in this plate were treated with 50 mM DTT prior to dilution. Row A, CytA; row B, derivatized CytA; row C, PBS; row D, MRC-OX7 monoclonal antibody; row E, DTT-treated CytA-MRC-OX7 conjugate (Figure 1, bottom panel, lane 6); row F, DTT-treated CytA-MRC-OX7 conjugate (Figure 1, bottom panel, lanes 7 and 8); and row G, purified CytA-anti-Thy 1 rat IgG2c conjugate (Figure 1, top panel, lane 3) at 6 μ g/mL in the first well.

mL conjugate (rows G and H) or IgG (row E), while the same concentration of CytA (row C) and derivatized CytA (row D) completely lysed rat erythrocytes up to well 7 (column 7). When these samples were initially treated with DTT, hemolysis was seen with the conjugate (Figure 4, plate II, row E up to column 3 and row F up to column 5) but not with the IgG (Figure 4, plate II, row D). It is interesting to note that the sample (bottom panel of Figure 1, lanes 7 and 8) which contained mainly the conjugate had more hemolytic activity (Figure 4, plate II, row F with hemolysis up to column 5) than the sample (bottom panel of Figure 1, lane 6) from peak I which contained free IgG in addition to the conjugate (Figure 4, plate II, row E with hemolysis up to column 3). Clearly, these data demonstrated that, in the conjugate form, CytA no longer shows its broad *in vitro* hemolytic activity. However, CytA was hemolytic once released from the ligand, which suggests that the loss of CytA activity in the conjugate form was not due to irreversible inactivation of CytA.

In Vitro Cytolytic Assays of the Conjugates

against Target and Nontarget Cells. The cytotoxicity of the purified IgG-CytA conjugates against target and nontarget cells was determined by Trypan blue dye uptake and compared with that of derivatized CytA, CytA, the derivatized antibody, and the antibody. The results from all of these assays established that none of the conjugates or the antibodies had any detectable cytolytic activity against the target cells even at concentrations as high as 500 μ g/mL. By comparison, CytA and derivatized CytA lysed 80% of EL-4 cells at 15 and 20 μ g/mL, respectively, while 20 μ g/mL CytA lysed 50% of AKR-A/2 cells after 1 h of incubation at room temperature.

Thy 1.2 positive EL-4 cells agglutinated when treated separately with the CytA-anti-Thy 1 rat IgG2c conjugate and the anti-Thy 1 rat IgG2c antibody. Similarly, the CytA-anti-Thy 1.1 MRC-OX7 conjugate and the MRC-OX7 were found to agglutinate the Thy 1.1 positive AKR-A/2 cells but not the Thy 1.2 positive EL-4 cells. Taken together, these results suggested that the conjugates were binding specifically to the target cells.

To test whether CytA is still active once released from the conjugates, 100 μ g/mL CytA-anti-Thy 1 rat IgG2c conjugate was treated with 50 mM DTT prior to incubation with the cells. Some toxicity was seen, albeit only 25% of that seen for CytA at 20 μ g/mL. Similarly, when 150 μ g/mL CytA-MRC-OX7 conjugate was initially treated with 50 mM DTT for 30 min before addition to the AKR-A/2 and EL-4 cells, it was found that after 2 h of incubation at room temperature over 50% of AKR-A/2 and 80% of EL-4 cells were killed. No toxicity was seen with the same concentration of the antibodies or derivatized antibodies treated with 50 mM DTT or PBS containing 50 mM DTT.

To extend the time of incubation, a different toxicity assay was conducted in which AKR-A/2 and EL-4 cells were incubated overnight at 37 $^{\circ}$ C with 20, 50, and 200 μ g/mL CytA-MRC-OX7 conjugate, MRC-OX7, the rat IgG2c, and CytA in a multiwell microtiter plate. This assay showed that neither the conjugate nor the IgGs were toxic to either of the two cell lines, while CytA killed over 75% of AKR-A/2 and virtually all of EL-4 cells at the lowest concentration tested (20 μ g/mL). A sample of the AKR-A/2 and EL-4 cells treated with 50 μ g/mL CytA-MRC-OX7 conjugate or IgG was then incubated with 50 mM DTT for 1 h at room temperature to examine whether CytA was still toxic. This treatment of the conjugate resulted in the death of 50 and 80% of AKR-A/2 and EL-4 cells, respectively, while the free IgG killed 20% of both types of cells.

The ability of the insulin-CytA conjugate to lyse target cells was tested against NIH 3T3 HIR3.5 and mouse Swiss 3T3 cell lines over a range of concentrations and incubation times. The results from NIH 3T3 HIR3.5 cells which express a high number of insulin receptors are presented in Figure 5A. This assay revealed that the percentage killing by the insulin-CytA conjugate increased in a dose- and time-dependent manner. For example, after 4 h of incubation with the NIH 3T3 HIR3.5 cells, 25 μ g/mL insulin-CytA conjugate killed about 35% of the cells, while 50 μ g/mL conjugate killed over 60% of the cells. The extension of the incubation time appeared to affect cell killing more significantly at low concentrations of the insulin-CytA conjugate than at high concentrations, since extending the time of incubation from 4 to 6 h increased cell killing by the insulin-CytA conjugate by 35 and 10% at 10 and 100 μ g/mL, respectively. This effect probably resulted from the down-regulation of insulin receptors (Khan, 1976).

Unlike the NIH 3T3 HIR3.5 cells, the mouse Swiss 3T3

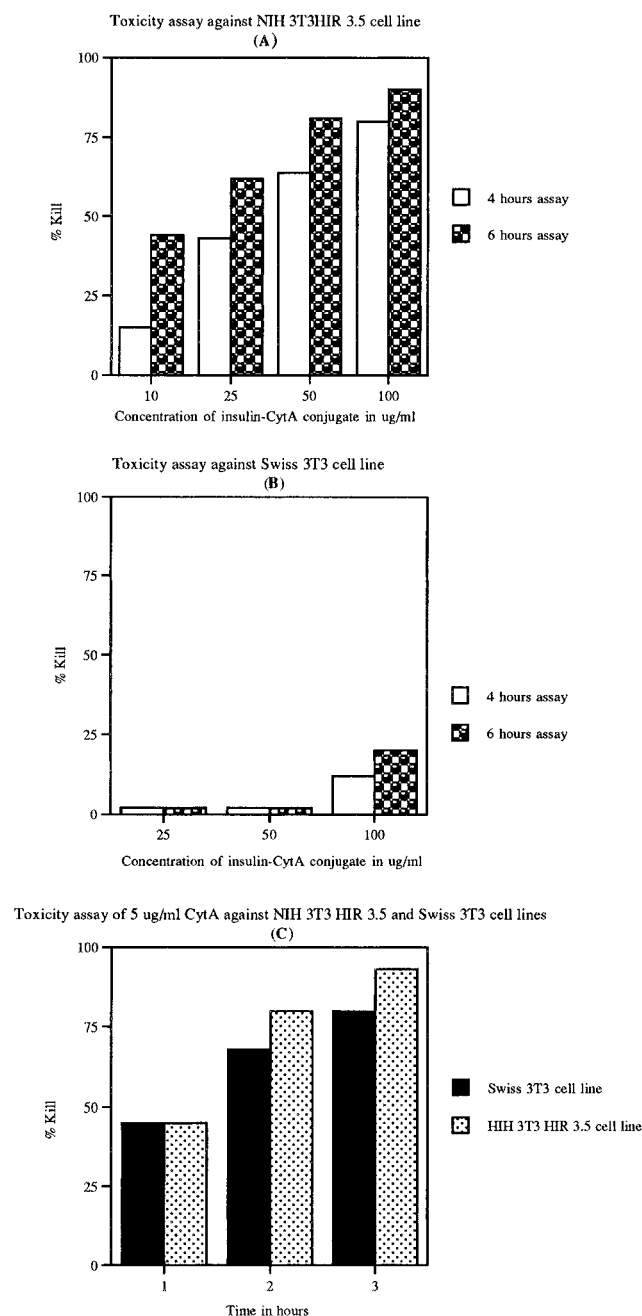


Figure 5. Toxicity assay of the insulin-CytA conjugate purified on a native gel (Figure 2) against the NIH 3T3 HIR3.5 (A) and the Swiss 3T3 (B) cell lines and of 5.0 $\mu\text{g/mL}$ CytA against both cell lines (C). To 47.5 μL aliquots of $1.0\text{--}1.5 \times 10^6$ cells/mL were added sample amounts in 2.5 μL to make the final concentrations shown in the graphs. Cells were incubated with the insulin-CytA conjugate for 4 and 6 h or with 5.0 $\mu\text{g/mL}$ CytA for 1, 2, and 3 h before being counted using Trypan blue staining. Data points are the means from three separate experiments. The mortality in control cells incubated with 100 $\mu\text{g/mL}$ insulin or the derivatized insulin added in equal volumes of PBS rarely exceeded 5%, and these values have been subtracted in the figure.

cells which express a low number of insulin receptors were much more resistant to killing by the insulin-CytA conjugate. Toxicity could only be seen with 100 $\mu\text{g/mL}$ insulin-CytA conjugate which killed 20% of the mouse Swiss 3T3 cells after 6 h of incubation, while 50 $\mu\text{g/mL}$ conjugate showed virtually no toxicity after 6 h of incubation (Figure 5B).

A control toxicity assay using 100 $\mu\text{g/mL}$ derivatized insulin or native insulin or 5 $\mu\text{g/mL}$ CytA was also performed against the NIH 3T3 HIR3.5 and Swiss 3T3

cells. The results from this assay showed that the derivatized insulin and insulin were not toxic to these cells (data not shown). In contrast, 5 $\mu\text{g/mL}$ CytA killed nearly 50% of both cell types after 1 h of incubation (Figure 5C). This figure also shows that the NIH 3T3 HIR3.5 cells were about 10% more susceptible to CytA killing than the mouse Swiss 3T3 cells after 2 and 3 h of incubation. This difference in susceptibility to CytA was, however, far less than that seen between these two cell lines to the insulin-CytA conjugate.

DISCUSSION

One of major problems still facing current ITs is that the toxic moiety must be translocated into the cytosol, a process that is suggested to be the rate-limiting step in achieving high selective killings. This may be overcome by constructing ITs with toxins that damage the membrane. Conjugates made with membrane-acting toxins may also be used to potentiate protein synthesis-inhibiting toxin ITs by facilitating their entry into the cytosol. In this report, we examine the use of CytA, a pore-forming toxin from Bti, in cell targeting. In addition to its broad cytolytic activity against a range of invertebrate and vertebrate cells (Thomas and Ellar, 1983; Armstrong et al., 1985; Chilcott and Ellar, 1988; Chilcott et al., 1990), CytA has other useful properties. Among these are the following. (a) Large quantities of CytA in the form of inclusion bodies from both native Bti (Thomas and Ellar, 1983) and cloned CytA in Bti IPS 78/11 (Crickmore et al., 1990) can easily be obtained. These inclusion bodies are readily soluble at alkaline pH, and the activated soluble toxin is fully active at neutral pH. (b) CytA is a relatively small protein (22 kDa once activated with proteinase K; S. A. S. Al-yahyaee, unpublished); thus, its accessibility to solid tumors may exceed that of other toxins. (c) CytA is one of the most extensively studied δ -endotoxins.

Chemical conjugation of CytA via disulfide bridges was achieved with two monoclonal antibodies. The first of these is a rat IgG2c directed to the mouse Thy 1 antigen (Cobbold et al., 1983); the second monoclonal antibody is a mouse IgG1 which recognizes the rat and the AKR mouse Thy 1.1 antigen (Mason and Williams, 1980). Thy 1 antigen was chosen in this model study because previous work with other toxins such as ricin and diphtheria had shown that the Thy 1 antigen is a good model for immunotoxin targeting and that the Thy 1 system is well established in terms of *in vitro* and *in vivo* evaluation of IT efficacy (Youle and Neville, 1980; Ramakrishnan and Houston, 1984; Thorpe et al., 1985, 1987b).

In vitro toxicity assays of the purified CytA-monoclonal antibody conjugates against target and nontarget cells revealed that the conjugates were not toxic to either target or nontarget cells. The conjugates did, however, appear to bind target cells, since they caused target cells but not nontarget cells to agglutinate. Furthermore, when the conjugates were treated with DTT, the released CytA was toxic to all cells tested. Taken together, these results suggested that the attachment of CytA to large molecules such as these antibodies prevented CytA from penetrating the membrane and/or forming a pore. This could have resulted from the inability of CytA to insert or oligomerize. CytA oligomerization was suggested to be essential for cytotoxicity (Chow et al., 1989; Maddrell et al., 1988). It was estimated from the molecular mass of aggregates recovered from cells treated with CytA that 16 molecules of CytA formed one oligomer (Chow et al., 1989). Using a mathematical model, Maddrell et al.

(1988) estimated that one CytA oligomeric pore is made of 12 molecules of CytA.

If oligomerization is required for CytA cytotoxicity, then it is conceivable that the IgG molecules attached to CytA hindered such oligomerization and/or oligomerization was not achieved due to a low number of antigens per cell since a threshold level of CytA is essential for CytA oligomerization (Chow et al., 1989). To test this hypothesis, CytA was conjugated to a much smaller sized molecule than IgG (i.e. insulin) and tested against cells expressing a higher and a lower number of receptors per cell than IgG target cells. The data obtained from these experiments showed that the insulin-CytA conjugate was toxic to cells expressing insulin receptors (Figure 5A,B). One interpretation of this finding is that, by being smaller than IgG, insulin had less inhibitory effect on oligomerization and/or insertion of CytA. Furthermore, the difference in number between Thy 1 antigens and insulin receptors per cell is less likely to influence CytA cytotoxicity since 100 $\mu\text{g/mL}$ insulin-CytA conjugate intoxicated about 25% of mouse Swiss 3T3 cells (Figure 5B) which express around 3000–5000 insulin receptors per cell, while 500 $\mu\text{g/mL}$ CytA-IgG conjugates were not toxic to cells expressing about 500 000 Thy 1 antigens per cell (Mason and Williams, 1980; Professor P. E. Thorpe, personal communication).

An alternative explanation is that the difference in toxicity between the CytA-IgG and the insulin-CytA conjugates may be due to differences in the distribution and mobility of the target molecules. If, for instance, the insulin receptors cluster more closely than the Thy 1 antigens, this could facilitate oligomerization. Furthermore, differences in intracellular trafficking between the CytA-IgG conjugates and the insulin-CytA conjugate might also influence CytA toxicity. Differences in intracellular trafficking have been suggested to affect the toxicity and effectiveness of ricin A chain-IgG conjugates (Bjorn et al., 1985). Although CytA is a membrane-acting toxin, it is still possible that CytA in the conjugate form becomes internalized and routed differently according to the attached ligand. Internalization and subsequent release of a membrane-acting toxin has recently been suggested to be important for the toxicity of conjugates containing a membrane-acting toxin (Pederzoli et al., 1995). Clearly, these speculations await further experiments to establish whether CytA toxicity in the conjugate forms is influenced by the size of the ligands, the number, distribution, and mobility of the target molecules, and/or intracellular trafficking.

The toxicity exhibited by the insulin-CytA conjugate appeared to be mediated through insulin since the insulin-CytA conjugate was far more toxic to NIH 3T3 HIR3.5 cells which express a higher number of insulin receptors than Swiss 3T3 cells, while both cell lines showed very similar susceptibilities to CytA (Figure 5). Furthermore, 50 $\mu\text{g/mL}$ insulin-CytA conjugate was not toxic to *A. aegypti* cells which are known to be a target of CytA (Chilcott and Ellar, 1988). The insulin-CytA conjugate also had no hemolytic activity against rat erythrocytes at the highest concentration tested (200 $\mu\text{g/mL}$, data not shown). The fact that the conjugate lost the ability to intoxicate nontarget cells clearly demonstrated the specificity of this conjugate and suggests that, in the conjugate form, CytA no longer shows its broad *in vitro* cytolytic activity.

However, competition experiments with NIH 3T3 HIR3.5 cells between the insulin-CytA conjugate and an excess of native insulin indicated that the intoxication of the NIH 3T3 HIR3.5 cells by the insulin-CytA conjugate was not inhibited by insulin even at a 50-fold

molar excess over the insulin-CytA conjugate. Although this lack of competition may mean that the toxicity exerted by the insulin-CytA conjugate was not mediated through insulin, it could also be explained by the fact that bound insulin is known to be internalized very rapidly by receptor-mediated endocytosis (Marshall, 1985a,b). This explanation is supported by the finding that internalized insulin becomes degraded very rapidly, while the insulin receptor has a half-life of up to 10 h and may recycle up to 200 times before it is finally degraded (Krupp and Lane, 1982). Furthermore, since insulin had a 7-fold higher capacity than the insulin-CytA conjugate to bind insulin receptors on the NIH 3T3 HIR3.5 cells (Figure 3), the native insulin may be internalized much faster than the insulin-CytA conjugate which as a result is able to accumulate on the membrane over the incubation time to reach the threshold level required to intoxicate the cells. In a separate experiment using an anti-insulin polyclonal antibody (1E2) (a kind gift from Professor K. Siddle, Department of Clinical Biochemistry, University of Cambridge), the toxicity of the insulin-CytA conjugate against the NIH 3T3 HIR3.5 cells was completely inhibited by a 3-fold molar excess of the antibody over the conjugate but not by a 1:1 molar ratio (data not shown). This result further supports the suggestion that intoxication of the NIH 3T3 HIR3.5 cells by the insulin-CytA conjugate was mediated via insulin.

The insulin-CytA conjugate was less toxic to target cells than CytA itself, although it was hoped that CytA potency might be increased by targeting it through a cell-reactive ligand. This expectation was based on the finding that CytA is more toxic to mosquito cells than erythrocytes, probably due to the presence of specific CytA receptors on mosquito cells. The lower toxicity of the insulin-CytA conjugate to the NIH 3T3 HIR3.5 cells compared to that of CytA might be due to one or more of the following. (a) Insulin molecules may interfere with or slow CytA oligomerization since *in vitro* toxicity assays of the insulin-CytA conjugate with NIH 3T3 HIR3.5 cells showed that the conjugate acted much slower than CytA, especially at low conjugate concentrations (Figure 5). (b) Some of the insulin-CytA conjugate might have been internalized before it was able to form a pore. (c) Because the assayed conjugate was heterogeneous in composition, only a fraction of the conjugate may be toxic, and therefore, the toxicity observed is not proportional to the total amount of the conjugate assayed. It is possible that some fraction of the conjugate did not bind to cells because it contained only a single chain of insulin. This may also explain why the insulin-CytA conjugate bound the NIH 3T3 HIR3.5 cells at $1/7$ of the level of native insulin (Figure 3).

The recent solution of the X-ray structure of a Cyt toxin (Li et al., 1996) may well stimulate their further use in bioconjugates. The potential use of δ -endotoxins for IT constructs may not be limited to the Cyt class. Although the Cry toxins are not cytolytic to animal cells, they may be made toxic if they are targeted. The specificity of the Cry toxins is due to high-affinity binding to specific membrane receptors on the target insect cells. By the same analogy, a targeting ligand may allow these toxins to produce cytolysis of the cells of interest.

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LITERATURE CITED

- Alouf, J. E. (1976) Cell membrane and cytolytic bacterial toxins. In *The Specificity and Action of Animal, Bacterial, and Plant Toxins* (P. Cuatrecasas, Ed.) pp 221–270, Chapman and Hall, London.
- Al-yahyaee, S. A. S., and Ellar, D. J. (1995) Maximal toxicity of cloned cytA δ -endotoxin from *Bacillus thuringiensis* subsp. *israelensis* requires proteolytic processing from both the N- and C-termini. *Microbiology* 141, 3141–3148.
- Armstrong, J. L., Rohrmann, G. F., and Beaudreau, G. S. (1985) Delta endotoxin of *Bacillus thuringiensis* subsp. *israelensis*. *J. Bacteriol.* 161, 39–46.
- Aronson, A. I. (1993) The two faces of *Bacillus thuringiensis*: insecticidal proteins and post-exponential survival. *Mol. Microbiol.* 7, 489–496.
- Bjorn, M. J., Ring, D., and Frankel, A. E. (1985) Evaluation of monoclonal antibodies for the development of breast cancer immunotoxins. *Cancer Res.* 45, 1214–1221.
- Blakey, D. C., Wawrzynczak, E. J., Wallace, P. M., and Thorpe, P. E. (1988) Antibody toxin conjugates: a perspective. *Prog. Allergy* 45, 50–90.
- Brinkmann, U., and Pastan, I. (1994) Immunotoxins against cancer. *Biochim. Biophys. Acta* 1198, 27–45.
- Byers, V. S., and Baldwin, R. W. (1991) Rationale for clinical use of immunotoxins in cancer and autoimmune disease. *Semin. Cell Biol.* 2, 59–70.
- Cantor, C. R., and Schimmel, R. R. (1980) *Biophysical Chemistry, Part II: Techniques for the Study of Biological Structure and Function*, W. H. Freeman and Co., San Francisco.
- Carlsson, J., Drevin, H., and Axen, R. (1978) Protein thiolation and reversible protein-protein conjugation. N-succinimidyl 3-(2-pyridyldithio) propionate, a new heterobifunctional reagent. *Biochem. J.* 173, 723–737.
- Chilcott, C. N., and Ellar, D. J. (1988) Comparative toxicity of *Bacillus thuringiensis* var. *israelensis* crystal proteins *in vivo* and *in vitro*. *J. Gen. Microbiol.* 134, 2551–2558.
- Chilcott, C. N., Knowles, B. H., Ellar, D. J., and Drobniewski, F. A. (1990) Mechanism of action of *Bacillus thuringiensis israelensis* parasporal body. In *Bacterial Control of Mosquitoes and Blackflies* (H. de Barjac and D. J. Sutherland, Eds.) pp 45–65, Rutgers University Press, New Brunswick, NJ.
- Chow, E., Singh, G. J. P., and Gill, S. S. (1989) Binding and aggregation of the 25-kilodalton toxin of *Bacillus thuringiensis* subsp. *israelensis* to cell membranes and alteration by monoclonal antibodies and amino acid modifiers. *Appl. Environ. Microbiol.* 55, 2779–2788.
- Clarck, S., Eckardt, G., Siddle, G., and Harrison, L. C. (1991) Changes in insulin-receptor structure associated with trypsin-induced activation of the receptor tyrosine kinase. *Biochem. J.* 26, 27–33.
- Cobbold, S. P., Thierfelder, S., and Waldmann, H. (1983) Immunosuppression with monoclonal antibodies: a model to determine the rules for effective serotherapy. *Mol. Biol. Med.* 1, 285–304.
- Crickmore, N., Bone, E. J., and Ellar, D. J. (1990) Genetic manipulation of *Bacillus thuringiensis*: towards an improved pesticide. *Aspects Appl. Biol.* 24, 17–24.
- Cumber, A. J., Forrester, J. A., Foxwell, B. M. J., Ross, W. C. J., and Thorpe, P. E. (1985) Preparation of antibody-toxin conjugates. *Methods Enzymol.* 112, 207–225.
- Drobniewski, F. A. (1989) Immunotoxins up to the present day. *Biosci. Rep.* 9, 139–156.
- Drobniewski, F. A., and Ellar, D. J. (1989) Purification and properties of a 28-kilodalton haemolytic and mosquitocidal protein toxin of *Bacillus thuringiensis* subsp. *darmstadtensis* 73-E10-2. *J. Bacteriol.* 171, 3060–3067.
- Drobniewski, F. A., Thorpe, P. E., Wallace, P. M., and Wawrzynczak, E. (1988) The potential of membrane-acting toxins for targeted cancer therapy. In *Targeting of Drugs: Anatomical and Physiological Considerations* (G. Gregoriadis, Ed.) pp 103–108, NATO ASI Series, Plenum Press, New York.
- Grossbard, M. L., Press, O. W., Appelbaum, F. R., Bernstein, I. D., and Nadler, L. M. (1992) Monoclonal antibody-based therapies of leukaemia and lymphoma. *Blood* 80, 863–878.
- Harlow, E., and Lane, D. (1988) *Antibodies: a Laboratory Manual*, Cold Spring Harbor Laboratory Press, Plainview, NY.
- Hofte, H., and Whiteley, H. R. (1989) Insecticidal crystal proteins of *Bacillus thuringiensis*. *Microbiol. Rev.* 53, 242–255.
- Khan, C. R. (1976) Membrane receptors for hormones and neurotransmitters. *J. Cell. Biol.* 70, 261–286.
- Knowles, B. H., and Ellar, D. J. (1986) Characterisation and partial purification of a plasma membrane receptor for *Bacillus thuringiensis* var. *kurstaki* lepidopteran-specific δ -endotoxin. *J. Cell. Sci.* 83, 89–101.
- Knowles, B. H., and Ellar, D. J. (1987) Colloid-osmotic lysis is a general feature of the mechanism of action of *Bacillus thuringiensis* δ -endotoxins with different specificity. *Biochim. Biophys. Acta* 924, 509–518.
- Knowles, B. H., and Dow, J. A. T. (1993) The crystal δ -endotoxins of *Bacillus thuringiensis*: models for their mechanism of action on the insect gut. *BioEssays* 15, 469–476.
- Knowles, B. H., White, P. J., Nicholls, C. N., Ellar, D. J. (1992) A broad spectrum cytolytic toxin from *Bacillus thuringiensis* var. *kyushuensis*. *Proc. R. Soc. London, Ser. B* 248, 1–7.
- Koni, P. A., and Ellar, D. J. (1994) Biochemical characterisation of *Bacillus thuringiensis* cytolytic δ -endotoxins. *Microbiology* 140, 1869–1880.
- Krupp, M. N., and Lane, M. D. (1982) Evidence for different pathways for the degradation of insulin and insulin receptor in the chick liver cell. *J. Biol. Chem.* 257, 1372–1377.
- Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227, 680–685.
- Li, J., Koni, P. A., and Ellar, D. J. (1996) Structure of the mosquitocidal δ -endotoxin from *Bacillus thuringiensis* ssp. *kyushuensis*. and implications for membrane pore formation. *J. Mol. Biol.* 257, 129–152.
- Lord, J. M., Hartley, M. R., and Roberts, L. M. (1991) Ribosome inactivating proteins of plants. *Semin. Cell Biol.* 2, 15–22.
- Maddrell, S. H. P., Lane, N. J., Harrison, J. B., Overton, J. A., and Moreton, R. B. (1988) The initial stages in the action of an insecticidal δ -endotoxin of *Bacillus thuringiensis* var. *israelensis* on the epithelial cells of the Malpighian tubules of the insect, *Rhodnius prolixus*. *J. Cell. Sci.* 90, 131–144.
- Marshall, S. (1985a) Degradative processing of internalised insulin in isolated adipocytes. *J. Biol. Chem.* 260, 13517–13523.
- Marshall, S. (1985b) Dual pathways for the intracellular processing of insulin. Relationship between retroendocytosis of intact hormone and the recycling of insulin receptors. *J. Biol. Chem.* 260, 13524–13531.
- Mason, D. W., and Williams, A. F. (1980) The kinetics of antibody binding to membrane antigens in solution and at the cell surface. *Biochem. J.* 187, 1–20.
- Millazzo, G., Yip, C. C., Maddux, B. A., Vigneri, R., and Goldfine, I. D. (1992) High-affinity insulin binding to an atypical insulin-like growth factor-I receptor in human breast cancer cells. *J. Clin. Invest.* 89, 899–907.
- Olsnes, S., Kozlov, J. K., van Duers, B., and Sandvig, K. (1991) Bacterial protein toxins acting on intracellular targets. *Semin. Cell Biol.* 2, 7–14.
- Pastan, I., Chaudhary, V., and FitzGerald, D. J. (1992) Recombinant toxins as novel therapeutic agents. *Annu. Rev. Biochem.* 61, 331–354.
- Pederzoli, C., Belmonte, G., Dalla Serra, M., Macek, P., and Menestrina, G. (1995) Biochemical and cytotoxic properties of conjugates of transferrin with equinatoxin II, a cytolytic from a sea anemone. *Bioconjugate Chem.* 6, 166–173.
- Ramakrishnan, S., and Houston, L. L. (1984) Prevention of growth of leukaemia cells in mice by monoclonal antibodies directed against Thy 1.1 antigen disulphide linked to two ribosomal inhibitors: pokeweed antiviral protein or ricin A chain. *Cancer Res.* 223, 58–61.
- Roth, R. A., Maddux, B. A., Wong, K. Y., Iwamoto, Y., and Goldfine, I. D. (1981) Insulin-ricin B chain conjugate; a hybrid molecule with ricin-binding activity and insulin biological activity. *J. Biol. Chem.* 256, 5350–5354.
- Schagger, H., and von Jagow, G. (1987) Tricine-sodium dodecyl sulphate-polyacrylamide gel electrophoresis for the separation

- of proteins in the range from 1 to 100 kDa. *Anal. Biochem.* 166, 368–379.
- Stewart, G. S. A. B., Johnstone, K., Hagelberg, E., and Ellar, D. J. (1981) Commitment of bacterial spores to germinate. *Biochem. J.* 198, 101–106.
- Thomas, W. E., and Ellar, D. J. (1983) *Bacillus thuringiensis* var. *israelensis* crystal δ -endotoxin: effects on insect and mammalian cells *in vitro* and *in vivo*. *J. Cell Sci.* 60, 181–197.
- Thorpe, P. E., Brown, A. F., Bremmer, J. A. G., Foxwell, B. M. J., and Stirpe, F. (1985) An immunotoxin composed of monoclonal anti-Thy 1.1 antibody and a ribosome-inactivating protein from *Saponaria officinalis*: potent antitumour effects *in vitro* and *in vivo*. *J. Natl. Cancer Inst.* 75, 151–159.
- Thorpe, P. E., Blakey, D. C., Brown, A. N., Knowles, P. P., Knyba, R. E., Wallace, P. M., Watson, G. J., and Wawrzynczak, E. J. (1987a) Comparison of two anti-Thy 1.1-abrin A-chain immunotoxins prepared with two different cross-linking agents: antitumour effects *in vivo*, fate, and tumour cell mutants. *J. Natl. Cancer Inst.* 79, 1101–1112.
- Thorpe, P. E., Wallace, P. M., Knowles, P. P., Relf, M. G., Brown, A. N. F., Watson, G. J., Knyba, R. E., Wawrzynczak, E. J., and Blakey, D. C. (1987b) New coupling agents for the synthesis of immunotoxins containing a hindered disulphide bond with improved stability *in vivo*. *Cancer Res.* 47, 5924–5931.
- Todaro, G. J., and Green, H. (1963) Quantitative studies of the growth of mouse embryo cells in culture and their development into established lines. *J. Cell Biol.* 17, 299–313.
- Uckun, F. M. (1993) Immunotoxins for the treatment of leukaemia. *Br. J. Haematol.* 85, 435–438.
- Uhr, J. W. (1991) Immunotoxins: an overview. *Semin. Cell Biol.* 2, 1–6.
- Van Rie, J., Jansens, S., Hofte, H., Degheele, D., and Van Mellaert, H. (1990) Receptors on the brush border membranes of the insect midgut as determinants of the specificity of *Bacillus thuringiensis* delta-endotoxins. *Appl. Environ. Microbiol.* 56, 1378–1385.
- Vitetta, E. S., and Thorpe, P. E. (1991) Immunotoxins containing ricin or its A chain. *Semin. Cell Biol.* 2, 47–58.
- Vitetta, E. S., Thorpe, P. E., and Uhr, J. W. (1993) Immunotoxins: magic bullets or misguided missiles? *Immunol. Today* 14, 252–259.
- Ward, E. S., Ellar, D. J., and Todd, J. A. (1984) Cloning and expression in *Escherichia coli* of the insecticidal δ -endotoxin gene of *Bacillus thuringiensis* var. *israelensis*. *FEBS Lett.* 175, 377–382.
- Ward, E. S., Ridley, A. R., Ellar, D. J., and Todd, J. A. (1986) *Bacillus thuringiensis* var. *israelensis* δ -endotoxin: cloning and expression of the toxin in sporogenic and asporogenic strains of *Bacillus subtilis*. *J. Mol. Biol.* 191, 13–22.
- Whittaker, J., Okamoto, A. K., Thys, R., Bell, G., Steiner, D. F., and Hoffmann, C. A. (1987) High-level expression of human insulin receptor cDNA in mouse NIH 3T3 cells. *Proc. Natl. Acad. Sci. U.S.A.* 84, 5237–5241.
- Youle, R. J., and Neville, D. M., Jr. (1980) Anti-Thy 1.2 monoclonal antibody linked to ricin is a potent cell-type-specific toxin. *Proc. Natl. Acad. Sci. U.S.A.* 77, 5483–5486.

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Synthesis and *nca*-Radioiodination of Arylstannyl–Cobalamin Conjugates. Evaluation of Aryliodo–Cobalamin Conjugate Binding to Transcobalamin II and Biodistribution in Mice

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A new method of preparing radiolabeled cobalamin derivatives has been developed. The method involves the use of cobalamin–tri-*n*-butylstannyl hippurate conjugates as intermediates to obtain radioiodinated cobalamin–iodohippurate conjugates. The arylstannyl functionality was used as an exchangeable group to obtain high specific activity radioiodinations and to circumvent some deleterious side reactions common to cobalamins under electrophilic iodination conditions. The first step in the synthesis of tri-*n*-butylstannyl hippurate conjugates was to obtain free carboxylate groups on the cobalamin moiety. This was accomplished by mild acid hydrolysis of the *b*-, *d*-, or *e*-propionamide side chains on the corrin ring, followed by careful separation of the isomeric products. The second step was to couple a linking molecule (diaminododecane) to the carboxylate. The final step was to conjugate *p*-tri-*n*-butylstannyl hippurate to the cobalamin–diaminododecane adduct. All three isomeric cobalamin–*p*-tri-*n*-butylstannyl hippurate conjugates were prepared, as were the corresponding cobalamin–*p*-iodohippurate conjugates (HPLC standards). Radioiodination reactions were conducted with *N*-chlorosuccinimide and Na[*I]I in MeOH using conditions previously developed for arylstannylations. However, unlike the previous reactions, a key factor in obtaining the desired radioiodinated cobalamins was that the reaction be conducted under neutral conditions. Isolated yields of 40–65% were obtained for all three cobalamin isomers. Specific activities of 10–33% theoretical were obtained for the radioiodinated cobalamins. Evaluation of competitive binding of (nonradioactive) cobalamin–iodohippurate conjugates with recombinant human transcobalamin II showed that the *e*-isomer bound nearly as well as [⁵⁷Co]cyanocobalamin (50%), whereas the *b*-isomer had decreased binding (6%) and the *d*-isomer was significantly decreased in its binding (0.7%). Two biodistributions of the radioiodinated *e*-isomer were conducted in athymic mice. One biodistribution investigated tissue localization in mice bearing a renal cell carcinoma xenograft, and the other biodistribution investigated tissue localization when the radioiodinated cyanocobalamin was mixed with 1% BSA prior to injection. A comparison of the results of the two biodistributions and a discussion of how they relate to previous [^{57/60}Co]cyanocobalamin biodistributions are provided.

INTRODUCTION

Cobalamin conjugates are being synthesized for evaluation as cellular growth blockers for the treatment of AIDS¹-related lymphoma. This therapeutic approach is

based on the concept that cobalamin derivatives can be used to block entry of cyanocobalamin, **1** (vitamin B₁₂), and related cobalamins into cells, thus depleting cells of this essential vitamin. Previous studies using the general anesthetic nitrous oxide (N₂O) have shown that inactivation of cobalamin in rapidly proliferating cells, such as leukemias and lymphomas, can result in a therapeutic effect *in vitro* (1, 2) and in patients (3, 4). Depletion of cobalamin in cells is expected to provide results similar to those found when cobalamin was inactivated with N₂O. While oxidation of cobalamin with N₂O does not require specific biochemical/cellular mechanisms to obtain its effect, development of cobalamin derivatives that are designed to alter the entry of cobalamin into cells will require certain constraints. For instance, to be effective, cobalamin derivatives must bind avidly with transcobalamin II, the cobalamin-binding protein in plasma (5). This is due to the fact that at physiological concentrations cyanocobalamin does not enter cells by itself, nor does it bind with the cell surface receptor molecule (6). Further, design of optimal cobalamin conjugates will require that the cobalamin derivatives be evaluated with regard to interaction of the cobalamin/TCII complex with the cell-surface receptor and with regard to their *in vivo* pharmacokinetics.

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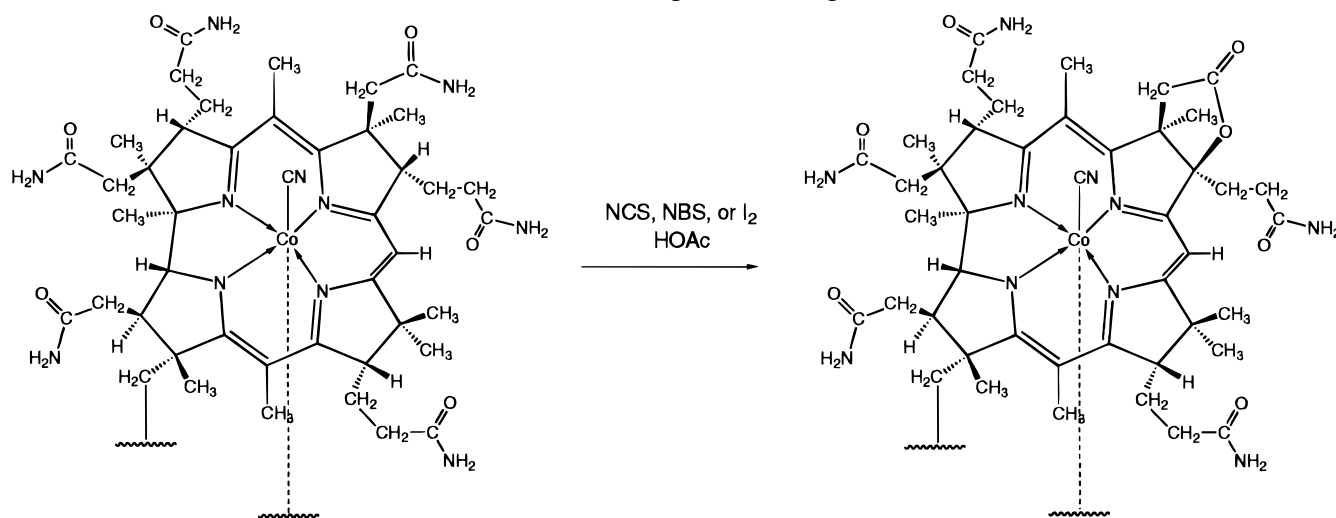
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¹ Abbreviations: AIDS, acquired immunodeficiency syndrome; BSA, bovine serum albumin; DCC, dicyclohexylcarbodiimide; DMF, dimethylformamide; DMSO, dimethyl sulfoxide; EDC, 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide; 2HEDS, 2-hydroxyethyl sulfide; HOAc, acetic acid; HSA, human serum albumin; IF, intrinsic factor; LC, liquid chromatography; *nca*, no-carrier-added; NaOAc, sodium acetate; 3NBA, 3-nitrobenzyl alcohol; NCS, *N*-chlorosuccinimide; NHS, *N*-hydroxysuccinimide; PBS, phosphate-buffered saline; RCC, renal cell carcinoma; TCII, transcobalamin II; rhTCII, recombinant human transcobalamin II; TFA, trifluoroacetic acid; TFP, tetrafluorophenyl; TFP-OH, tetrafluorophenol.

Scheme 1. *c*-Lactone Formation in the Presence of Halogenation Reagents and Acid

To assist in the development of therapeutic cobalamin conjugates, a method for radiolabeling the synthesized cobalamin derivatives was sought. Early investigators demonstrated that cyanocobalamin could be labeled with cobalt radionuclides (Co-57, -58, -60) (7–14) and carbon-14 (15). Tritium has also been used to radiolabel cyanocobalamin (16). While these labeling methods are adequate for obtaining information on cyanocobalamin, they were unattractive for our applications due to the potential difficulties with multistep derivatization of radioactive molecules and (in the case of C-14) the low specific activity that would be obtained. Also, the cost of radiolabeled starting materials in a multistep synthesis was considered a problem. Therefore, we chose to develop a new method of radiolabeling cyanocobalamin derivatives. An important consideration in developing a radiolabeling method is the need to incorporate the radionuclide in the last step of the synthesis. Additionally, we were interested in utilizing radionuclides of iodine, as they are readily available and relatively inexpensive. A further attraction of radioiodination was the fact that dual label experiments (e.g. I-125, -131) could be performed.

Previous studies have shown that direct iodination of cyanocobalamin under neutral or acidic conditions results in an unstable adduct (17), and reaction with halogens or *N*-haloamides under acidic conditions results in formation of the *c*-lactone (Scheme 1) or *c*-lactam (18–21). Thus, we sought to develop a method for high specific activity radioiodination of cobalamin derivatives which would be facile and could be accomplished in a manner that did not alter the cobalamin portion of the conjugate. The approach chosen to prepare radioiodinated cobalamin derivatives was to use arylstannane derivatives as intermediates. These intermediates have been shown to provide rapid incorporation of no-carrier-added (*nca*) radioiodine into a large variety of compounds under mild reaction conditions (22, 23). The radioiodination of arylstannanes proceeds through an electrophilic substitution reaction, resulting in high radiochemical yields of compounds with high specific activity. On the basis of previous studies with cobalamin derivatives, we chose to conjugate the requisite arylstannane to the cobalamin moiety through a diaminododecane linking molecule attached to the *b*-, *d*-, and *e*-propionamide side chains of the corrin ring (24–27). Those studies had shown that conjugation of the diaminododecane with the three monocarboxylate derivatives, formed from mild acid hydrolysis of the corrin ring propionamide side groups, provided

cobalamin derivatives with various binding affinities for the cobalamin binding protein transcobalamin II (TCII) (28).

Our approach to prepare the arylstannane derivatives involved three chemical steps: (1) hydrolysis of the corrin ring *b*-, *d*-, and *e*-propionamide side chains; (2) conjugation of the separate monocarboxylates with diaminododecane; and (3) conjugation of each cobalamin–diaminododecane adduct with an activated arylstannane. Once synthesized, radioiodination of the cobalamin–arylstannane conjugates was conducted by modification of conditions previously used for radioiodination of other arylstannane-containing molecules. Details of the synthesis and radioiodination of three isomeric stannylhippuryl–cobalamin conjugates, evaluation of TCII binding with three isomeric iodohippuryl–cobalamin conjugates, and two biodistributions of one radioiodinated isomer in athymic mice are reported herein.

EXPERIMENTAL PROCEDURES

General. All chemicals purchased from commercial sources were of analytical grade or better and were used without further purification. Cyanocobalamin (vitamin B₁₂) was obtained from Sigma Chemical Co. (St. Louis, MO). *N*-Hydroxysuccinimide was purchased from Lancaster Synthesis Inc. (Windham, NH). All other reagents were obtained from Aldrich Chemical Co. (Milwaukee, WI), except where noted. Solvents for HPLC analysis were obtained as HPLC grade and were filtered (0.2 μ m) prior to use. Ion exchange chromatography was conducted with 200–400 mesh strongly basic anion, 2% cross-linked Dowex 1 chloride (Aldrich). Amberlite XAD-2 nonionic polymeric adsorbent and octadecyl functionalized silica gel for column chromatography were obtained from Aldrich. Bio-Sil NH₂ (aminopropyl bonded silica) (40–63 μ m) for column packing was purchased from Bio-Rad Laboratories (Hercules, CA). Phosphate-buffered saline (PBS), pH 7.4, was prepared as a solution containing 8.1 mM Na₂PO₄, 1.2 mM KH₂PO₄, and 138 mM NaCl. Human serum albumin (HSA) was obtained from Miles, Inc. (Elkhart, IN). Bovine serum albumin (BSA) was obtained from ICN Biomedicals, Inc. (Costa Mesa, CA), and was diluted to 2% in PBS (10 mM sodium phosphate/150 mM NaCl), pH 7.4.

Elemental analyses were obtained from Desert Analytics (Tucson, AZ). Cobalamin derivatives retain various quantities of water even after drying under high vacuum. Data obtained from elemental analysis were evaluated

by calculating the elemental abundance for various quantities of water. The calculated carbon abundance closest to that obtained in the analysis was used to determine the number of water molecules associated with the compound. Even though all analytical samples were >98% pure by HPLC analysis, on the basis of experience with other stannyl compounds, we anticipated difficulties in obtaining satisfactory elemental analyses of stannyl derivatives **11**–**13**. Therefore, HPLC chromatograms and NMR spectra have been submitted in support of the identity and purity of these compounds (see Supporting Information).

Na[¹²⁵I]I and Na[¹³¹I]I were purchased from NEN/DuPont (Billerica, MA) as high concentration/high specific activity radioiodide in 0.1 N NaOH. Measurement of ¹²⁵I and ¹³¹I was accomplished on the Capintec CRC-15R or a Capintec CRC-6A radioisotope calibrator. Tissue samples were counted in a LKB 1282 gamma counter. [⁵⁷Co]Cyanocobalamin was obtained from Amersham Corp. (Amersham, U.K.) at a specific activity of 210 μ Ci/ μ g (7.78 MBq/ μ g). Counting of [⁵⁷Co]cyanocobalamin-containing samples was conducted with a Beckman 300 gamma counter.

Handling of Radioactivity. Reactions involving radioactive materials were conducted in a charcoal-filtered Plexiglas enclosure within a fume hood. All radioactive materials were opened, handled, and stored in the Plexiglas enclosure until used in studies or removed as radiation waste. Radioiodination reactions were conducted in 1 mL vials with Teflon-coated septa. The reaction mixtures were vented through a 10 mL charcoal-filled syringe, and additions of reagents or removal of materials was conducted by passing a syringe needle through the septum. Standard radiation safety procedures, including double gloves, were used. Radiation monitoring followed approved procedures.

Spectroscopic Data. ¹H NMR spectra were obtained on a Bruker AC-500 (500 MHz) instrument. The chemical shifts are expressed as parts per million using tetramethylsilane as an internal standard (δ = 0.0 ppm). IR data were obtained on a Perkin-Elmer 1420 infrared spectrophotometer. UV data were obtained on a Perkin-Elmer Lambda 2 UV-vis spectrophotometer or a Shimadzu UV 160U spectrophotometer. UV absorbances were obtained by dissolving a weighed sample (approximately 1.00 mg) into MeOH or H₂O in a 10 mL volumetric flask. A 1 mL aliquot of this solution was diluted to 2 mL in a volumetric flask. An aliquot of the diluted solution was placed in a quartz cuvette and the absorption was recorded over the wavelength range of 200–1100 nm. Extinction coefficients (ϵ) were calculated from the cobalamin's absorbance at the 360 nm peak using the Beer–Lambert equation. Mass spectral data were obtained on a VG 70SEQ mass spectrometer with 11250J data system. Fast atom bombardment (FAB⁺) mass spectral data were obtained at 8 kV using a matrix of 3-nitrobenzyl alcohol (3NBA) or 90% thioglycerol, 9% DMSO, and 1% TFA (DMIX). Obtaining mass spectral data for the stannyl derivatives **11**–**13** proved to be difficult using standard mass spectra instrumental methods. However, fully opening the mass spectrometer collector slit resulted in the obtainment of spectra consistent with the proposed structures.

Analytical Chromatography. HPLC separations of (nonradioactive) compounds were obtained on a Hewlett-Packard 1050 quaternary gradient pumping system with a multiple-wavelength UV detector (360 nm) and a Vares ELSD MKIII evaporative light-scattering detector. Analysis of the HPLC data were conducted on Hewlett-Packard HPLC Chemstation software. All reactions were moni-

tored by HPLC. Separations of the hippurates **16**–**18** were accomplished on a reversed-phase C₁₈ column (4.6 mm $\times$ 250 mm, 5 μ m, Alltima) using a gradient elution. The solvents in the gradient were acetonitrile (A) and aqueous 1% HOAc (B). The gradient began with 70% A/30% B for 2 min, and then it was increased to 100% A over the next 13 min, where it was held for an additional 10 min. Retention times for the hippurates were as follows: **16** = 3.1 min; **17** = 7.2 min; and **18** = 21.7 min.

Separations of the cyanocobalamin, **1**, and cobalamin derivatives **2**–**13** were conducted on two different columns, an aminopropyl–silica column and a C₁₈ reversed-phase column at a flow rate of 1 mL/min. The methods employed for each of these columns are described separately below. *Method 1:* The HPLC separations of the precursor compounds **1**–**7** were conducted on a 5 μ m, 4.6 mm $\times$ 250 mm aminopropyl column (Rainin microsorb-MV amino column) eluting with 58 mM pyridine acetate, pH 4.4, in H₂O/THF (96:4) solution (28). Retention times for the cobalamins evaluated with this system were as follows: **1** = 4.3 min; **2** = 6.5 min; **3** = 8.0 min; **4** = 8.8 min; **5** = 3.0 min; **6** = 2.9 min; and **7** = 2.9 min. *Method 2:* The iodohippuryl- and stannylhippuryl-cobalamin conjugates **8**–**13** were evaluated by reversed-phase chromatography. Reversed-phase HPLC chromatography was carried out on a Hewlett-Packard LiChrospher 100 RP-18 (5 μ m; 4.6 mm $\times$ 125 mm) C₁₈ column using a gradient solvent system. Solvent A in the gradient was MeOH. Solvent B was H₂O. Starting from 70% A, the initial solvent mixture was held for 2 min, then the gradient was increased to 100% A over the next 10 min, and 100% A was held for 5 min. Retention times under these conditions were as follows: **8** = 8.1 min; **9** = 7.5 min; **10** = 7.7 min; **11** = 14.7 min; **12** = 14.7 min; and **13** = 14.7 min.

RadioHPLC. Chromatographic separations involving radiolabeled cobalamins were conducted on a system consisting of two Beckman Model 110B pumps, a Beckman 420 controller, a Beckman Model 153 UV detector (254 nm), and a Beckman Model 170 radioisotope detector. Separations were accomplished using two different columns at a flow rate of 1 mL/min. *Method A:* Initial studies used an Alltima C₁₈ column (5 μ m; 4.6 mm $\times$ 250 mm) with a gradient separation. Solvent A in the gradient was MeOH. Solvent B was H₂O. Starting from 70% A, the initial solvent mixture was held for 2 min, then the gradient was increased to 100% A over the next 10 min, and then 100% A was held for 5 min. Retention times under these conditions were as follows: **8** = 9.2 min; **9** = 9.3 min; **10** = 9.2 min; **11** = 18.4 min; **12** = 18.3 min; and **13** = 18.3 min. *Method B:* This method was used to obtain purified samples of [^{*}I]-**10** from the radioiodination of *e*-isomer **13**. For the separation, a Hamilton PRP-1 column (10 μ m; 4.6 mm $\times$ 250 mm; Alltech) was used with a gradient of MeOH/H₂O as in method A. Under these conditions the retention times were as follows: **10** = 12.7 min and **13** = 18.4 min.

Preparative LC. A preparative LC system containing a Rainin Rabbit-plus peristaltic pumping system, a Dynamax (Model UV-1) UV-visible absorbance detector, and a Dynamax Model FC1 fraction collector was used to obtain pure samples of **8**–**13**. Preparative purifications of cobalamin–stannylhippuryl conjugates **11**–**13** for use in radioiodination reactions were conducted by reversed-phase chromatography on an analytical system. The system was as described under Analytical Chromatography (above), except a 100 μ L injection loop was used. The purification was conducted on an Alltima C₁₈ column (5 μ m; 4.6 mm $\times$ 250 mm; Alltech, Deerfield, IL) employing a gradient of MeOH/H₂O. The gradient was as

described for method 2 (above). Retention times were as follows: **11** = 8.1 min; **12** = 7.7 min; and **13** = 8.1 min.

Preparation of Cyanocobalamin Monocarboxylic Acids 2, 3, or 4. The *b*-, *d*-, and *e*-cobalamin monocarboxylates (**2**, **3**, and **4**, respectively) were prepared as previously described (28). Briefly, cyanocobalamin was hydrolyzed in 0.1 N HCl over 10 days at room temperature. Following the hydrolysis reaction, the isomeric monocarboxylates were separated from cyanocobalamin and from di- and triacids by ion exchange chromatography. Separation of the individual carboxylate isomers was accomplished by preparative liquid chromatography on an aminopropyl-silica column (25 mm × 1000 mm) at a flow rate of 0.15 mL/min.

General Procedure for Conjugation of 2, 3, or 4 with 1,12-Diaminododecane; Preparation of 5–7. The conjugation of the cobalamin monocarboxylates with diaminododecane was accomplished as previously described (28). Briefly, reaction of **2**, **3**, or **4** with diaminododecane, EDC, and NHS in a 1:1 mixture of DMF and H₂O for 4 days yielded the desired compounds after purification by ion exchange chromatography.

Preparation of *p*-Iodohippuric Acid–Cobalamin Conjugates 8–10. To a solution containing 0.30 g (0.192 mmol) of **5**, **6**, or **7** and 18 μ L of triethylamine in 40 mL of DMF was added 0.105 g (0.231 mmol) of *p*-iodohippurate TFP ester, **17**, over a 5–10 min period. The reaction mixture was stirred at room temperature for 2 h (HPLC monitored) and then evaporated to dryness. The resultant solid residue was dissolved in 20 mL of 80% methanol/H₂O and loaded on a preparative liquid chromatography system. The product was eluted on a reversed-phase column (500 mm × 25 mm, Alltech, 150 psi) (octadecyl) with the same solvent mixture as used for loading the column. The fractions containing the final product were evaporated to dryness to yield **8**, **9**, or **10**.

b-Isomer (**8**): yield, 94 mg (80%); mp 198–204 °C (dec); ¹H NMR (MeOH) δ 0.43 (s, 3H), 1.18 (s, 4H), 1.24 (d, 5H), 1.28 (m, 22H), 1.36 (m, 16H), 1.42 (s, 6H), 1.86 (s, 3H), 2.25 (d, 6H), 2.5 (d, 10H), 2.8 (s, 4H), 2.97 (s, 5H), 3.15 (m, 3H), 3.2 (s, 4H), 3.6 (m, 1H), 3.68 (d, 1H), 3.75 (m, 1H), 3.9 (d, 1H), 3.97 (d, 1H), 4.04 (m, 1H), 4.1 (d, 1H), 4.19 (m, 1H), 4.3 (m, 1H), 4.47 (m, 1H), 4.65 (m, 1H), 6.0 (s, 1H), 6.3 (d, 1H), 6.5 (s, 1H), 7.1 (s, 1H), 7.2 (s, 1H), 7.6 (d, 2H), 7.8 (d, 2H); MS (FAB⁺), mass calcd for C₈₄H₁₁₉N₁₆O₁₆CoPI 1825, found 1825; IR (KBr) 3400, 3200, 2950, 2060, 1660, 1570, 1490, 1060 cm⁻¹; UV (MeOH) λ 360 nm (ϵ = 20 400). Anal. Calcd for C₈₄H₁₁₉N₁₆O₁₆CoPI·9H₂O: C, 50.72; H, 6.89; N, 11.27. Found: C, 50.92; H, 6.86; N, 11.55.

d-Isomer (**9**): yield, 100 mg (85%); mp 236–238 °C (dec); ¹H NMR (MeOH) δ 0.42 (s, 3H), 1.18 (s, 4H), 1.3 (m, 22H), 1.36 (m, 16H), 1.45 (s, 6H), 1.9 (s, 6H), 2.14 (s, 3H), 2.25 (d, 8H), 2.56 (d, 10H), 2.8–3.0 (m, 6H), 3.3 (s, 6H), 3.6 (m, 1H), 3.68 (d, 1H), 3.75 (m, 1H), 3.9 (d, 1H), 4.0 (d, 1H), 4.07 (m, 1H), 4.12 (d, 1H), 4.17 (d, 1H), 4.3 (m, 1H), 4.47 (m, 1H), 4.65 (m, 1H), 6.0 (s, 1H), 6.2 (d, 1H), 6.5 (s, 1H), 7.1 (s, 1H), 7.2 (s, 1H), 7.6 (d, 2H), 7.8 (d, 2H); MS (FAB⁺), mass calcd for C₈₄H₁₁₉N₁₆O₁₆CoPI 1825, found 1825; IR (KBr) 3400, 3200, 2950, 2060, 1660, 1570, 1490, 1060 cm⁻¹; UV (MeOH) λ 360 nm (ϵ = 17 000). Anal. Calcd for C₈₄H₁₁₉N₁₆O₁₆CoPI·8H₂O: C, 51.19; H, 6.85; N, 11.37. Found: C, 51.27; H, 6.84; N, 11.33.

e-Isomer (**10**): yield, 79 mg (67%); mp 229–232 °C (dec); ¹H NMR (MeOH) δ 0.45 (s, 3H), 1.18 (s, 4H), 1.25 (d, 5H), 1.29 (m, 22H), 1.36 (m, 16H), 1.4 (s, 6H), 1.87 (s, 3H), 2.25 (d, 6H), 2.36 (d, 4H), 2.55 (d, 10H), 2.8 (m, 4H), 2.97 (s, 5H), 3.15 (m, 3H), 3.3 (s, 4H), 3.6 (m, 1H), 3.68 (d, 1H), 3.75 (m, 1H), 3.9 (d, 1H), 4.0 (d, 1H), 4.07

(m, 1H), 4.1 (d, 1H), 4.18 (m, 1H), 4.3 (m, 1H), 4.5 (m, 1H), 4.65 (m, 1H), 6.0 (s, 1H), 6.3 (d, 1H), 6.5 (s, 1H), 7.1 (s, 1H), 7.2 (s, 1H), 7.6 (d, 2H), 7.8 (d, 2H); MS (FAB⁺), mass calcd for C₈₄H₁₁₉N₁₆O₁₆CoPI 1825, found 1825; IR (KBr) 3400, 3200, 2950, 2060, 1660, 1570, 1490, 1060 cm⁻¹; UV (MeOH) λ 360 nm (ϵ = 17 900). Anal. Calcd for C₈₄H₁₁₉N₁₆O₁₆CoPI·9H₂O: C, 50.72; H, 6.89; N, 11.27. Found: C, 50.80; H, 6.48; N, 11.10.

Preparation of *p*-Tri-*n*-butylstannylhippuric Acid–Cobalamin Conjugates 11–13. The same procedure was used as described for the synthesis of **8**, **9**, or **10** (above), except 0.141 g (0.231 mmol) of *p*-tributylstannyl hippurate TFP ester, **18**, was added in the place of **17**.

b-Isomer (**11**): yield, 89 mg (80%); mp 182–185 °C (dec); ¹H NMR (MeOH) δ 0.43 (s, 3 H), 0.88 (t, 15 H), 1.15 (t, 12 H), 1.17 (s, 4 H), 1.22 (d, 4 H), 1.29 (s, 24 H), 1.36 (br s, 6 H), 1.4 (s, 6 H), 1.6 (m, 3 H), 1.87 (s, 8 H), 1.9 (s, 1 H), 2.05 (m, 2 H), 2.25 (s, 6 H), 2.36 (m, 3 H), 2.55 (d, 10 H), 2.8 (s, 4 H), 2.97 (s, 5 H), 3.06 (t, 2 H), 3.1 (m, 3 H), 3.3 (s, 9 H), 3.34 (m, 1 H), 3.4 (m, 1 H), 3.58 (m, 1 H), 3.65 (m, 1 H), 3.75 (d, 1 H), 3.9 (d, 1 H), 4.00 (s, 2 H), 4.07 (m, 1 H), 4.1 (d, 1 H), 4.16 (m, 1 H), 4.3 (m, 2 H), 4.48 (m, 2 H), 4.6 (m, 1 H), 6.0 (s, 1 H), 6.3 (d, 1 H), 6.5 (s, 1 H), 7.0 (s, 1 H), 7.2 (s, 1 H), 7.6 (d, 2 H), 7.8 (d, 2 H); MS (FAB⁺), mass calcd for C₉₆H₁₄₆N₁₆O₁₆CoPSn 1988, found 1988, 2010 (M + Na); IR (KBr) 3400, 3200, 2950, 2060, 1660, 1570, 1490, 1060 cm⁻¹; UV (MeOH) λ 360 nm (ϵ = 17 000).

d-Isomer (**12**): yield, 95 mg (82%); mp 188–200 °C (dec); ¹H NMR (MeOH) δ 0.43 (s, 3 H), 0.88 (t, 15 H), 1.15 (t, 12 H), 1.18 (s, 4 H), 1.3 (m, 13 H), 1.39 (m, 13 H), 1.45 (s, 5 H), 1.6 (m, 4 H), 1.72 (m, 2 H), 1.9 (s, 6 H), 2.25 (d, 6 H), 2.35 (m, 5 H), 2.56 (m, 5 H), 2.8–3.0 (m, 8 H), 3.15 (m, 4 H), 3.3 (m, 2 H), 3.4 (m, 2 H), 3.6 (m, 1 H), 3.68 (m, 1 H), 3.75 (m, 1 H), 3.9 (d, 1 H), 4.00 (d, 2 H), 4.07 (m, 1 H), 4.12 (d, 1 H), 4.2 (br s, 1 H), 4.3 (m, 1 H), 4.47 (m, 1 H), 4.7 (m, 1 H), 6.0 (s, 1 H), 6.2 (d, 1 H), 6.5 (s, 1 H), 7.1 (s, 1 H), 7.2 (s, 1 H), 7.6 (d, 2 H), 7.8 (d, 2 H); MS (FAB⁺), mass calcd for C₉₆H₁₄₆N₁₆O₁₆CoPSn 1988, found 1988, 2010 (M + Na); IR (KBr) 3400, 3200, 2950, 2060, 1660, 1570, 1490, 1060 cm⁻¹; UV (MeOH) λ 360 nm (ϵ = 16 600).

e-Isomer (**13**): yield, 78 mg (70%); mp 178–182 °C (dec); ¹H NMR (MeOH) δ 0.43 (s, 3 H), 0.88 (t, 15 H), 1.15 (t, 12 H), 1.17 (m, 3H), 1.2 (d, 4 H), 1.27 (m, 15 H), 1.35 (br s, 9 H), 1.42 (s, 3 H), 1.53 (m, 2 H), 1.6 (m, 4 H), 1.86 (s, 4 H), 1.9 (s, 1 H), 2.25 (d, 6 H), 2.5 (d, 10 H), 2.8 (s, 3 H), 2.9 (m, 6 H), 2.97 (s, 5 H), 3.15 (m, 3 H), 3.2 (m, 4 H), 3.3 (s, 9 H), 3.4 (m, 3 H), 3.6 (d, 1 H), 3.75 (d, 1 H), 3.96 (d, 1 H), 4.0 (s, 2 H), 4.08 (m, 2 H), 4.19 (m, 1 H), 4.3 (m, 2 H), 4.65 (m, 1 H), 6.0 (s, 1 H), 6.3 (d, 1 H), 6.5 (s, 1 H), 7.1 (s, 1 H), 7.2 (s, 1 H), 7.6 (d, 2 H), 7.8 (d, 2 H); MS (FAB⁺), mass calcd for C₉₆H₁₄₆N₁₆O₁₆CoPSn 1988, found 1988, 2010 (M + Na); IR (KBr) 3400, 3200, 2950, 2060, 1660, 1570, 1490, 1060 cm⁻¹; UV (MeOH) λ 360 nm (ϵ = 17 700).

Synthesis of *p*-Iodohippuric Acid TFP Ester, **17.** A 5.3 g (7.1 mmol) quantity of glycine was dissolved in 100 mL of 10% NaOH. To this solution was added 19.4 g (7.3 mmol) of *p*-iodobenzoyl chloride in several portions. The reaction mixture was allowed to stir for 10 min and then cooled in an ice–H₂O bath. To facilitate stirring, 100 mL of water was added. The solution was then acidified to pH 5 with dropwise addition of 6 N HCl. The thick faint yellow precipitate was collected by vacuum filtration and dried. Recrystallization from boiling MeOH yielded 15.3 g (70%) of off-white crystals of **16**, mp 228–230 °C, with noticeable decomposition beginning at 202 °C; ¹H NMR (DMSO-*d*₆) δ 3.77 (d, 2H, *J* = 2.7 Hz), 7.65

(d, 2H, $J = 4.4$ Hz), 7.85 (d, 2H, $J = 4.4$ Hz), 8.57 (t, 1H, $J = 5.4$ Hz); IR (nujol) 3260, 1740, 1635, 1570, 1535, 995, 830 cm^{-1} ; HRMS, calcd for $\text{C}_9\text{H}_9\text{NO}_3\text{I}$ ($M + \text{H}$) 305.9627, found 305.9615.

A solution containing 1.0 g (3.3 mmol) of **16** suspended in 30 mL of anhydrous EtOAc was cooled in an ice– H_2O bath. To this solution was added 1.6 g (9.6 mmol) of 2,3,5,6-tetrafluorophenol (TFP-OH) in 5 mL of anhydrous EtOAc, followed by 0.74 g (3.6 mmol) of 1,3-dicyclohexylcarbodiimide (DCC). The reaction was stirred for 1 h at 0 °C and then allowed to warm to room temperature while stirring overnight. The EtOAc was removed by rotary evaporation to yield a tacky white solid. This material was dissolved in 10 mL of hexanes, filtered, and dried to yield 0.89 g of a light yellow solid. The solid was eluted on a 2.5 cm $\times$ 40 cm silica gel 60 column with a 25% EtOAc/1% HOAc/74% hexanes mixture. Fractions of 100 mL were collected. The desired product eluted in fractions 6–11. Those fractions were combined, and the solvent was removed to yield 0.79 g (53%) of **17** as a white solid: mp 152–154 °C; ^1H NMR (CDCl_3) δ 4.63 (d, 2H, $J = 2.7$ Hz), 6.72 (t, 1H, $J = 4.4$ Hz), 7.05 (m, 1H), 7.55 (d, 2H, $J = 4.3$ Hz), 7.82 (d, 2H, $J = 4.3$ Hz); IR (nujol) 3270, 1775, 1630, 1525, 1190, 1180, 1150, 1060, 950, 840, 835, 775 cm^{-1} ; HRMS, calcd for $\text{C}_{15}\text{H}_9\text{NO}_3\text{F}_4\text{I}$ ($M + \text{H}$) 453.9563, found 453.9564.

Synthesis of *p*-Stannylhippuric Acid TFP Ester, **18.** A 2.0 g (4.4 mmol) quantity of **17** was dissolved in 40 mL of anhydrous toluene under argon. To this solution was added 8.0 mL (15.8 mmol) of hexabutyltin and 100 mg (0.09 mmol) of tetrakis(triphenylphosphine)-palladium(0). After stirring at room temperature for 30 min, the mixture was heated to reflux for 2 h and another 100 mg of the catalyst was added. Within 30 min, the mixture turned black. The reaction mixture was allowed to cool and toluene was removed by rotary evaporation, yielding a black oil. This oil was applied to a 250 g (4 cm $\times$ 45 cm) silica gel 60 column and eluted with 100% hexanes; 100 mL fractions were collected. At fraction 11, the eluting solvent was changed to 10% EtOAc in hexanes. The fractions were examined by HPLC, and fractions 9–15 were combined. The solvent was removed from the combined fractions to yield 1.82 g (68% yield) of a colorless oil: ^1H NMR (CDCl_3) δ 0.88 (t, 9H, $J = 7.3$ Hz), 1.08 (m, 6H), 1.32 (m, 6H), 1.54 (m, 6H), 4.65 (d, 2H, $J = 2.6$ Hz), 6.78 (t, 1H, $J = 5.4$ Hz), 7.03 (m, 1H), 7.56 (d, 2H, $J = 4.0$ Hz), 7.76 (d, 2H, $J = 4.0$ Hz); IR (neat) 3300, 1800, 1785, 1635, 1520, 1480, 1175, 1105, 950 cm^{-1} ; HRMS, calcd for $\text{C}_{27}\text{H}_{36}\text{NO}_3\text{F}_4\text{Sn}$ 618.1653 ($M + \text{H}$, Sn–120), found 618.1655.

Preparation of Cyanocobalamin–Diaminododecane–*p*-[*I]Iodohippurate Derivatives [*I]11–[*I]13. *Purification of Cyanocobalamin–Stannyl Hippurate Conjugates 8–10 by HPLC.* A saturated solution of the conjugate was prepared in methanol. This solution was passed through a 45 μm filter, and three to five 20 μL aliquots were injected on the HPLC (C_{18} column). The peak at 8–9.2 min was collected from each injection, and the collected fractions were combined. The concentration of cobalamin derivative in the combined fractions was estimated by UV. The cobalamin-containing solution was then divided into aliquots containing 0.5 mg, and a stream of argon was passed over each aliquot to remove the solvent. The samples were stored in a refrigerator, covered from light with aluminum foil.

*Method A (Example for *e*-Isomer).* This procedure was followed to obtain [^{131}I]-**10** for the first biodistribution study. In a small vial was placed 50 μL of a 1 mg/mL solution of **13** (HPLC purified) in MeOH. To this solution was added 1.5 μL (1.5 mCi) of $\text{Na}[^{131}\text{I}]\text{I}$ in 0.1 N NaOH,

followed by the addition of 10 μL of a 1 mg/mL solution of *N*-chlorosuccinimide (NCS) in MeOH containing 5% HOAc. After 5 min, 5 μL of a 0.72 mg/mL solution of sodium metabisulfite in H_2O was added to quench the reaction. A total of 1.21 mCi in an approximately 65 μL volume was removed from the reaction vessel and injected on the HPLC (C_{18} column). Collection of the desired peak (6–7 min) yielded 390 μCi (32%).

*Method B (*e*-Isomer).* In a small vial was placed 50 μL of a 1 mg/mL solution of **13** (HPLC purified) in MeOH. To this solution was added 4.5 μL (977 μCi) of $\text{Na}[^{125}\text{I}]\text{I}$ in 0.1 N NaOH, followed by the addition of 4.5 μL of 0.1 N HOAc. To the resulting solution was added 10 μL of a 1 mg/mL solution of NCS in MeOH. After 15 min, 10 μL of a 1.0 mg/mL solution of sodium metabisulfite in H_2O was added to quench the reaction. A total of 951 μCi in approximately 75 μL was removed from the reaction vessel by syringe and injected on the HPLC (PRP-1 column). The iodinated product was collected from the HPLC effluent (12.4–13.6 min), yielding 581 μCi (61%) of [^{125}I]-**10**. The radioiodinated product was evaluated by HPLC and set in the refrigerator for storage in the HPLC solvent (Figure 4).

*Procedure Followed To Obtain [^{125}I]-**10** for the Second Biodistribution Study.* This reaction was conducted as method B above with the following changes. A 6 μL aliquot of $\text{Na}[^{125}\text{I}]\text{I}$ in 0.1 N NaOH was added to the cobalamin solution. To that solution was added 6 μL of 0.1 N HOAc in MeOH. The entire reaction mixture (1.93 mCi) was drawn into a syringe and injected onto an HPLC (PRP column). The desired peak (12 min) was collected to yield 0.74 mCi (38%).²

Binding of Cobalamin–Iodohippurate Derivatives with Recombinant Human Transcobalamin II (rhTCII). rhTCII was obtained as previously reported (29). Samples (2.0 mg) of **8–10** were dissolved in 100–200 μL of DMSO and diluted to 1.0 mL with H_2O . Quantification of the cobalamin derivatives was accomplished by UV absorbance based on the extinction coefficients at 360 nm (data provided with individual compounds). Thus, a 20 μL aliquot was diluted to 1 mL with H_2O , and the absorbance at 360 nm was determined. An aliquot of the original solution was diluted to obtain a 1 μM solution, which was further diluted to 0.1 μM for use in the assay.

Apo-rhTCII was partially purified on a cation exchange column as previously described (30). The rhTCII was diluted in PBS containing 0.025% HSA to bind approximately 10 pmol of cyanocobalamin (**1**)/mL. The assay tubes contained 0.01 pmol of [^{57}Co]-**1**, 0.1–30 pmol of unmodified **1** or cobalamin derivatives **8–10**, and 100 μL of the diluted rhTCII solution. The solution volume in each tube was adjusted to 1.0 mL with PBS, and the samples were incubated at room temperature for 1 h. The free [^{57}Co]-**1** and protein-bound [^{57}Co]-**1** were separated by adsorption of free cobalamin to hemoglobin-coated charcoal (31), and the amount of radioactivity in each fraction was determined in a gamma counter. The decrease in binding of [^{57}Co]-**1** in the presence of various amounts of a cobalamin derivative was calculated and graphed (Figure 5). The quantity of each derivative required to inhibit binding of the [^{57}Co]-**1** to rhTCII by 50% was estimated, and the binding affinity of each compound relative to cyanocobalamin was determined.

² These radioiodination conditions generally result in 45–60% isolated radiochemical yields. The low yield obtained in this experiment appeared to be due to having more base present than anticipated, resulting in more free iodide being observed than in other similar experiments.

Animals and Tumor Model. Male athymic mice (*nu/nu*), obtained from Simonson Laboratories (Gilroy, CA), were housed for 1 week in the isolator facility prior to the beginning of the study. For those mice bearing tumor xenografts, tumor pieces (5–10 mg) of TK-82 renal cell carcinoma (RCC) (32) were implanted (subcutaneously) above the right shoulder using a protocol approved by the Animal Care Committee at the University of Washington. Mice in the first biodistribution had an average weight of 26.1 ± 1.9 g, and the TK-82 tumor xenografts had an average weight of 160 ± 116 mg. Mice in the second biodistribution had an average weight of 24.94 ± 1.80 g.

Biodistribution Studies. *Biodistribution 1.* A 500 μ L aliquot of sterile saline was added to the [^{131} I]-**10** fraction collected from the HPLC, and the solution was reduced to 500 μ L under argon flow. The solution was further diluted with sterile saline to prepare an injectate of 5 μ Ci/100 μ L. Twenty-four athymic mice with TK-82 RCC xenografts were injected with 5.1 μ Ci each of [^{131} I]-**10** (specific activity of 190 μ Ci/ μ g) via the lateral tail vein. The actual amount of injectate administered to each animal was determined by weighing the syringe before and after injection (range 96–103 μ L). The amount of radioactivity administered to each animal was determined by counting quadruplicate standards of 1 μ L volume of the injectate and multiplying the average standard by the actual volume of injectate. Groups of eight mice were sacrificed by cervical dislocation at 1, 4, and 24 h postinjection. The biodistribution data obtained are given in Table 2, group 1. In the biodistribution, tissues were excised, blotted free of blood, weighed, and counted. The muscle in front of the neck was excised to obtain the thyroid gland. This was done to evaluate whether free radioiodide was present. The stomach was evaluated for the same reason. Urine samples were collected by a bladder tap (using a syringe) after sacrifice. The urine samples were placed in preweighed tubes and weighed after collection to quantify each sample. It is not uncommon in this procedure to obtain urine samples from some, but not all, animals. At the 1 h sacrifice time, urine samples were collected from all eight animals in the group; at 4 h, urine samples were obtained from only two animals; and at 24 h, urine samples were collected from five animals. Calculation of the percent injected dose per gram (% ID/g) and standard deviation ($\pm$ SD) in the tissues was accomplished with an Excel spreadsheet. All calculations involving iodine-131 were corrected for radioactive decay.

Biodistribution 2. This biodistribution was conducted as described for biodistribution 1, with the following differences. The [^{125} I]-**10** fraction isolated from the HPLC was diluted with 300 μ L of DMSO, and the mixture was reduced in volume to approximately 300 μ L under a stream of argon. Half of the solution (355 μ Ci) was transferred to a new vial, and 1.5 mL of sterile saline was added. After mixing, 1 mL was drawn into a syringe and the amount of activity in the syringe was checked. Less than 10% of the expected dose was in the syringe. The contents of the syringe was returned to the vial, and 1.5 mL of a 2% BSA/PBS solution was added, making a 1% BSA solution. Upon checking the amount of activity in a 1 mL syringe, we found that the expected amount was obtained. The 1% BSA solution was then diluted with additional 1% BSA to obtain a concentration of 7 μ Ci/100 μ L for injection. Eighteen athymic mice were injected with 7.1 μ Ci each of [^{125} I]-**10** (specific activity of 0.29 μ Ci/ μ g) in approximately 100 μ L (range 98–105 μ L) of 1% BSA. Groups of six mice were sacrificed at 1, 4, and 24 h postinjection. The mice did not have tumor xenografts, but additional tissues were evaluated. Those

tissues were skin (not reported), heart, and brain. The brain was obtained by cutting the back of the skull for removal. The data obtained is provided in Table 2, group 2.

RESULTS

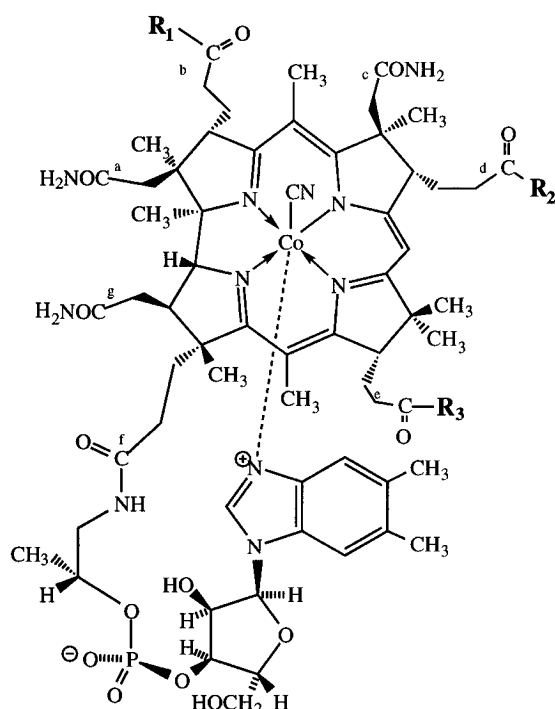
Synthesis of Arylstannane Conjugates. The syntheses of stannyl hippurate conjugates **11–13**, and the corresponding iodohippuryl conjugates **8–10**, were accomplished by first preparing the cobalamin monocarboxylic acids **2–4** (Table 1) (28). Preparation of monocarboxyl cyanocobalamin derivatives was accomplished by hydrolysis of the corrin ring propionamides at room temperature in 0.1 N HCl over a 10 day period. After separation from nonhydrolyzed cyanocobalamin and the di- and tricarboxylates by ion chromatography, the *b*-, *d*-, and *e*-monocarboxylates (**2**, **3**, and **4**, respectively) were separated by preparative liquid chromatography using an aminopropyl-silica column. The individual monocarboxylates **2–4** were coupled with diaminododecane by reaction with a carbodiimide (EDC) in DMF/H₂O containing NaCN/NHS to give compounds **5–7**.

The carboxylate-activated hippurate derivatives **17** and **18** were prepared as depicted in Scheme 2. Preparation of iodohippurate was readily accomplished by reaction of glycine in 10% NaOH with *p*-iodobenzoyl chloride. Formation of the TFP ester was accomplished by reaction of tetrafluorophenol (TFP-OH) and dicyclohexylcarbodiimide (DCC) in THF. Difficulties in purification resulted in a moderate yield of **17**. Stannylation was accomplished on the TFP ester containing **17** by reaction of hexabutyltin using the palladium catalyst tetrakis(triphenylphosphine)palladium(0). Iodination of **18** with NaI and NCS in MeOH resulted in conversion to the iodohippurate **17**. The iodohippuryl conjugates **8–10** and stannylhippuryl conjugates **11–13** were obtained by reaction of **5**, **6**, or **7** with the hippuric acid derivative **17** or **18**. After purification, analytical HPLC indicated that single compounds were obtained (e.g. Figure 1; Supporting Information).

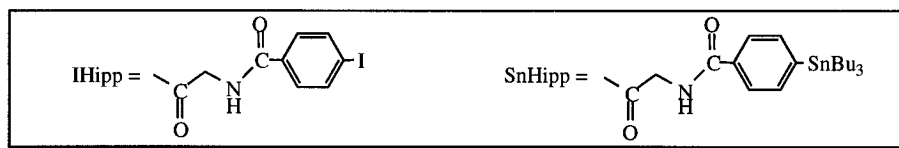
Radioiodination Studies.³ Initial radioiodination reactions of **11–13** were carried out in MeOH/1% HOAc solution employing NCS/Na[^{125} I]I. Reaction with each of the three isomers provided a radioiodinated compound in high yield, but the retention time of the radiolabeled species did not correspond to that of the co-injected iodo standards (**8**, **9**, or **10**). An example is shown in Figure 2, where the radioHPLC had a major radioactivity peak at 8.4 min and a minor peak at 9.5 min (Figure 2A). After co-injection of the radiolabeled material and the iodo standard (Figure 2B), it became apparent that the radioactivity peak at 8.4 min was not the desired product, as the iodo standard peak had a retention time of 9.2 min (with shoulder at 8.4 min) by UV detection, and the UV detector comes before the radioactivity detection in the effluent flow. Thus, it seemed likely that the radioactivity peak corresponding to the desired product had a retention time of 9.5 min.

A concern about oxidative side products, particularly lactone formation (Scheme 1), produced under acidic conditions, led to an investigation of (nonradioactive) iodination reactions under acidic, basic, and neutral conditions. To be more reflective of radiolabeling conditions, the studies were conducted at 0.1 equiv of NCS and NaI. Under acidic conditions, a radioiodinated product that did not correspond to the iodo standard was

³ Note that three different HPLC systems and conditions were used to monitor radioactive and nonradioactive reactions. The equipment and conditions are described under Experimental Procedures.

Table 1. Synthesized Derivatives of Cyanocobalamin for Radioiodination Studies

- 1 $R_1 = \text{NH}_2$; $R_2 = \text{NH}_2$; $R_3 = \text{NH}_2$
- 2 $R_1 = \text{OH}$; $R_2 = \text{NH}_2$; $R_3 = \text{NH}_2$
- 3 $R_1 = \text{NH}_2$; $R_2 = \text{OH}$; $R_3 = \text{NH}_2$
- 4 $R_1 = \text{NH}_2$; $R_2 = \text{NH}_2$; $R_3 = \text{OH}$
- 5 $R_1 = \text{NH}(\text{CH}_2)_{12}\text{NH}_2$; $R_2 = \text{NH}_2$; $R_3 = \text{NH}_2$
- 6 $R_1 = \text{NH}_2$; $R_2 = \text{NH}(\text{CH}_2)_{12}\text{NH}_2$; $R_3 = \text{NH}_2$
- 7 $R_1 = \text{NH}_2$; $R_2 = \text{NH}_2$; $R_3 = \text{NH}(\text{CH}_2)_{12}\text{NH}_2$
- 8 $R_1 = \text{NH}(\text{CH}_2)_{12}\text{NH-IHipp}$; $R_2 = \text{NH}_2$; $R_3 = \text{NH}_2$
- 9 $R_1 = \text{NH}_2$; $R_2 = \text{NH}(\text{CH}_2)_{12}\text{NH}_2\text{-IHipp}$; $R_3 = \text{NH}_2$
- 10 $R_1 = \text{NH}_2$; $R_2 = \text{NH}_2$; $R_3 = \text{NH}(\text{CH}_2)_{12}\text{NH-IHipp}$
- 11 $R_1 = \text{NH}(\text{CH}_2)_{12}\text{NH-SnHipp}$; $R_2 = \text{NH}_2$; $R_3 = \text{NH}_2$
- 12 $R_1 = \text{NH}_2$; $R_2 = \text{NH}(\text{CH}_2)_{12}\text{NH}_2\text{-SnHipp}$; $R_3 = \text{NH}_2$
- 13 $R_1 = \text{NH}_2$; $R_2 = \text{NH}_2$; $R_3 = \text{NH}(\text{CH}_2)_{12}\text{NH-SnHipp}$

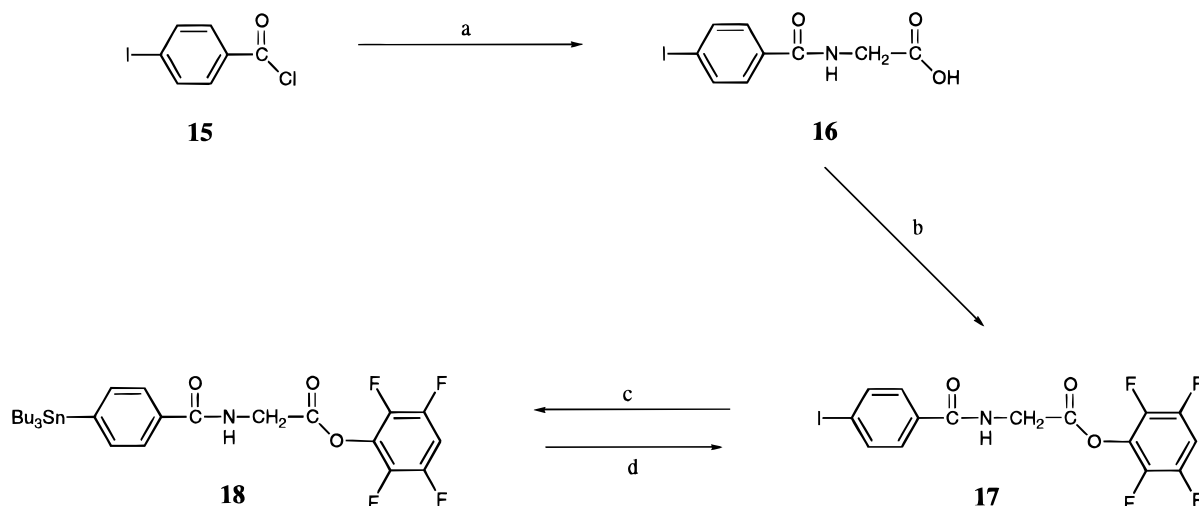


obtained. Under basic conditions, no reaction occurred. However, under neutral conditions, a product that coeluted with the iodo standard was obtained.

Although the arylstannanes **11–13** appeared to be >95% pure by HPLC after the first purification (e.g. Figure 1), the starting material was repurified by preparative HPLC prior to further radioiodinations. A second purification step is often conducted to assure that reactive minor impurities will not compete in *nca*-radioiodinations. Similar to the iodination reactions, radioiodination of the HPLC-purified starting material under neutral conditions provided the desired radioiodi-

nated product (i.e. 9.5 min). Unfortunately, the yield was low (25–30%), and an additional radioiodinated species which was similar in lipophilicity to the starting arylstannane (i.e. 19 min) was also obtained. The HPLC retention time for the new radioiodinated species indicated that the arylstannane was not substituted by iodine, suggesting that the radioiodide may have displaced the cyano group on the cobalt. To reverse this, the reaction was quenched with the addition of NaCN. Indeed, the lipophilic radioactivity peak previously seen at 19 min disappeared and a new radioactivity peak at 3.7 min appeared. Addition of NaCN to the reaction

Scheme 2. Synthesis of Hippuryl TFP Esters



^a Glycine, 10% NaOH, 76%. ^b TFP-OH, DCC, 52%. ^c *n*-Bu₃Sn₂, [(C₆H₅)₃P]₄Pd(0), 67%. ^d NaI, NCS, MeOH/5% HOAc.

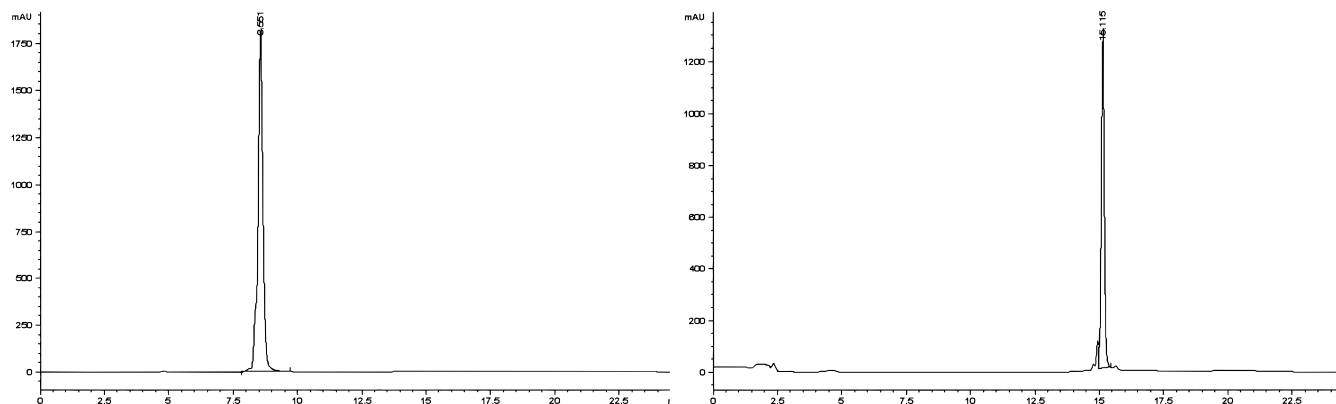


Figure 1. HPLC chromatograms (UV detection) for the isolated products from the reaction of aminododecylcobalamin, **7**, with TFP ester of *p*-iodohippurate, **17**, and *p*-tri-*n*-butylstannylhippurate, **18**, to yield cobalamin conjugates **10** (A, left) and **13** (B, right). A reversed-phase C_{18} column was used for the separation. Conditions are described under Experimental Procedures, Analytical Chromatography, Method 2.

mixture was not desired, and the radiolabeling yields were not optimal, so additional radiolabeling studies were conducted.

Subsequent radioiodination studies demonstrated that the reactions could be conducted under near-neutral conditions to give 38–65% yields (in nine syntheses) of the desired radioiodinated product. Furthermore, most reaction conditions did not produce the more lipophilic (later eluting) radioiodinated species. Isolation of the desired radioiodinated product was achieved by collection of the HPLC effluent at the appropriate time. However, initial attempts at isolation of the radioiodinated cobalamins from the HPLC effluent were inefficient, as it appeared that a large proportion of the radioactivity was being retained on the C_{18} HPLC column. While it was not apparent why this occurred, it appeared that the compound was binding nonspecifically to the column. This nonspecific binding was also observed with glass vials and the syringe needles used to administer the compound to mice.⁴ Use of a different HPLC column (Hamilton PRP-1) resulted in good recovery of the radio-labeled cobalamin. Thus, optimization of the radioiodination reactions included varying reaction conditions and using a column that did not retain the product. The *in vitro* stability of [¹²⁵I]-**10** was evaluated by re-examining the radioiodinated cobalamin after storage in a refrigerator for over 3 months. Radiochromatograms of the isolated product after storage for 1 h, 16 h, and 37 days are shown in Figure 4. Radiochromatograms taken at various times up to 99 days postisolation looked identical to those shown in Figure 4.

TCII Binding. Binding of cobalamin derivatives with TCII is essential to the receptor binding and cellular uptake of these compounds by cells. To assess the binding of nonradioactive iodohippuryl conjugates of cyanocobalamin, competitive binding assays were performed using [⁵⁷Co]cyanocobalamin, as the tracer, and partially purified recombinant human apo-rhTCII. The binding curves for compounds **8**–**10** are compared with that of cyanocobalamin in Figure 5. In comparison with cyanocobalamin, which binds rhTCII with high affinity ($K_a = 10^{-12}$ M), the iodohippurate derivatives

of the *e*-carboxylate isomer (**10**) and *b*-carboxylate isomer (**8**) bind rhTCII with 2- and 16-fold lower apparent affinities, respectively. The *d*-iodohippuryl-cobalamin derivative (**9**), on the other hand, binds with a 75-fold lower apparent affinity than [⁵⁷Co]-**1**, requiring 152 pmol to inhibit binding of [⁵⁷Co]-**1** to rhTCII by 50% (value derived by extrapolation of the binding data).

Biodistribution in Mice. Two biodistributions of radioiodinated **10** were carried out to determine the tissue distribution in mice and to assess the *in vivo* integrity of the radioiodine label. In one distribution, mice bearing human renal cell carcinoma (RCC) tumor xenografts, TK-82 (32), were studied, as it was of interest to determine whether this radiolabeled derivative would localize, or be retained in, that tumor model. We chose to investigate the TK-82 tumor model even though data on the numbers of cobalamin/TCII receptor sites on this tumor xenograft could not be readily obtained due to the fact that it is not propagated as a cell culture line.

In the first biodistribution, radiolabeling of **13** with *nca*-[¹³¹I]iodide resulted in the obtainment of 390 μ Ci of [¹³¹I]-**10** with a specific activity of 0.19 Ci/mg (0.19 μ Ci/ng). A solution containing approximately 5 μ Ci/100 μ L was prepared, and this amount was injected via the lateral tail vein into 24 athymic mice bearing TK-82 tumor xenografts. Animals were sacrificed at 1, 4, and 24 h postinjection. Data obtained for the distribution of radioactivity (% ID/g) in nine tissues, blood, and urine are provided in Table 2, group 1. All tissue concentrations decreased with time, except the stomach, which increased at 4 h over that seen at 1 h. Kidney concentrations were the highest at the 1 h time point and, with the exception of neck (thyroid), retained the highest level of radioactivity per gram. Urine collection indicated that a large portion of the radioactivity may have been excreted by this route.⁵

The results from the first biodistribution led to an uncertainty regarding the stability of the radioiodine label *in vivo*, as high neck (thyroid) and stomach concentrations were observed. The fact that the concentra-

⁴ There were difficulties with an apparent nonspecific binding of radioiodinated **10**. Upon removal of the MeOH from the isolated HPLC solvent, the radioactivity began to adhere to glass surfaces and to syringe needle surfaces, making it difficult to obtain the quantities of radiolabeled material sought. This is thought to be related to the decreased water solubility of the iodohippuryldiaminododecane-linked cyanocobalamin from that of cyanocobalamin.

⁵ The total percentage of radioactivity being excreted in the urine was not determined. The feces were not collected, and therefore it is difficult to determine how much activity was excreted via that route. However, on the basis of the relatively low values (% ID/g) in the intestines versus the quantities seen in the kidneys and urine, it would seem that hepatobiliary was not the major route of excretion for the radioiodinated cyanocobalamin derivative.

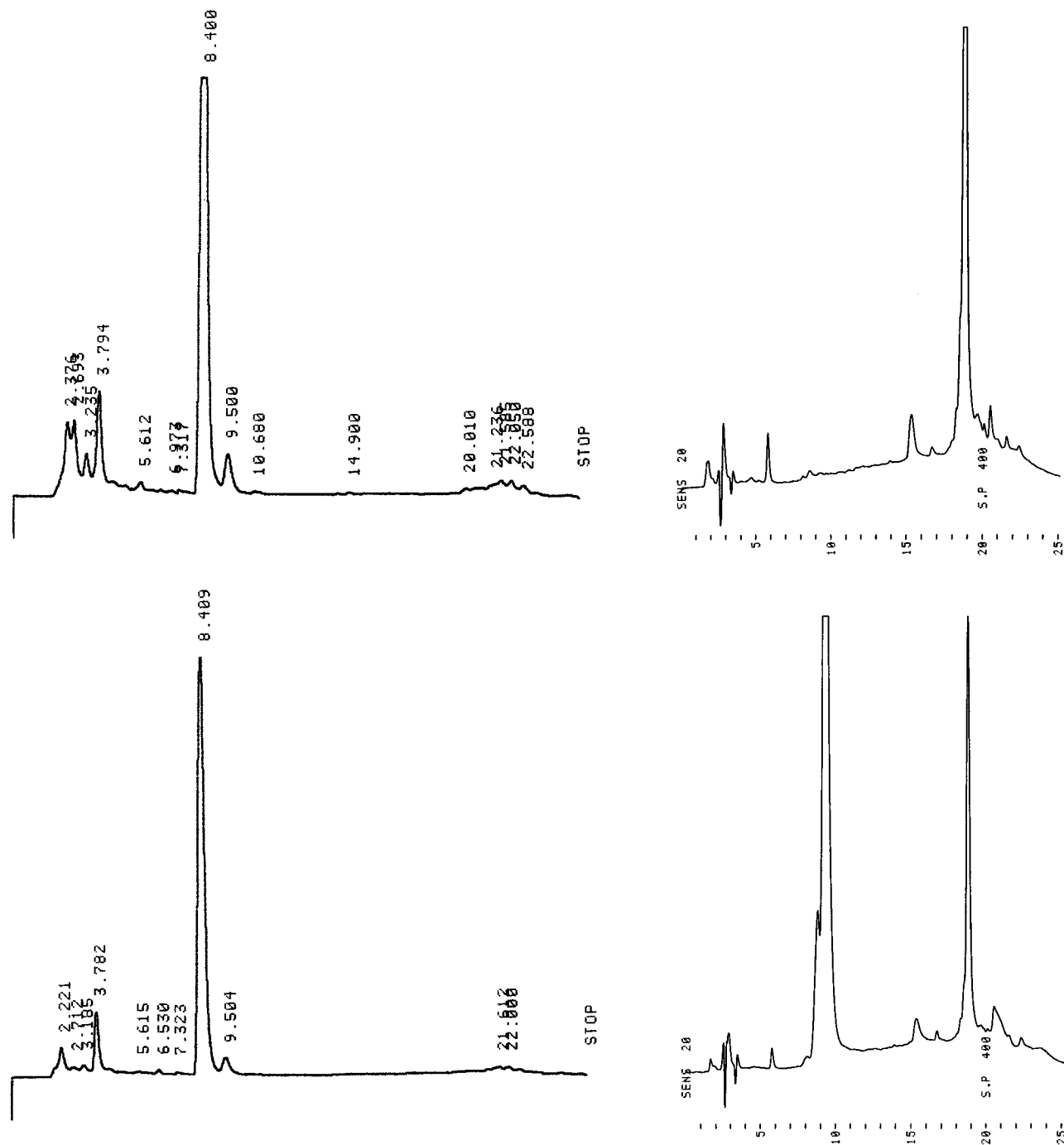


Figure 2. HPLC radiochromatograms (left) and UV chromatograms (right) of (A, top) the reaction mixture from radioiodination of **13** using NCS and Na^{[125]I} under acidic conditions and (B, bottom) the same reaction mixture with added iodo standard, **10** (9.23 min on UV trace). A reversed-phase C₁₈ column was used for the separation with conditions described under Experimental Procedures, RadioHPLC, Method A.

tion of radioiodine in the neck decreased at 4 and 24 h was suggestive of having a bolus of radioiodine injected with the preparation, rather than *in vivo* degradation to free iodide. To better assess this, a second biodistribution was conducted in athymic mice. Because previous investigators had reported that high concentrations of vitamin B₁₂ (cyanocobalamin) were found in rat hearts and pituitary glands (33), we chose to include heart and brain tissues in the study.

In the second biodistribution, **13** was radiolabeled with *nca*-[¹²⁵I]iodide, resulting in the obtainment of 740 μ Ci of [¹²⁵I]-**10** having a specific activity of 0.29 Ci/mg. Due to difficulties with the radiolabeled cobalamin adhering to the glass vial in the previous biodistribution study, the fraction containing [¹²⁵I]-**10** collected from the HPLC

effluent was diluted with 300 μ L of DMSO prior to removal of the MeOH/H₂O under a stream of N₂. This procedure worked well, but dilution to a concentration of DMSO of <10% resulted in [¹²⁵I]-**10** coming out of solution, and adherence to the glass vessel was noted. Fortunately, addition of 2% BSA in PBS to the vial removed all of the radioactivity from the vessel.⁶ A solution containing 7.1 μ Ci of [¹²⁵I]-**10** in 100 μ L of 1% BSA was injected via the lateral tail vein into 18 athymic

⁶ Evaluation of [¹²⁵I]-**10** in 1% BSA/PBS by size exclusion chromatography (Waters Protein Pak 200 SW, eluting with 75 mM NaCl/25 mM Tris/1 mM EDTA, pH 7.2) showed that <20% of the radioactivity was associated with the BSA. This indicated that the cobalamin was not tightly bound with BSA.

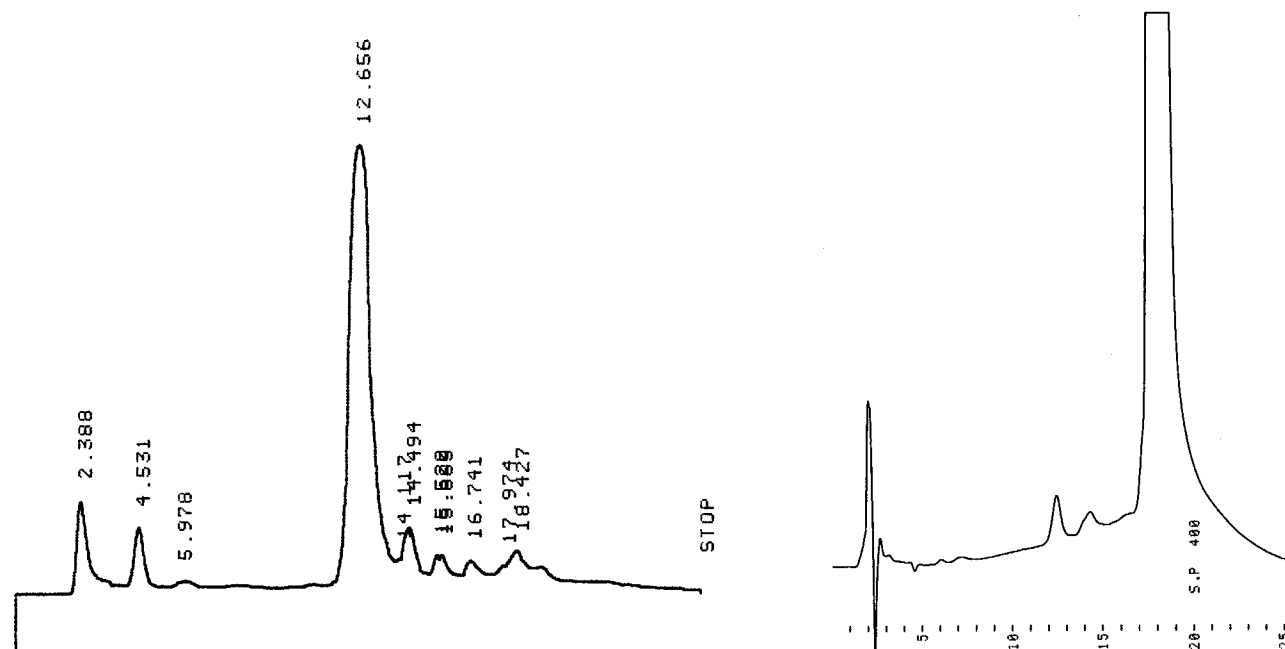


Figure 3. Radiochromatogram (left) and UV chromatogram (right) of reaction mixture from radioiodination of **13** using NCS and Na^{[125]I} under neutral conditions. A Hamilton PRP-1 column was used for the separation with conditions described under Experimental Procedures, RadioHPLC, Method B.

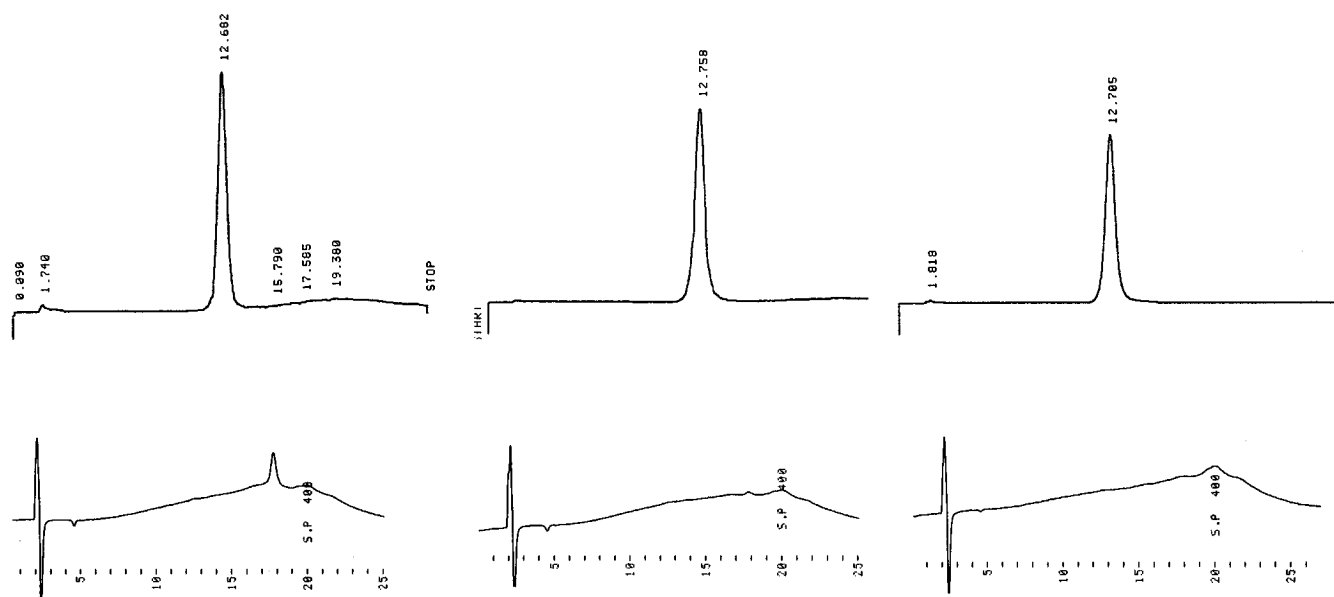


Figure 4. HPLC radiochromatograms (upper) and UV chromatograms (lower) of isolated [¹²⁵I]-**10** at 1 h postisolation (left), 16 h postisolation (center), and 37 days postisolation (right).

mice. Groups of six mice were sacrificed at 1, 4, and 24 h postinjection. The distribution data obtained from 11 tissues, blood, and urine are provided in Table 2, group 2. Importantly, the concentration of radioactivity in the neck and stomach indicated that the radiolabel was not being dissociated from the cobalamin *in vivo*. As with the first biodistribution (group 1), the highest concentration of radioactivity was found in the kidneys. While the 1 h tissue concentrations of the two biodistributions were appreciably different, the 4 and 24 h tissue concentrations were quite similar. When the tissue-to-blood ratios were evaluated (Table 3), even the 1 h data were similar. Examination of the heart and blood tissue concentrations did not show high accumulations of [¹²⁵I]-**10**. Heart tissue was found to be 3–4 times that of muscle but was less than that of blood at all time points. Brain tissue had low concentrations of radioactivity at all time points.

DISCUSSION

Radiocobalt-labeled cyanocobalamin has been, and continues to be, routinely used for many different types of investigations involving cobalamins. For our studies, radiocobalt labeling appeared to be problematic, due primarily to the fact that [⁶⁰Co]cyanocobalamin would have to be used as the starting material in a multistep synthesis. Our desire to conduct the syntheses and introduce the radiolabel in a final step led to an investigation of incorporation of radioiodine into cobalamin derivatives. Literature reports indicated that direct radioiodination of cobalamins could not be accomplished (17) and that side reactions involving formation of B-pyrrolidine ring lactone and lactam rings (18–21) would have to be circumvented. Our previous experience involving radioiodinations using organometallic inter-

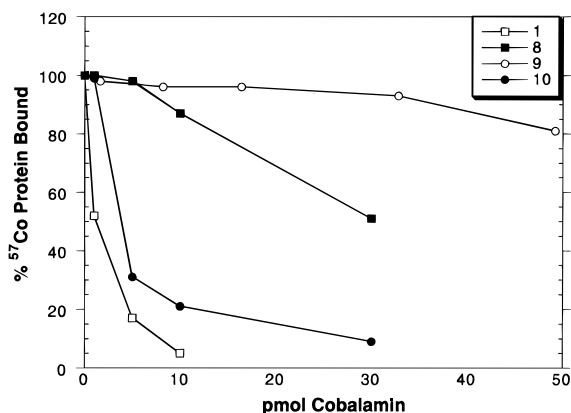


Figure 5. Competitive binding curve for [⁵⁷Co]cyanocobalamin, **1**, and iodohippurycobalamin derivatives with rhTCII. [⁵⁷Co]-Cyanocobalamin (0.01 pmol) was mixed with increasing concentrations of **1** or cobalamin derivatives **8–10**. Diluted rhTCII (100 μ L) that could bind approximately 1 pmol of **1** was then added to each tube. The volume was adjusted to 1 mL with PBS and incubated at room temperature for 1 h. Free and protein-bound [⁵⁷Co]-**1** was separated by adsorption to hemoglobin-coated charcoal, and the radioactivity in each fraction was measured.

mediates (**22**) made it apparent that cobalamin conjugates containing organometallic functional groups might be used to get around the difficulties encountered in iodination of cobalamins. Although a number of different organometallic intermediates have been shown to be effective for incorporation of radioiodine into organic compounds, arylstannane conjugates were chosen for investigation as these intermediates have been found to incorporate radioiodine efficiently under mild reaction conditions.

A major consideration in the conjugation of the requisite arylstannane to the cobalamin was that the radioiodinated moiety be stably attached to it. Evaluation of a number of different locations for conjugation on the cobalamin molecule led to the conclusion that corrin ring side chains were the preferred sites of attachment (**28**). While stability of the radiolabel was considered critical, it could not be obtained at the expense of loss of binding with the essential cobalamin binding protein TCII. Studies by a number of investigators, as well as in our own laboratory, have found that derivatization of the corrin ring *b*-, *c*-, *d*-, and *e*-propionamides results in variable binding to TCII (**24–28**). Fortunately, binding characteristics that are similar to those of cyanocobalamin have been obtained with *e*-propionamide derivatives. Although the arylstannane might be coupled directly to the corrin side group (carboxylate), it was thought that a spacer moiety between the cyanocobalamin molecule and the radioiodination moiety might be better. Diaminododecane was chosen as a molecule that would link the arylstannane with the cobalamin. This spacer moiety had been previously used in cyanocobalamin derivatives being attached to a column matrix (**34**) and has been employed in the preparation of biotin derivatives (**28**).

Initial targeted cobalamin conjugates had a *p*-tri-*n*-butylstannylbenzoate as the arylstannane moiety. Although preparation of one of the requisite nonradioactive standards was accomplished readily using *p*-iodobenzoyl chloride, reaction with the corresponding TFP ester of *p*-tri-*n*-butylstannylbenzoate did not give the desired arylstannane conjugate. Under the reaction conditions employed, the stannylbenzoate conjugation gave a product that incorporated two molecules of the stannylbenzoate rather than one. Rather than developing reaction

conditions to achieve the conjugation with *para*-stannylbenzoate, we chose to prepare and use the TFP ester of *p*-tri-*n*-butylstannylhippurate, **18**. In the latter compound, the reacting carboxylate is removed from the influence of the stannyl group. The requisite TFP esters of *p*-iodohippurate and *p*-tri-*n*-butylstannylhippurate, **17** and **18**, respectively, were prepared as shown in Scheme 2. These compounds had been previously synthesized because of our interest in their use for radioiodination of monoclonal antibodies (**35**). Once prepared, conjugation of **17** and **18** with the isomeric diaminododecane cobalamin adducts, **5–7**, provided the iodo standards **8–10** and the stannylhippurates **11–13** in good yields.

Developing optimal conditions for radioiodination of **11–13** was fraught with difficulties. However, once it was determined that the iodinations had only minor side reactions when conducted under neutral conditions, good radiochemical yields were obtained. As with many *nca*-radioiodinations, optimal conditions included the attainment of highly purified starting materials before radioiodination. Somewhat unexpected was the requirement to switch from a silica-based C₁₈ column to a polymeric PRP-1 column to minimize the amount of radiolabeled compound being retained on the column. It was not apparent why this change in columns made such a significant improvement in the isolated yields. However, adherence to other surfaces such as glass tubes and metal in syringe needles when the amount of organic modifier in the solvent was decreased indicates that it could be related to the lipophilicity of the compounds. The fact that BSA could be used to solublize radioiodinated **10** seems to further support the concept that hydrophobicity of these compounds is a problem. Additional studies are planned to further evaluate this aspect of the radioiodinated cobalamins.

High specific activity radioiodination was sought in our studies. To understand how radioiodinated cobalamin conjugates compared to radiocobalt-labeled cyanocobalamin, a comparison of specific activities was done. There are two radionuclides of iodine that are routinely used: iodine-125 ($t_{1/2}$ = 60.14 days) and iodine-131 ($t_{1/2}$ = 8.04 days). The theoretical specific activity of iodine-125 is 17.4 mCi/ μ g (2180 mCi/ μ mol) and of iodine-131 is 124 mCi/ μ g (16 400 mCi/ μ mol). The actual specific activity of iodine-131 is about 10% of theoretical as stable iodine (iodine-127) is formed in the reaction used to produce it. Thus, it is expected that specific activities of the two radioiodines are similar when received. There are also two radionuclides of cobalt that can be obtained from commercial sources: cobalt-57 ($t_{1/2}$ = 270.9 days) and cobalt-58 ($t_{1/2}$ = 70.8 days). The theoretical specific activity of cobalt-57 is 8.5 mCi/ μ g (or 480 mCi/ μ mol) and of cobalt-58 is 32.3 mCi/ μ g (1840 mCi/ μ mol). Specific activities of [⁵⁷Co]cyanocobalamin obtained from commercial sources range from 0.10 to 0.30 mCi/ μ g, which is 28–85% of theoretical (0.355 mCi/ μ g).⁷

The specific activity of radioiodinated cobalamin conjugates could be obtained directly from HPLC data by dividing the isolated radioactivity by the quantity of cobalamin associated with the UV peak area on the HPLC chromatogram. Because the molar extinction coefficients are relatively high for cobalamins, the area under the UV peak could be used to assess the amount of compound present. Conversion of the area under the UV peak to micrograms of cobalamin derivative was readily accomplished from a standard curve previously determined for the compound. The specific activities

⁷ No information was obtained on the specific activity of cobalt-58 from current commercial sources.

Table 2. Distribution of Radioactivity for [¹³¹I]-10** in Athymic Mice as Percent of Injected Dose per Gram of Tissue**

tissue/fluid	1 h ^a		4 h		24 h	
	group 1 ^b	group 2 ^c	group 1	group 2	group 1	group 2
blood	21.82 ± 4.04	12.02 ± 1.72	6.03 ± 1.47	5.05 ± 0.53	0.53 ± 0.17	0.61 ± 0.09
muscle	2.16 ± 0.49	1.22 ± 0.36	1.07 ± 0.28	1.06 ± 0.14	0.14 ± 0.03	0.16 ± 0.05
lung	11.24 ± 2.10	6.32 ± 1.08	5.04 ± 1.48	4.24 ± 0.65	0.53 ± 0.16	0.72 ± 0.18
kidney	69.27 ± 29.15	46.74 ± 8.82	24.92 ± 10.60	31.67 ± 5.65	1.98 ± 0.67	2.97 ± 0.52
spleen	10.83 ± 1.80	6.55 ± 0.86	3.85 ± 0.94	2.75 ± 0.32	0.57 ± 0.14	0.56 ± 0.11
liver	30.61 ± 7.05	12.21 ± 1.67	8.56 ± 3.06	5.66 ± 1.24	0.83 ± 0.24	1.06 ± 0.08
intestines	5.89 ± 1.64	3.50 ± 1.22	5.16 ± 1.65	3.60 ± 1.15	0.52 ± 0.23	0.57 ± 0.09
neck	33.28 ± 6.25	5.49 ± 1.12	23.60 ± 11.43	3.81 ± 0.54	10.88 ± 14.24	0.98 ± 0.50
stomach	18.99 ± 4.90	4.48 ± 0.93	26.33 ± 5.23	4.25 ± 0.67	0.87 ± 0.36	0.91 ± 0.14
urine ^d	76.6 ± 40.29	21.23 ± 3.63	131.42 ± 111.44 ^e	107.86 ± 73.17	14.02 ± 7.22 ^f	18.86 ± 2.67
tumor ^g	15.29 ± 4.02	— ^h	6.15 ± 1.98	—	0.91 ± 0.34	—
heart	— ⁱ	4.21 ± 2.38	—	3.12 ± 0.34	—	0.45 ± 0.07
brain	— ⁱ	0.73 ± 0.20	—	0.35 ± 0.07	—	0.13 ± 0.02

^a Time of sacrifice from injection of [¹³¹I]-**10**. ^b Group 1 results were obtained for *n* = 8 mice at each time point; average animal weight, 26.14 ± 1.91 g; 5.2 μCi of [¹³¹I]-**10**/animal in 100 μL of 0.9% saline; approximately 26 ng of **10** injected/animal. ^c Group 2 results were obtained for *n* = 6 mice at each time point; average animal weight, 24.94 ± 1.80 g; 7.1 μCi of [¹²⁵I]-**10**/animal in 100 μL of 1% BSA/PBS (also contained <2% DMSO); approximately 24 ng of **10** injected/animal. ^d Urine was collected by syringe bladder tap immediately after sacrifice. ^e Urine was collected from two of eight animals. ^f Urine was collected from five of eight animals. ^g Tumor xenografts were TK-82 renal cell carcinoma; average tumor weight, 0.160 ± 0.116 g. ^h Animals did not have tumor xenografts. ⁱ Not determined.

Table 3. Tissue-to-Blood Ratios for Biodistribution of [¹³¹I]-10** in Athymic Mice^a**

tissue	1 h		4 h		24 h	
	group 1 ^b	group 2 ^c	group 1	group 2	group 1	group 2
muscle	0.10 ± 0.01	0.10 ± 0.03	0.18 ± 0.02	0.21 ± 0.03	0.27 ± 0.04	0.16 ± 0.07
lung	0.52 ± 0.03	0.53 ± 0.05	0.83 ± 0.07	0.84 ± 0.06	1.07 ± 0.24	1.18 ± 0.12
kidney	2.23 ± 1.21	3.87 ± 0.29	4.02 ± 0.69	6.27 ± 0.95	3.72 ± 0.37	4.95 ± 0.99
spleen	0.50 ± 0.04	0.55 ± 0.04	0.64 ± 0.05	0.55 ± 0.06	1.20 ± 0.30	0.92 ± 0.09
liver	1.39 ± 0.10	1.02 ± 0.03	1.40 ± 0.17	1.11 ± 0.19	1.58 ± 0.18	1.77 ± 0.24
intestines	0.27 ± 0.07	0.29 ± 0.11	0.86 ± 0.19	0.72 ± 0.22	0.94 ± 0.23	0.94 ± 0.15
neck	1.55 ± 0.23	0.45 ± 0.05	4.21 ± 2.41	0.75 ± 0.09	16.46 ± 21.82	1.57 ± 0.53
stomach	0.92 ± 0.38	0.33 ± 0.05	4.58 ± 1.27	0.84 ± 0.07	1.68 ± 0.38	1.50 ± 0.09
tumor	0.69 ± 0.10		1.01 ± 0.15		1.75 ± 0.22	
heart		0.43 ± 0.12		0.62 ± 0.02		0.74 ± 0.05
brain		0.06 ± 0.02		0.07 ± 0.01		0.21 ± 0.04

^a Values shown were calculated by dividing the % injected dose/g of tissue by % injected dose/g of blood in each animal, then the ratios were averaged and standard deviation was calculated for the sets of animals at each time point. ^b Group 1 results were obtained from *n* = 8 mice at each time point. ^c Group 2 results were obtained from *n* = 6 mice at each time point.

obtained in five *nca*-radioiodinations (I-125) were 0.39 mCi/μg (712 mCi/μmol), 0.36 mCi/μg (657 mCi/μmol), 0.32 mCi/μg (584 mCi/μmol), 0.29 mCi/μg (529 mCi/μmol), and 0.22 mCi/μg (402 mCi/μmol), which are 18–33% of the theoretical specific activities. Three additional *nca*-radioiodinations employing I-131 had somewhat lower specific activities [i.e. 0.19 mCi/μg (347 mCi/μmol), 0.13 mCi/μg (237 mCi/μmol), and 0.12 mCi/μg (219 mCi/μmol)]. These results indicate that the specific activity obtainable for the radioiodinated cobalamin derivatives is in the same range as commercially available [⁵⁷Co]cyanocobalamin, but specific activities that are 2–3 times that of [⁵⁷Co]cyanocobalamin may be obtained. Although the specific activities of [⁵⁷Co]cyanocobalamin and the radioiodinated cobalamins are obtained as microcuries per microgram, due to the differences in weights, comparisons of specific activities between cobalamins must be made on the basis of the amount of activity per mole.

Our previous studies of cobalamin derivatives had shown that conjugation at the *b*-, *d*-, and *e*-propionamide side groups of the corrin ring gave compounds that differed greatly in binding with rhTCII. Although we expected to obtain cobalamin derivatives in which the relative order of binding affinity could be predicted, it was important to determine the magnitude of their binding compared to that of [⁵⁷Co]cyanocobalamin. Therefore, rhTCII binding studies were conducted, and the results are shown in Figure 5. In the assay, 2 pmol of cyanocobalamin was required to decrease the binding of the tracer [⁵⁷Co]cyanocobalamin, **1**, by 50%. The *e*-isomer, **10**, bound nearly as well as cyanocobalamin. It

took only 4 pmol of the *e*-isomer, **10**, to obtain 50% binding of [⁵⁷Co]-**1**; thus, it is estimated that the binding affinity for **10** is 50% of cyanocobalamin. Similarly, it took 32 pmol of the *b*-isomer, **8**, to obtain 50% binding of [⁵⁷Co]-**1**, indicating that the binding is approximately 6% that of cyanocobalamin. The *d*-isomer, **9**, had the lowest binding, taking (an estimated) 152 pmol to obtain 50% binding of [⁵⁷Co]-**1**, indicating that its binding is only 0.7% that of cyanocobalamin. Importantly, the conjugation of iodohippurate to the cobalamin molecule did not appear to alter its binding characteristics as the rhTCII binding curves shown in Figure 5 for **8**–**10** are similar to those obtained with the parent monocarboxylic acids **2**–**4** and the trioxadiazine adducts **5**–**7** (28).

Two biodistributions of radioiodinated cobalamin **10** in athymic mice were conducted to assess the tissue localization and pharmacokinetics of this new cobalamin derivative. Although the first biodistribution study was not conceived to assess tumor localization, we chose to include a solid tumor RCC xenograft (TK-82) that has been studied extensively by members of our group (32, 36, 37) in an attempt to gain information about localization of **10** to that tumor xenograft. Since the quantity of injected cobalamin derivative was considered critical in the *in vivo* evaluations, high specific activity derivatives were prepared. On the basis of the specific activity and amount injected, in the first biodistribution (Table 2, group 1) approximately 26 ng of [¹³¹I]-**10** was administered per animal, and in the second biodistribution (Table 2, group 2) approximately 24 ng of [¹²⁵I]-**10** was administered per animal. A direct comparison with quantity

of [$^{131/125}$ I]-**10** with [^{57} Co]-**1** cannot be made as the molecular weight of [$^{131/125}$ I]-**10** is 1831/1825 and that of [^{57} Co]-**1** is 1353 (based on radionuclides used). Thus, an equivalent amount of [^{57} Co]-**1** would be 18–19 ng. With the small administered quantities, it was expected that all of the [^{131} I]-**10** would be bound by mouse TCII in the blood.

It is interesting to note the high levels in the kidney observed in both biodistributions, particularly at the early time points. These findings are consistent with those of previous investigators (using [^{57,60} Co]cyanocobalamin), where the TCII/cobalamin complex was found to be taken up more avidly by the kidney than any other tissue, including the liver (38–40). As previously suggested (33, 39, 41), the data indicate that the kidney may have a regulatory role in maintaining the concentration of cyanocobalamin as the high kidney concentrations at 1 h appear to be rapidly excreted in the urine. In another study, investigators who employed extraction techniques rather than measurement of radioactivity reported that high concentrations of vitamin B₁₂ were found in the hearts of rats when vitamin B₁₂ was not withheld from the diet (33). The data from this investigation do not indicate that particularly high concentrations of radioiodinated **10** were found in the heart, although the concentrations were 3–4 times that in muscle at the times evaluated. Due to the difficulties in obtaining pituitary glands from athymic mice, the amount of radioactivity in the whole brain was measured. Concentrations of radioiodinated **10** which were less than that in muscle were noted for the 1 and 4 h times. Tumor uptake and/or retention of radioiodinated **10** was examined in one biodistribution. The amount of radioactivity in the tumor did appear to be high. At 1 h tumor concentration was less than that in blood but was 7 times that of muscle. Although the concentration of [^{131} I]-**10** dropped significantly as the blood concentration dropped, at 24 h the tumor concentration was nearly 2 times that of blood and over 6 times muscle concentration. It is difficult to draw conclusions about tumor uptake or retention from these preliminary data. Additional studies that employ less lipophilic radioiodinated derivatives and derivatives which have lower binding with TCII are being considered to gain a better understanding of the tumor localization/retention results obtained.

The concern about the *in vivo* stability of the radioiodine label raised by results from the first biodistribution was alleviated in the second biodistribution. It seems likely that, even though the preparation was isolated from the HPLC as a peak distant from where free iodide elutes (solvent front), there must have been some iodide adhering to the cobalamin molecule, or radioiodide was introduced through contamination. The application of the PRP HPLC column appears to have diminished this problem, as no free iodide has been observed in any preparation obtained from that column.

In summary, a method of radioiodination of cobalamin has been developed. To our knowledge, the described examples are the first to be reported. Importantly, one radioiodinated cobalamin conjugate binds with TCII with an affinity similar to that of [^{57} Co]cyanocobalamin and is stable *in vitro* and *in vivo*. However, comparisons with natural cobalamins should not be drawn from these experiments as the radioiodinated cobalamin derivative **10** is structurally different from the native compounds. A planned experiment will compare the *in vivo* distribution of [^{125} I]-**10** with [^{57} Co]-**1**. Other planned studies include radioiodination of cobalamin conjugates that have other moieties attached. However, it would seem that there may be other applications for the radioiodinated

compounds described here. For instance, they may be of value in evaluation of deficiencies of human cobalamin transport and metabolism (42). Different methods of assaying for cobalamins have been described (43–46), but methods employing direct measurement with radioisotopes appear to be the easiest to perform. One notable application is the Schilling test, which might be conducted as a dual isotope evaluation (46), and the requisite radiolabeled cobalamins may be more readily obtained with iodine-125/iodine-131 than those radiolabeled with cobalt-57/cobalt-58. Also, since some of the corrin ring side-chain isomers have considerably lower binding with TCII (and presumably IF), evaluations of specific versus nonspecific binding in the measured absorption, distributions, and metabolism might be possible.

ACKNOWLEDGMENT

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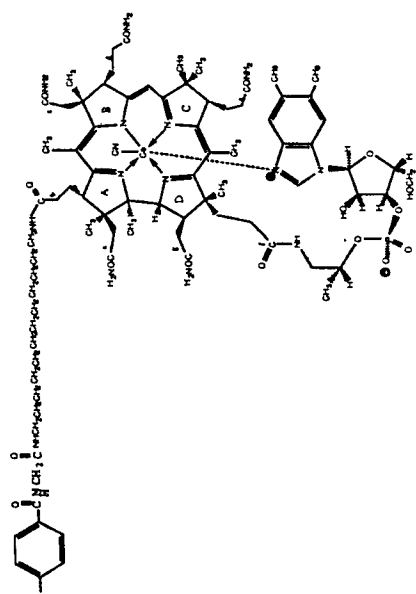
Supporting Information Available: HPLC chromatograms and ^1H NMR spectra of purified cyanocobalamin–iodohippurate conjugates (**8–10**) and cyanocobalamin–stannyl hippurate conjugates (**11–13**) and of hippurates **16–18** (18 pages). Ordering information is given on any current masthead page.

LITERATURE CITED

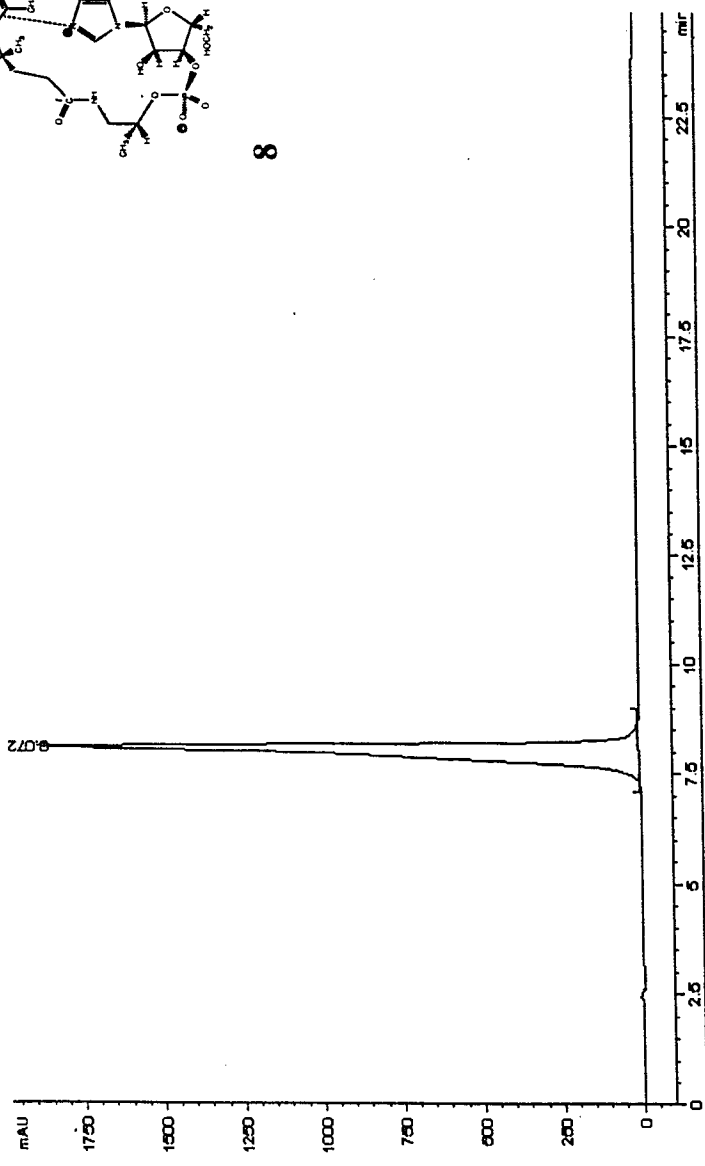
- (1) Kroes, A. C., Ermens, A. A., Lindemans, J., and Abels, J. (1986) Effects of 5-fluorouracil treatment of rat leukemia with concomitant inactivation of cobalamin. *Anticancer Res.* 6, 737–742.
- (2) Kroes, A. C., Lindemans, J., Schoester, M., and Abels, J. (1986) Enhanced therapeutic effect of methotrexate in experimental rat leukemia after inactivation of cobalamin (vitamin B₁₂) by nitrous oxide. *Cancer Chemother. Pharmacol.* 17, 114–120.
- (3) Kroes, A. C., Lindemans, J., and Abels, J. (1984) Synergistic growth inhibiting effect of nitrous oxide and cycloleucine in experimental rat leukaemia. *Br. J. Cancer* 50, 793–800.
- (4) Eastwood, D. W., Green, C. D., Lambdin, M. A., and Gardner, R. (1963) Effect of nitrous oxide on the white-cell count in leukemia. *New Engl. J. Med.* 268, 297–299.
- (5) Seetharam, B. (1994) Gastrointestinal absorption and transport of cobalamin (vitamin B₁₂). In *Physiology of the Gastrointestinal Tract* (L. R. Johnson, Ed.) pp 1997–2026, Raven Press, New York.
- (6) Hall, C. A. (1990) The role of transcobalamin II in the cellular uptake of cobalamin. In *Biomedicine and Physiology of Vitamin B₁₂* (J. C. Linnell, and H. R. Bhatt, Eds.) pp 239–253, Children's Medical Charities, London.
- (7) Anderson, R. C., and Delabarre, Y. (1951) The preparation of radioactive vitamin B₁₂ by direct neutron irradiation. *J. Am. Chem. Soc.* 73, 4051–4052.
- (8) Smith, E. L., Hockenhull, J. D., and Quilter, A. R. J. (1952) Tracer studies with the B₁₂ vitamins. 2. Biosynthesis of vitamin B₁₂ labelled with ^{60}Co and ^{32}P . *Biochem. J.* 52, 387–388.
- (9) Maddock, A. G., and Coelho, F. P. (1954) The retention of cobalt-60 in vitamin B₁₂. *J. Chem. Soc.*, 4702–4704.
- (10) Bradley, J. E., Smith, E. L., Baker, S. J., and Mollin, D. L. (1954) The use of the radioactive isotope of cobalt Co⁵⁸ for the preparation of labelled vitamin B₁₂. *Lancet* 267, 476–477.
- (11) Smith, E. L. (1952) Tracer studies with the B₁₂ vitamins. 1. Neutron irradiation of vitamin B₁₂. *Biochem. J.* 52, 384–387.

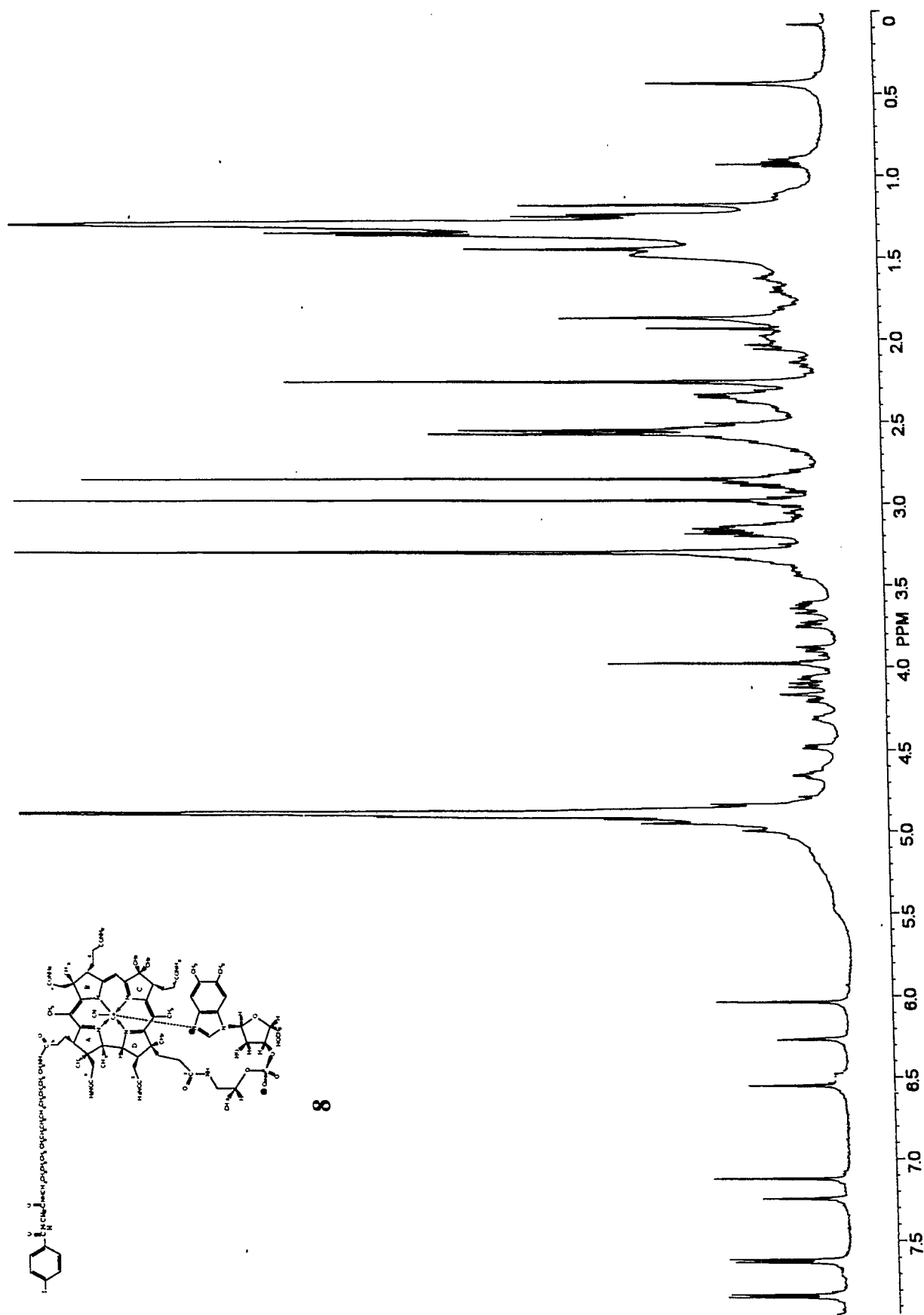
- (12) Smith, E. L., Fantes, K. H., Ball, S., Waller, J. G., Emery, W. B., Anslow, W. K., and Walker, A. D. (1952) B₁₂ vitamins (cobalamins). 1. Vitamins B_{12c} and B_{12d}. *Biochem. J.* 52, 389–395.
- (13) Rosenblum, C., and Woodbury, D. T. (1951) Cobalt 60 labeled vitamin B₁₂ of high specific activity. *Science* 113, 215.
- (14) Chalet, L., Rosenblum, C., and Woodbury, D. T. (1950) Biosynthesis of radioactive vitamin B₁₂ containing cobalt⁶⁰. *Science* 111, 601–602.
- (15) Boxer, G. E., Rickards, J. C., Rosenblum, C., and Woodbury, D. T. (1951) Preparation of C¹⁴-labeled vitamin B₁₂ (C¹⁴-cobalamin). *Arch. Biochem.* 30, 470–471.
- (16) Toraya, T., Ohashi, K., Ueno, H., and Fukui, S. (1975) Preparation, properties and biological activities of succinyl derivatives of vitamin B₁₂. *Bioinorg. Chem.* 4, 245–255.
- (17) Smith, E. L., Ball, S., and Ireland, D. M. (1952) B₁₂ vitamins (cobalamins). 2. Neutral, basic, and acidic cobalamins. *Biochem. J.* 52, 395–400.
- (18) Wagner, F. (1965) Reactions of the cyano and alkyl cobalamins. *Proc. R. Soc. London A* 288, 344–347.
- (19) Dolphin, D., Johnson, A. W., and Shaw, N. (1963) Sulphitocobalamin. *Nature* 199, 170–171.
- (20) Bonnett, R., Cannon, J. R., Clark, V. M., Johnson, A. W., Parker, L. F. J., Smith, E. L., and Todd, S. A. (1957) Chemistry of the vitamin B₁₂ group. Part V. The structure of the chromophoric grouping. *J. Chem. Soc.*, 1158–1168.
- (21) Gossauer, A., Heise, K.-P., Laas, H., and Inhoffen, H. H. (1976) Über die Chlorierung von Derivaten des Cobyrynsäureheptamethylesters (Contributions to the knowledge of the corrin chromophore. II. Chlorination of cobyrinic acid heptamethyl ester derivatives). *Liebigs Ann. Chem.*, 1150–1165.
- (22) Wilbur, D. S. (1992) Radiohalogenation of proteins: an overview of radionuclides, labeling methods, and reagents for conjugate labeling. *Bioconjugate Chem.* 3, 433–470.
- (23) Kabalka, G. W., and Varma, R. S. (1989) The synthesis of radiolabeled compounds via organometallic intermediates. *Tetrahedron* 45, 6601–6621.
- (24) Bonnett, R. (1982) Reactions of the corrin macrocycle. B₁₂: Vol. 1—Chemistry (D. Dolphin, Ed.) pp 201–243, Wiley, New York.
- (25) Morley, C. G. D., Blakley, R. L., and Hogenkamp, H. P. C. (1968) Analogs of deoxyadenosylcobalamin with alterations in a side chain of the corrin ring. *Biochemistry* 7, 1231–1239.
- (26) Kenley, J. S., Leighton, M., and Bradbeer, C. (1978) Transport of vitamin B₁₂ in *Escherichia coli*. Corrinoid specificity of the outer membrane receptor. *J. Biol. Chem.* 253, 1341–1346.
- (27) Toraya, T., Krodell, E., Mildvan, A. S., and Abeles, R. H. (1979) Role of peripheral side chains of vitamin B₁₂ coenzymes in the reaction catalyzed by dioldehydrase. *Biochemistry* 18, 417–426.
- (28) Pathare, P. M., Wilbur, D. S., Heusser, S., Quadros, E. V., McLoughlin, P., and Morgan, A. C. (1996) Synthesis of cobalamin-biotin conjugates which vary in position of coupling. An evaluation of the location of conjugated groups on transcobalamin II binding. *Bioconjugate Chem.* 7, 217–232.
- (29) Quadros, E. V., Sai, P., and Rothenberg, S. P. (1993) Functional human transcobalamin II isoproteins are secreted by insect cells using the Baculovirus expression system. *Blood* 81, 1239–1245.
- (30) Quadros, E. V., Rothenberg, S. P., Pan, Y. Ch. E., and Stein, S. (1986) Purification and molecular characterization of human transcobalamin II. *J. Biol. Chem.* 261, 15455–15460.
- (31) Lau, K. S., Gottlieb, C., Wasserman, L. R., and Herbert, V. (1965) Measurement of serum vitamin B₁₂ level using radioisotope dilution and coated charcoal. *Blood* 26, 202–214.
- (32) Vessella, R. L., Alvarez, V., Chiou, R.-K., Rodwell, J., Elson, M., Palme, D., Shafer, R., and Lange, P. (1987) Radioimmunoassay and radioimmunotherapy of renal cell carcinoma xenografts. *NCI Monogr. No. 3*, 159–167.
- (33) Dryden, L. P., and Hartman, A. M. (1966) Relative concentration of vitamin B₁₂ in the organs of the male rats as affected by its intake of the vitamin. *J. Nutr.* 90, 382–386.
- (34) Yamada, R., and Hogenkamp, H. P. C. (1972) The synthesis of a 5'-deoxyadenosylcobalamin-agarose adsorbent and its utility in the purification of ribonucleotide reductase. *J. Biol. Chem.* 247, 6266–6270.
- (35) Hamlin, D. K., Wilbur, D. S., Stray, J. E., Chitnis, A., Buhler, K. R., Daniel, J., and Vessella, R. L. (1995) Synthesis and evaluation of *para*-iodohippuric acid tetrafluorophenyl ester for radioiodination of antibodies. *J. Labelled Compds. Radiopharm.* 37, 557–559.
- (36) Wilbur, D. S., Vessella, R. L., Stray, J. E., Goffe, D. K., Blouke, K. A., and Atcher, R. W. (1993) Preparation and evaluation of *para*-[²¹¹At]astatobenzoyl labeled anti-renal cell carcinoma antibody A6H F(ab')₂. *In vivo* distribution comparison with *para*-[¹²⁵I]iodobenzoyl labeled A6H F(ab')₂. *Nucl. Med. Biol.* 20, 917–927.
- (37) Wilbur, D. S., Stray, J. E., Hamlin, D. K., Curtis, D. K., and Vessella, R. L. (1994) Monoclonal antibody Fab' fragment cross-linking using equilibrium transfer alkylation reagents. A strategy for site-specific conjugation of diagnostic and therapeutic agents with F(ab')₂ fragments. *Bioconjugate Chem.* 5, 220–235.
- (38) Salmi, H. A., and Lindgren, I. (1963) The localization of radiovitamin B₁₂ ⁵⁷Co in rat kidney. *Acta Physiol. Scand.* 213, 137.
- (39) Okuda, K. (1962) Relationship between intake of vitamin B₁₂ and its storage by the kidney in the rat. *J. Nutr.* 77, 131–136.
- (40) Okuda, K. (1962) Starvation and renal uptake of vitamin B₁₂. *Nature* 195, 292–293.
- (41) Scott, J. S. D., Treston, A. M., Bowman, E. P. W., Owens, J. A., and Cooksley, W. G. E. (1984) The regulatory roles of liver and kidney in cobalamin (vitamin B₁₂) metabolism in the rat: the uptake and intracellular binding of cobalamin and the activity of the cobalamin-dependent enzymes in response to varying cobalamin supply. *Clin. Sci.* 67, 299–306.
- (42) Beck, W. S. (1975) Metabolic features of cobalamin deficiency in man. *Cobalamin: Biochemistry and Pathophysiology* (B. M. Babior, Ed.) pp 403–450, Wiley, New York.
- (43) Skeggs, H. R. (1967) Vitamin B₁₂. *The Vitamins: Chemistry, Physiology, Pathology, Methods* (P. György and W. N. Pearson, Eds.) pp 277–301, Academic Press, New York.
- (44) Arnaud, J., Cotisson, A., Meffre, G., Bourgeay-Causse, M., Augert, C., Favier, A., Vuillez, J.-P., and Ville, G. (1994) Comparison of three commercial kits and a microbiological assay for the determination of vitamin B₁₂ in serum. *Scand. J. Clin. Lab. Invest.* 54, 235–240.
- (45) Lau, K.-S., Gottlieb, C., Wasserman, L. R., and Herbert, V. (1965) Measurement of serum vitamin B₁₂ level using radioisotope dilution and coated charcoal. *Blood* 26, 202–214.
- (46) Krynyckyi, B. R., and Zuckier, L. S. (1995) Accuracy in using dual-isotope Schilling test to measure urine samples: a multicenter study. *J. Nucl. Med.* 36, 1659–1665.

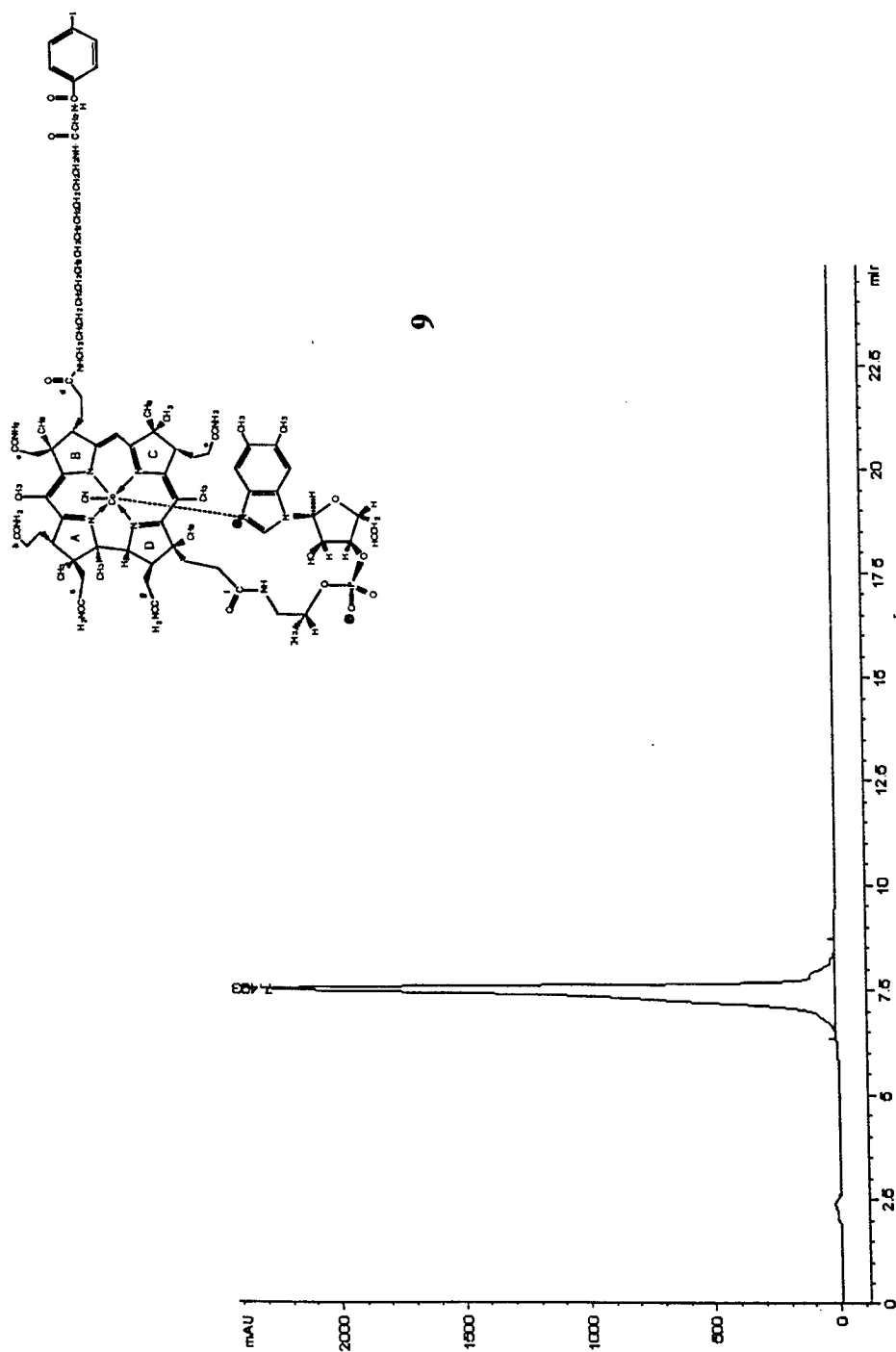
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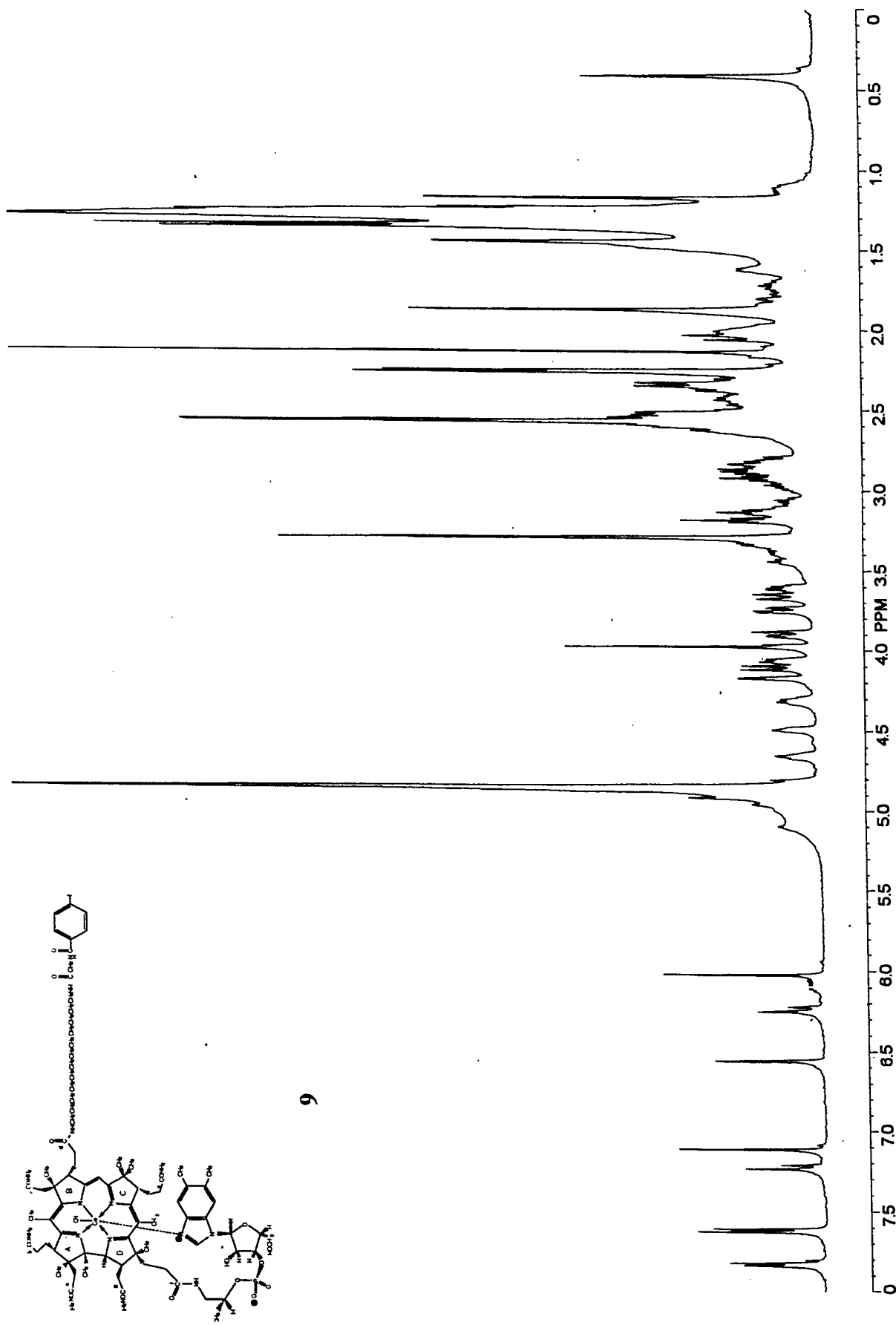
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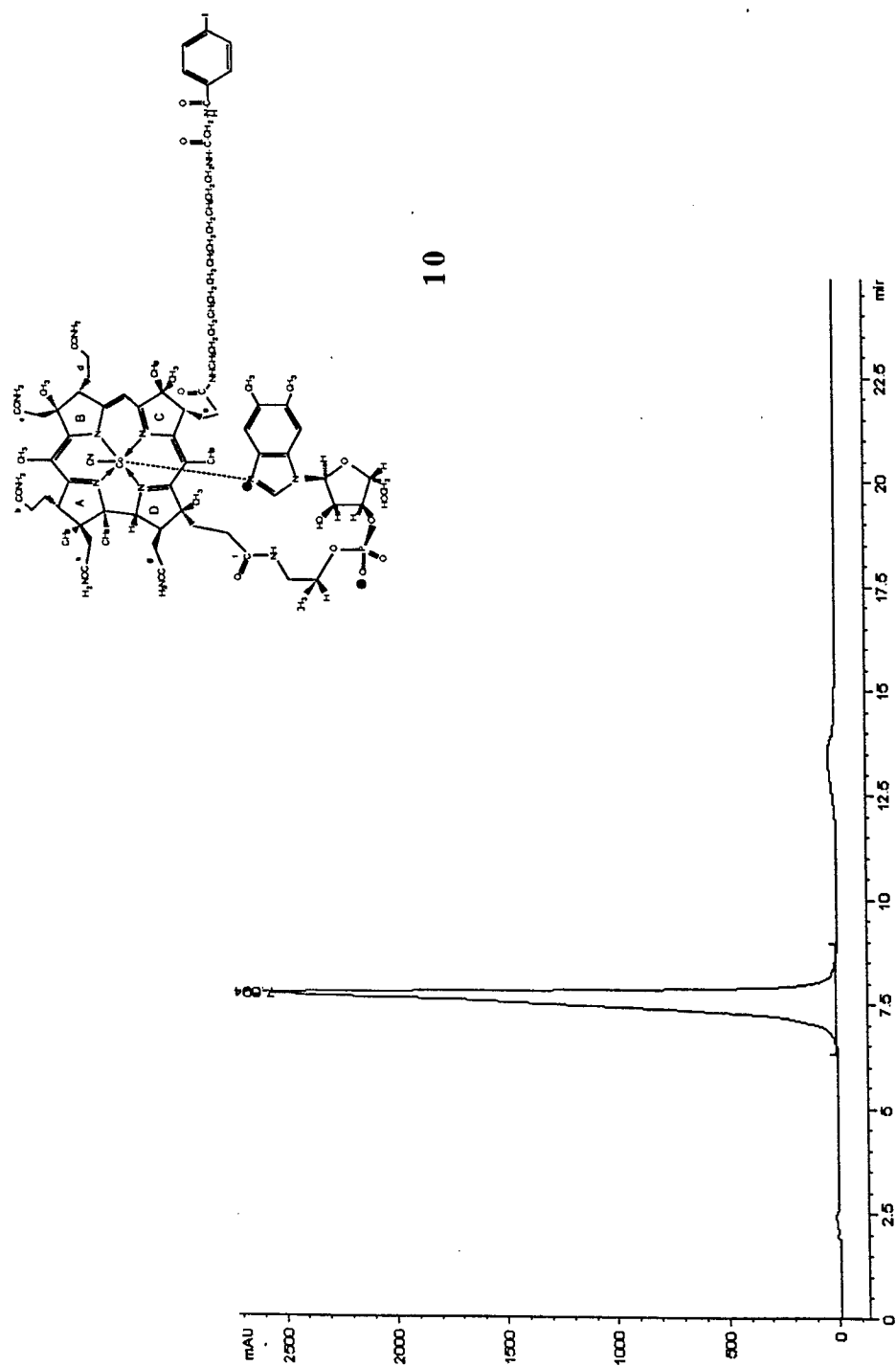


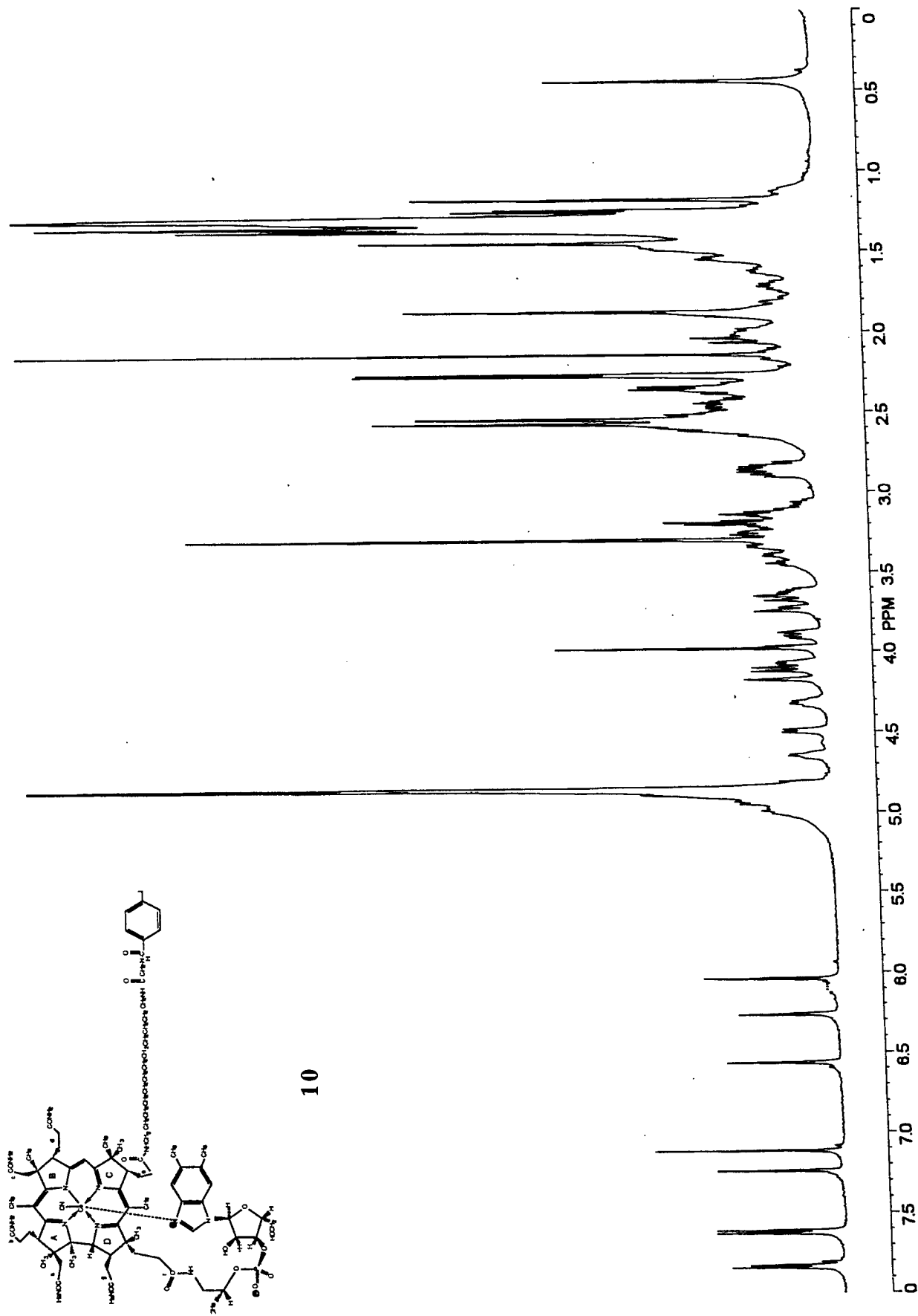


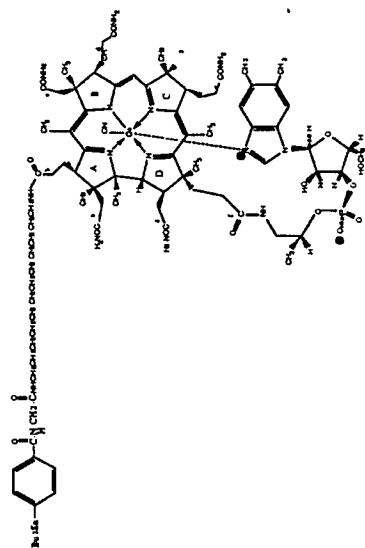


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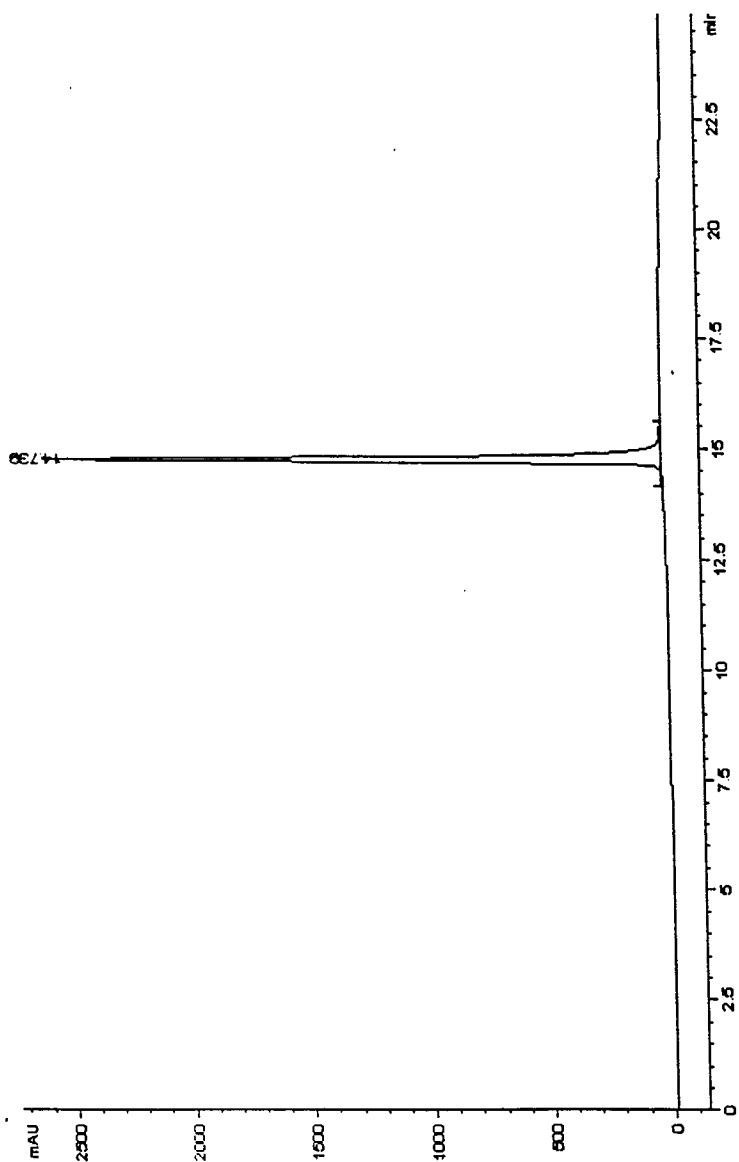


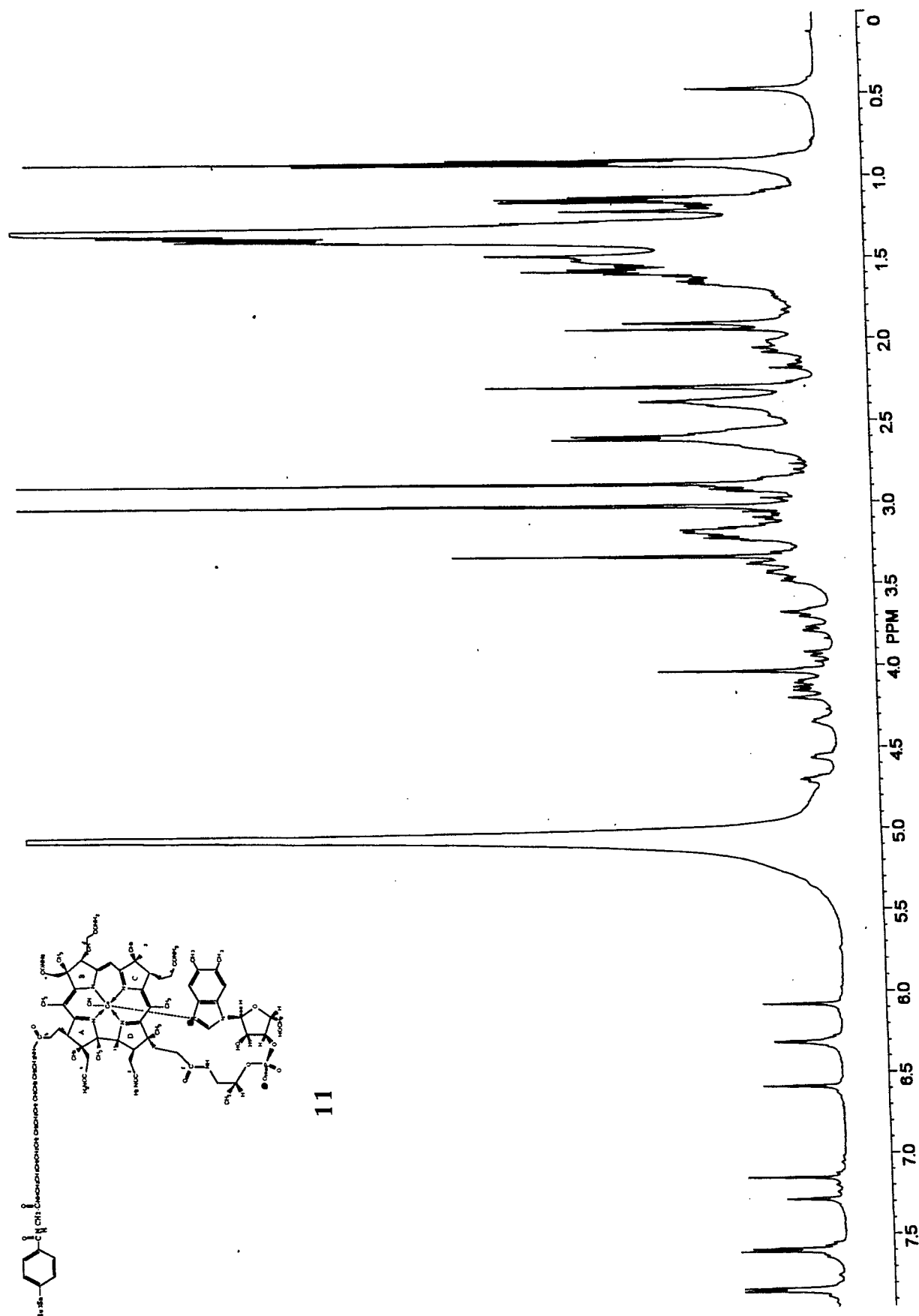




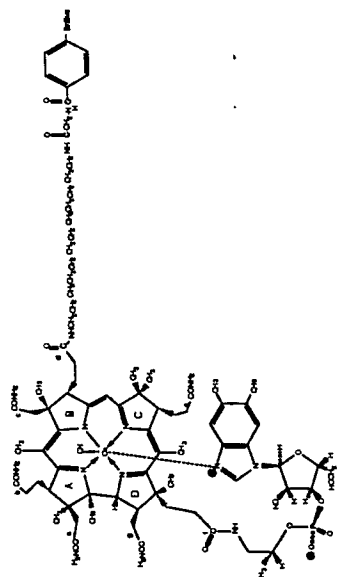


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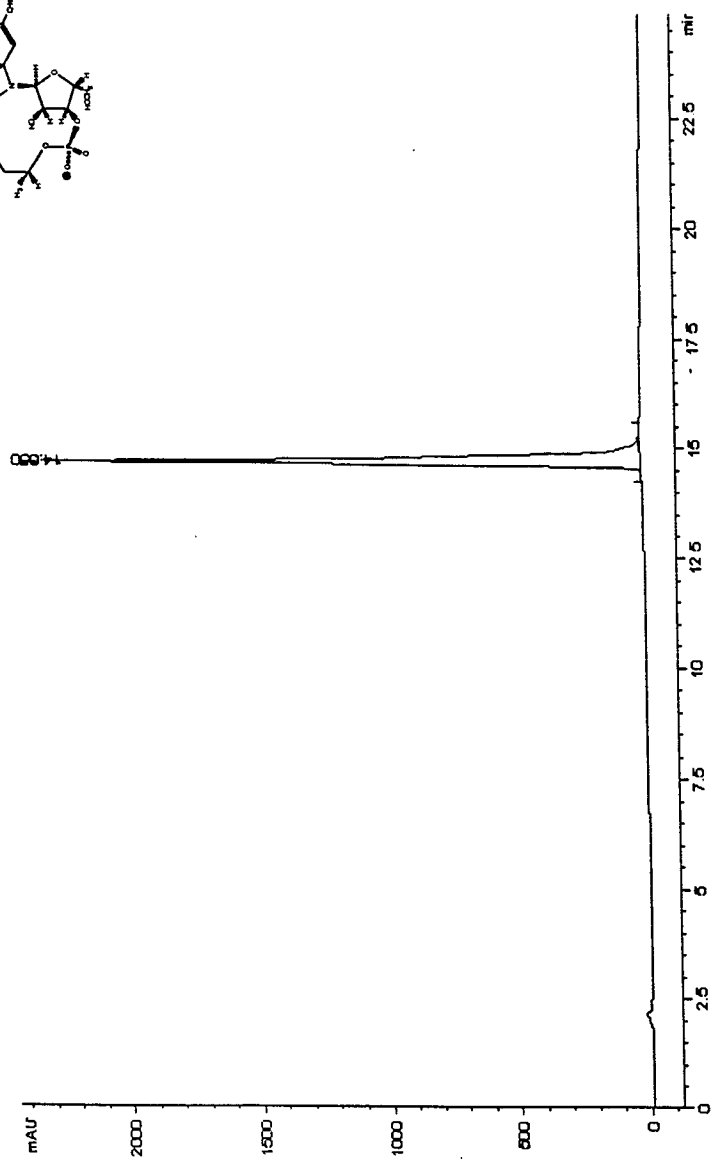


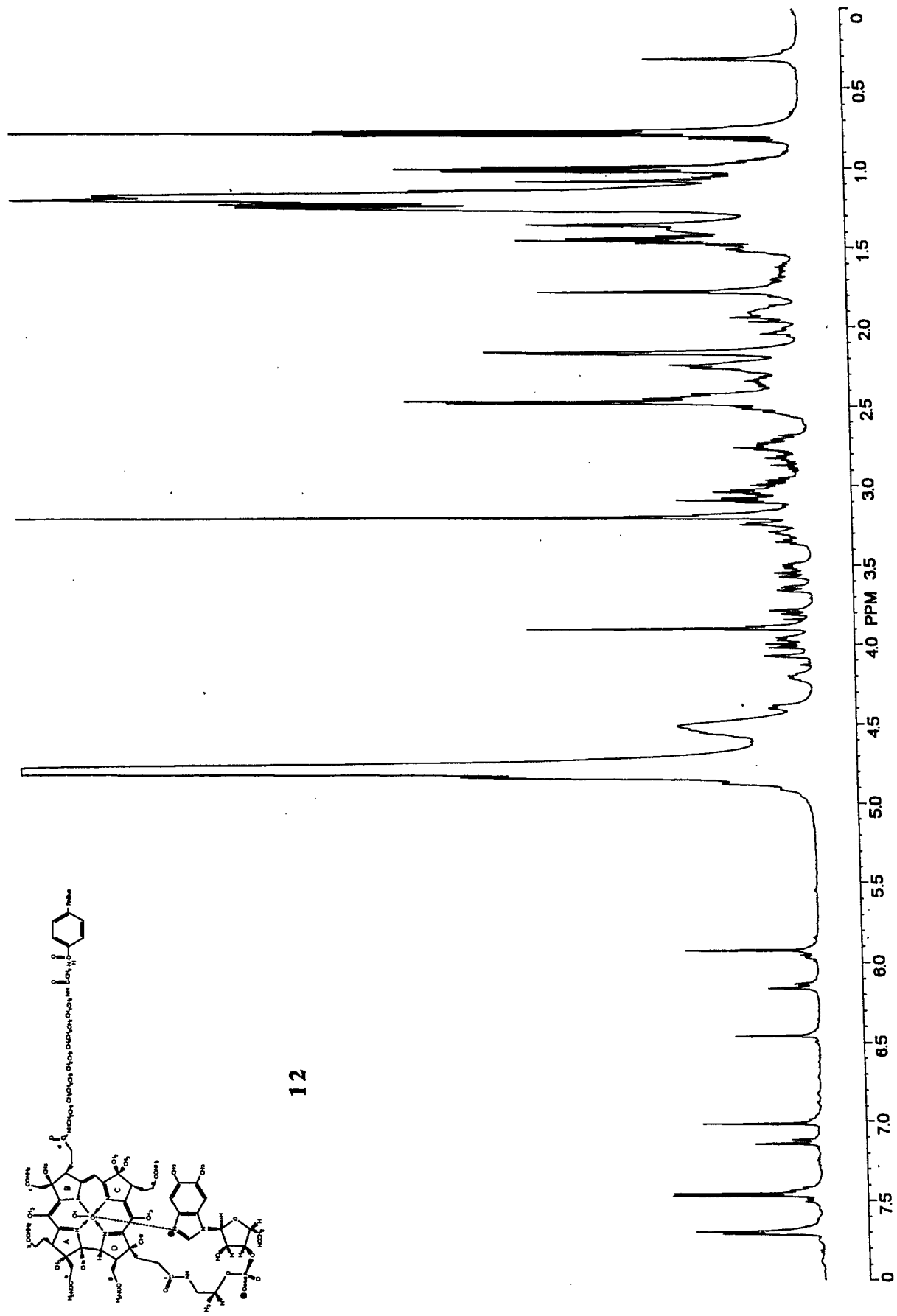


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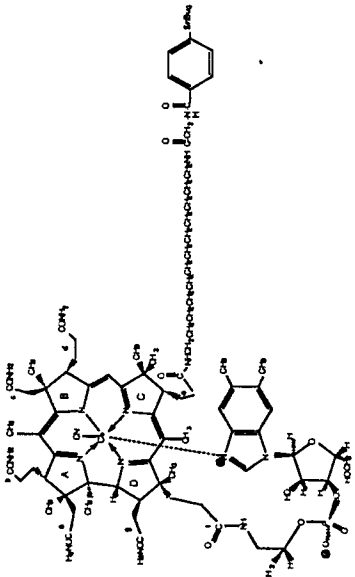


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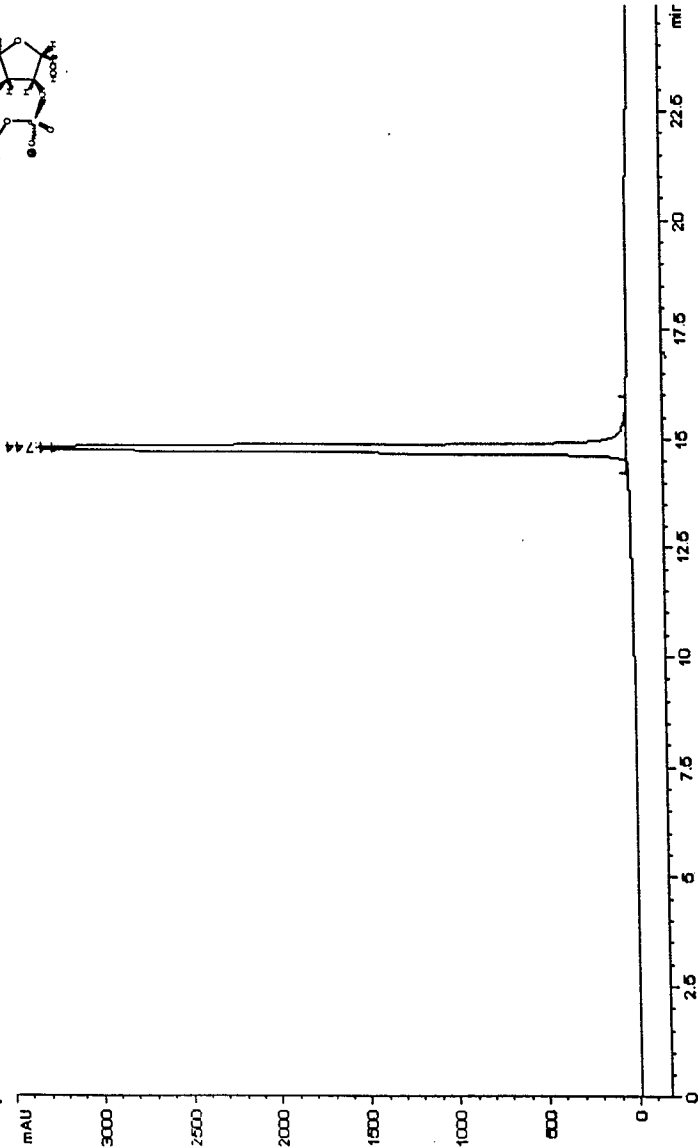


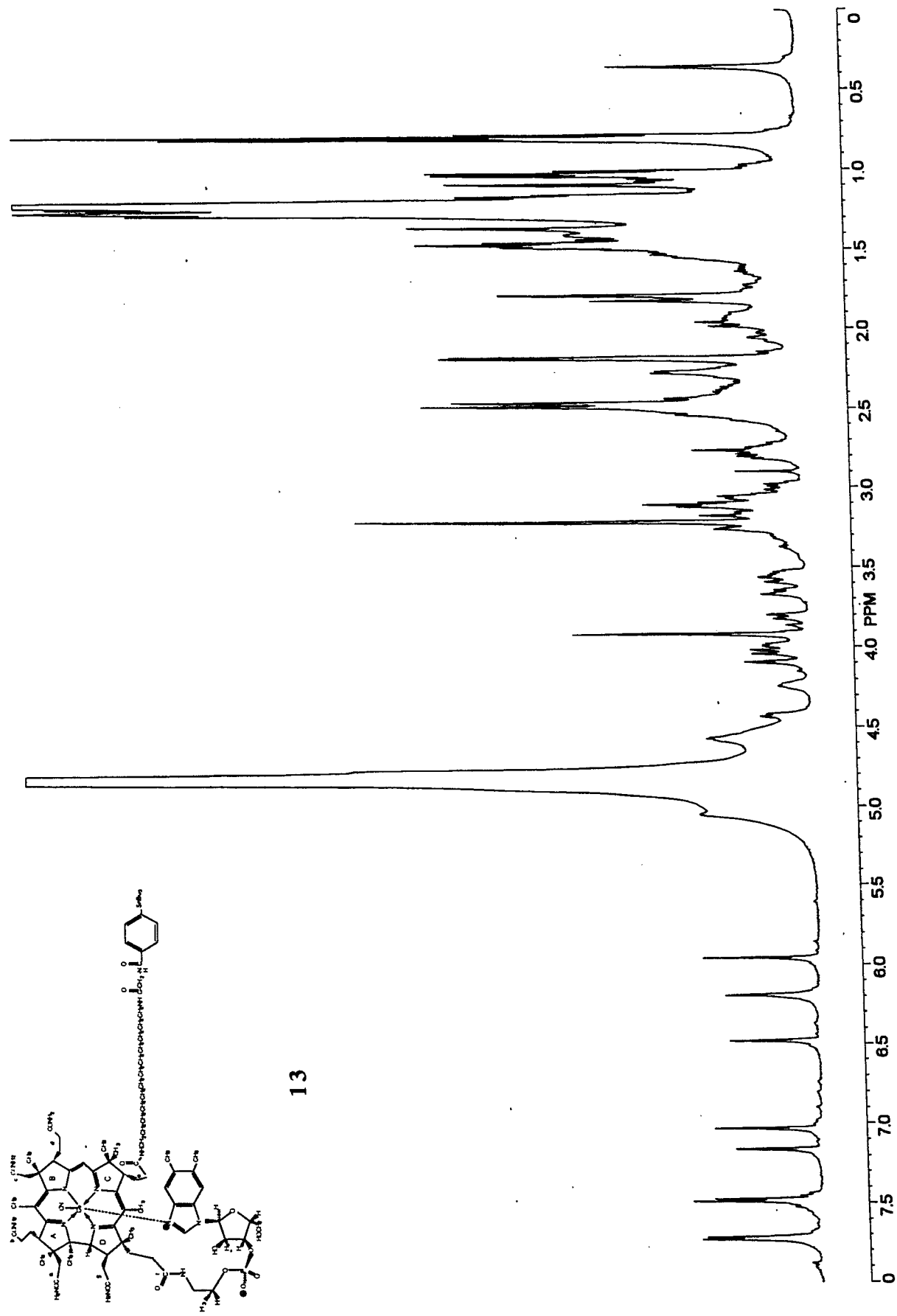


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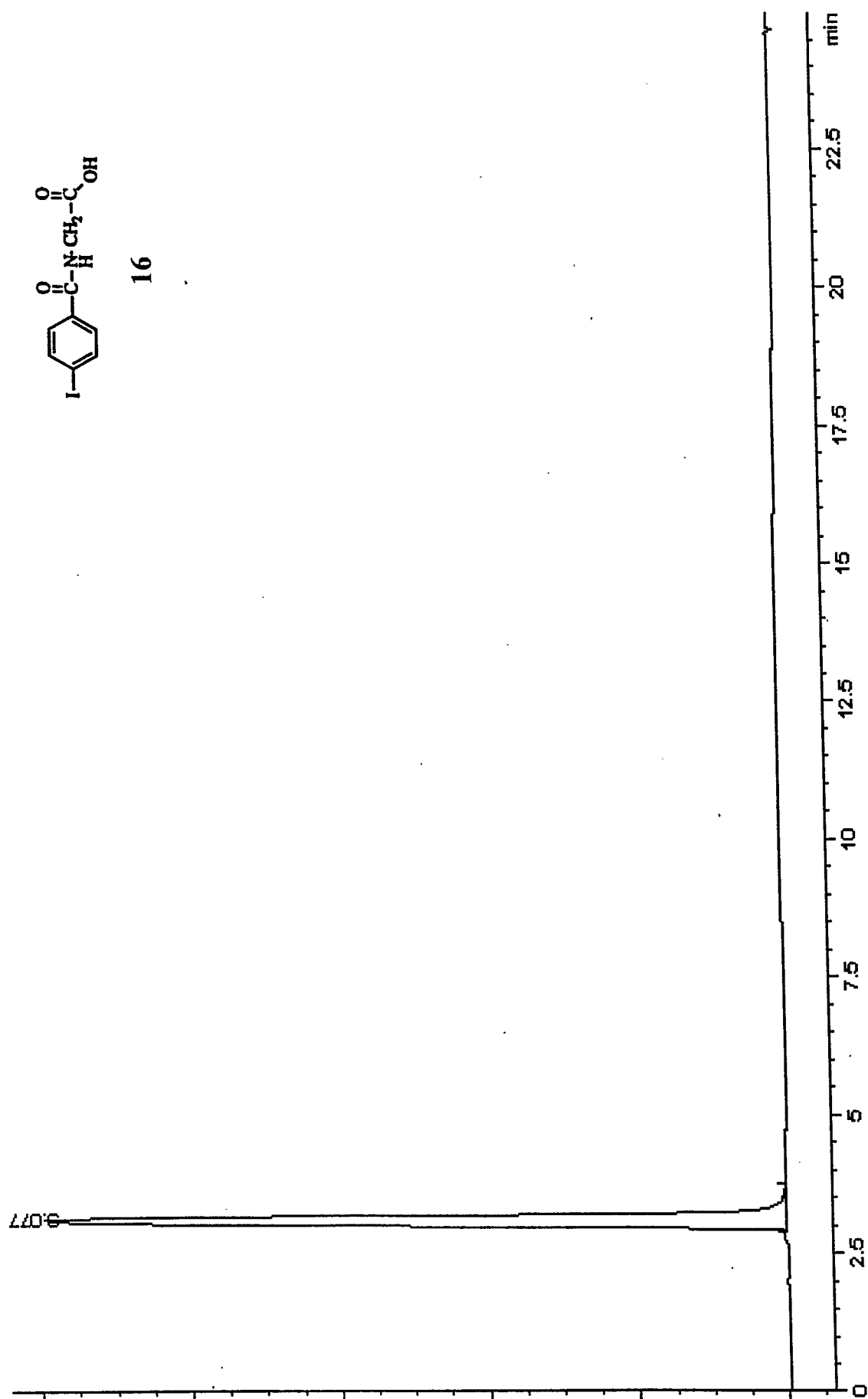


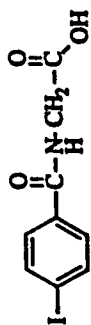
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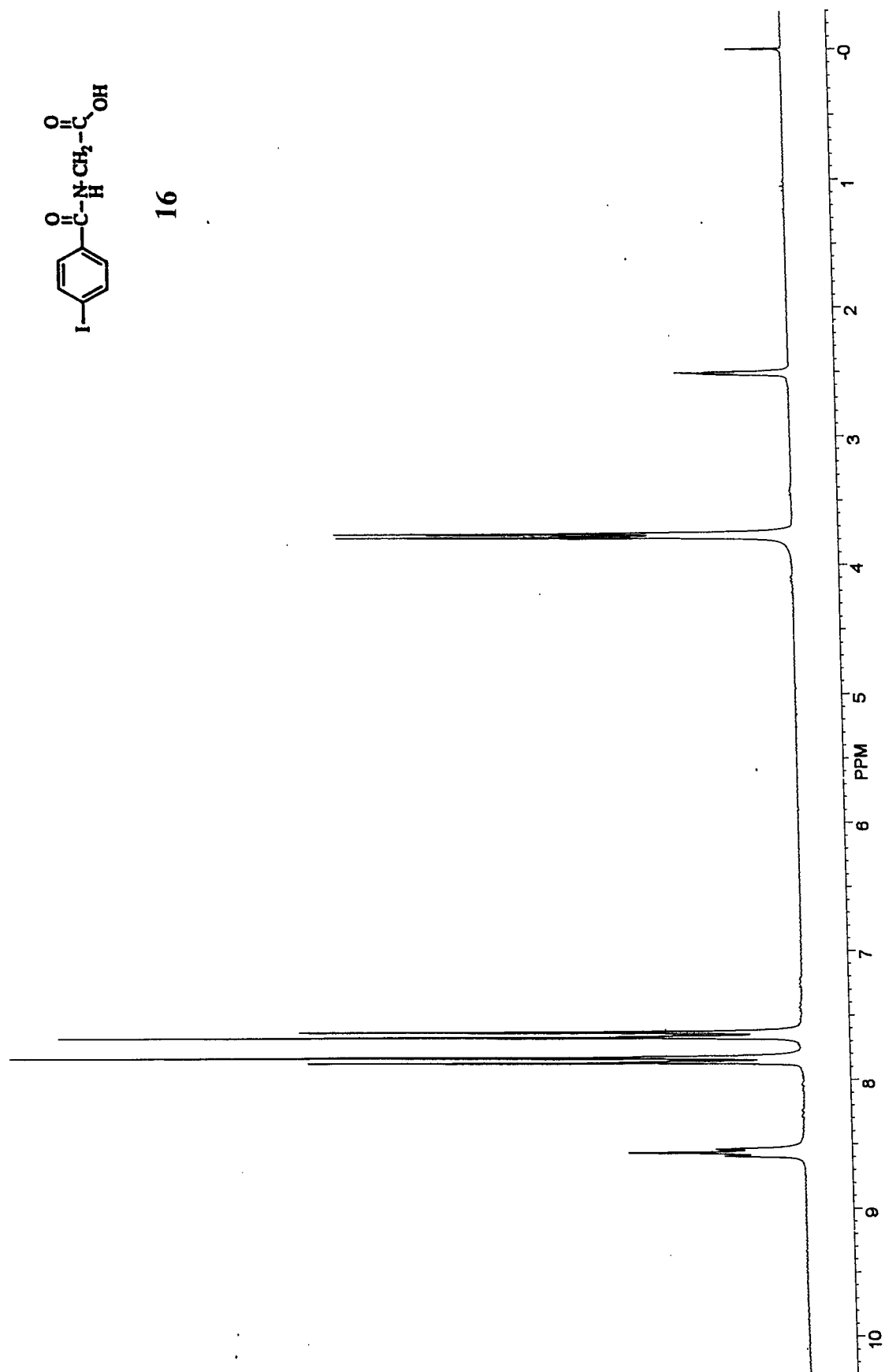


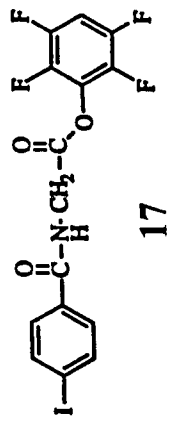
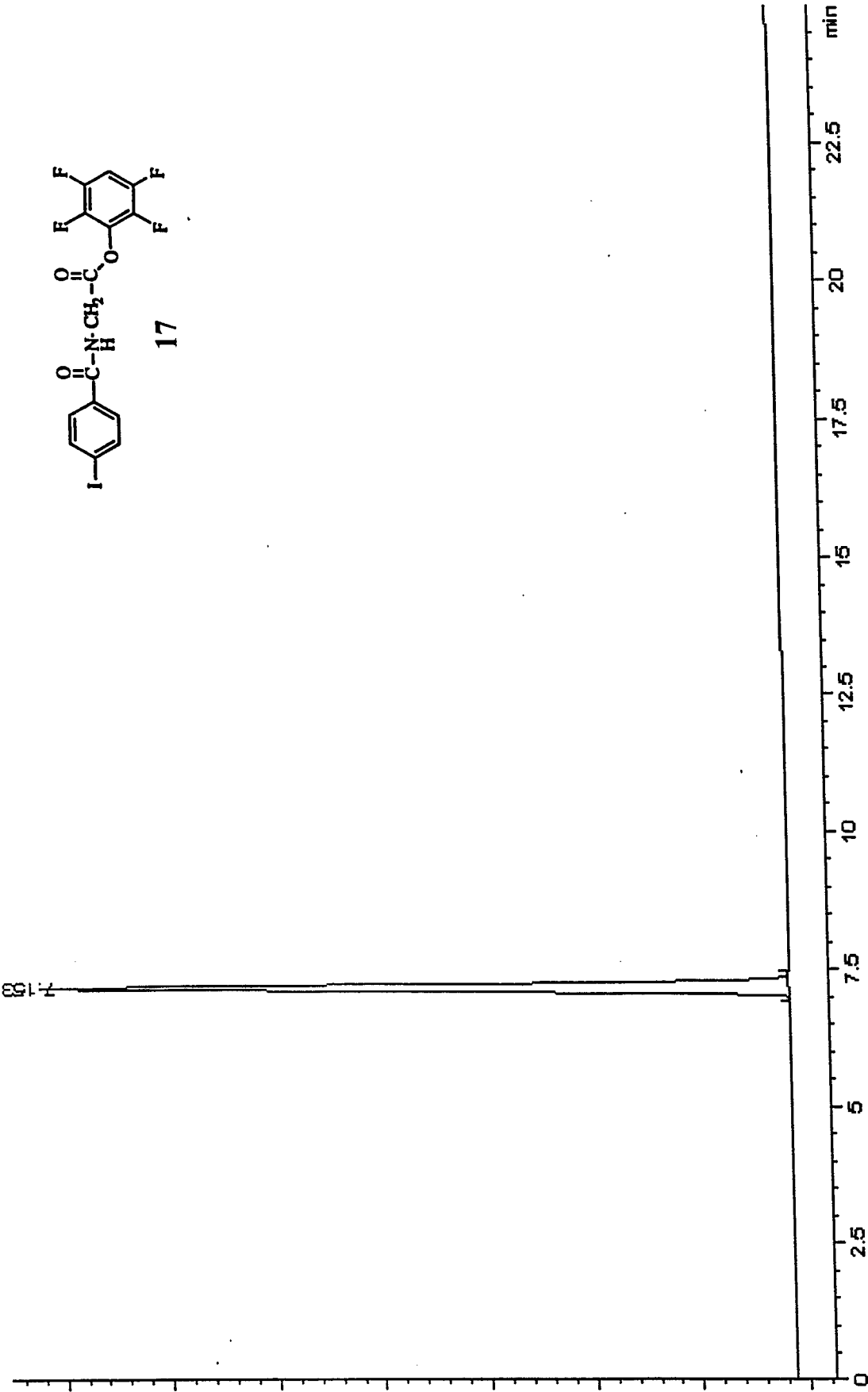
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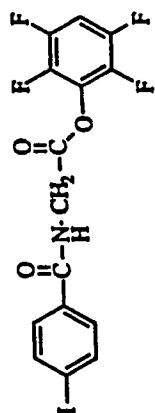




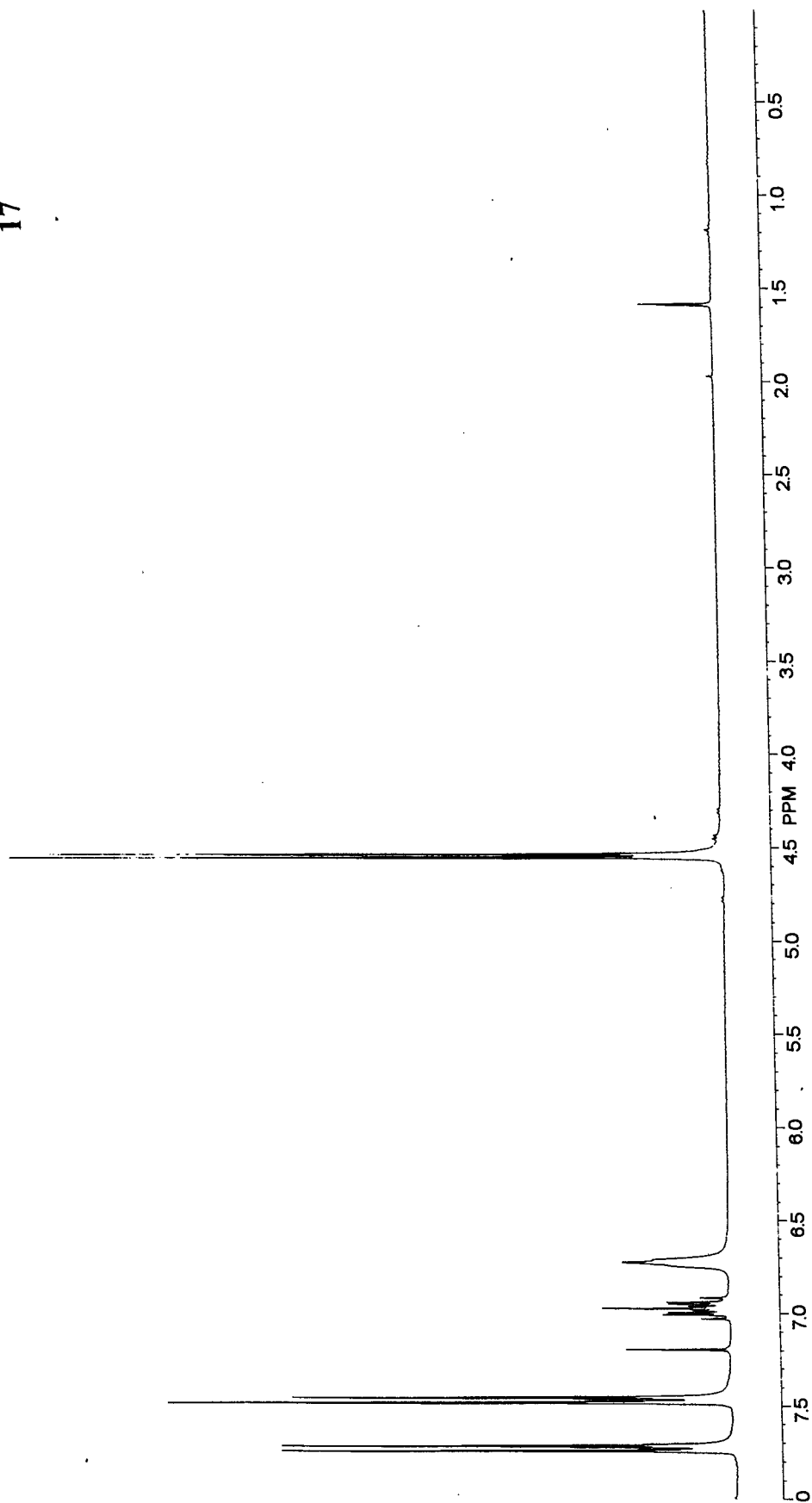
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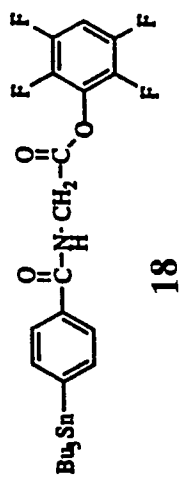
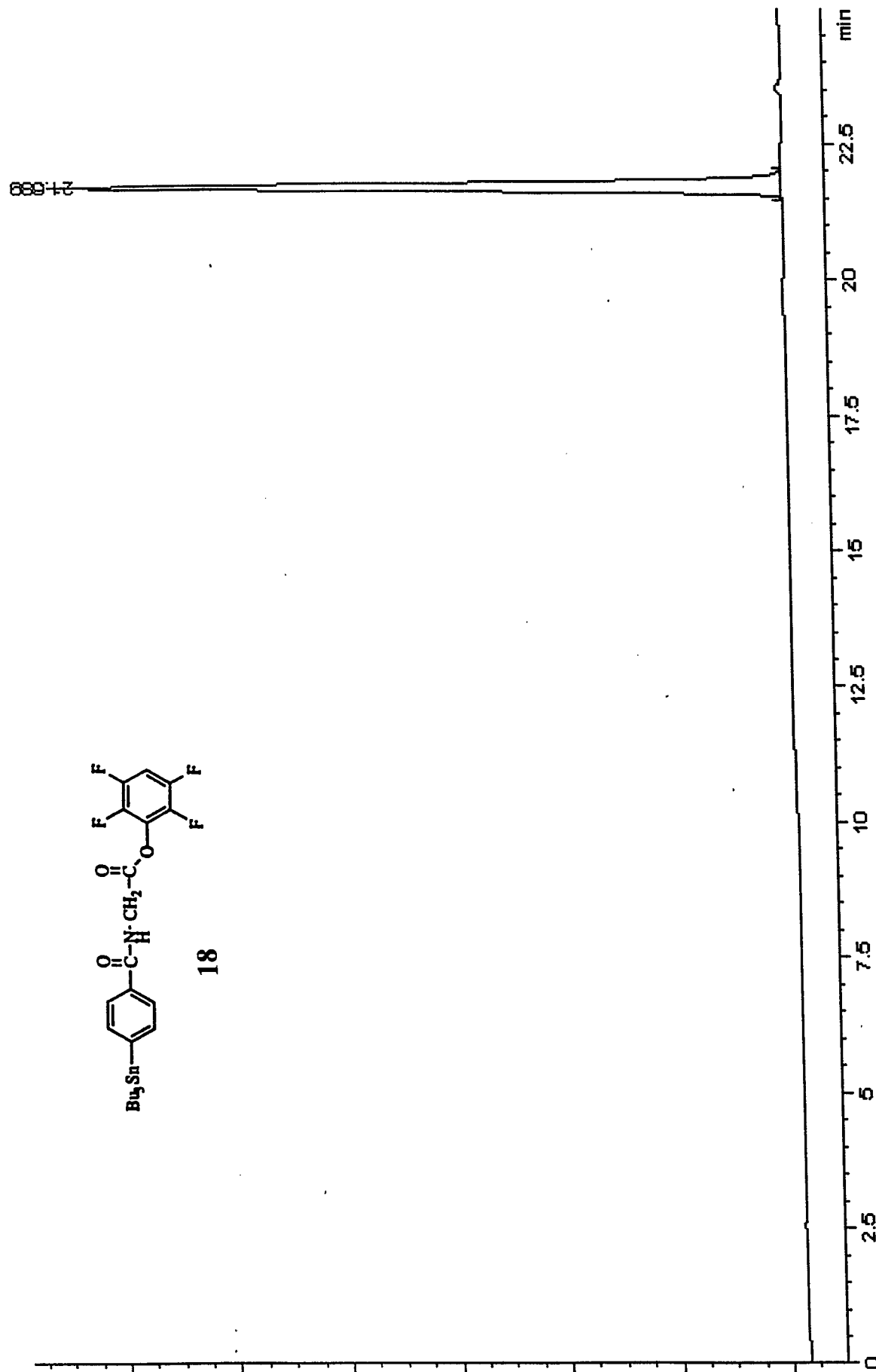


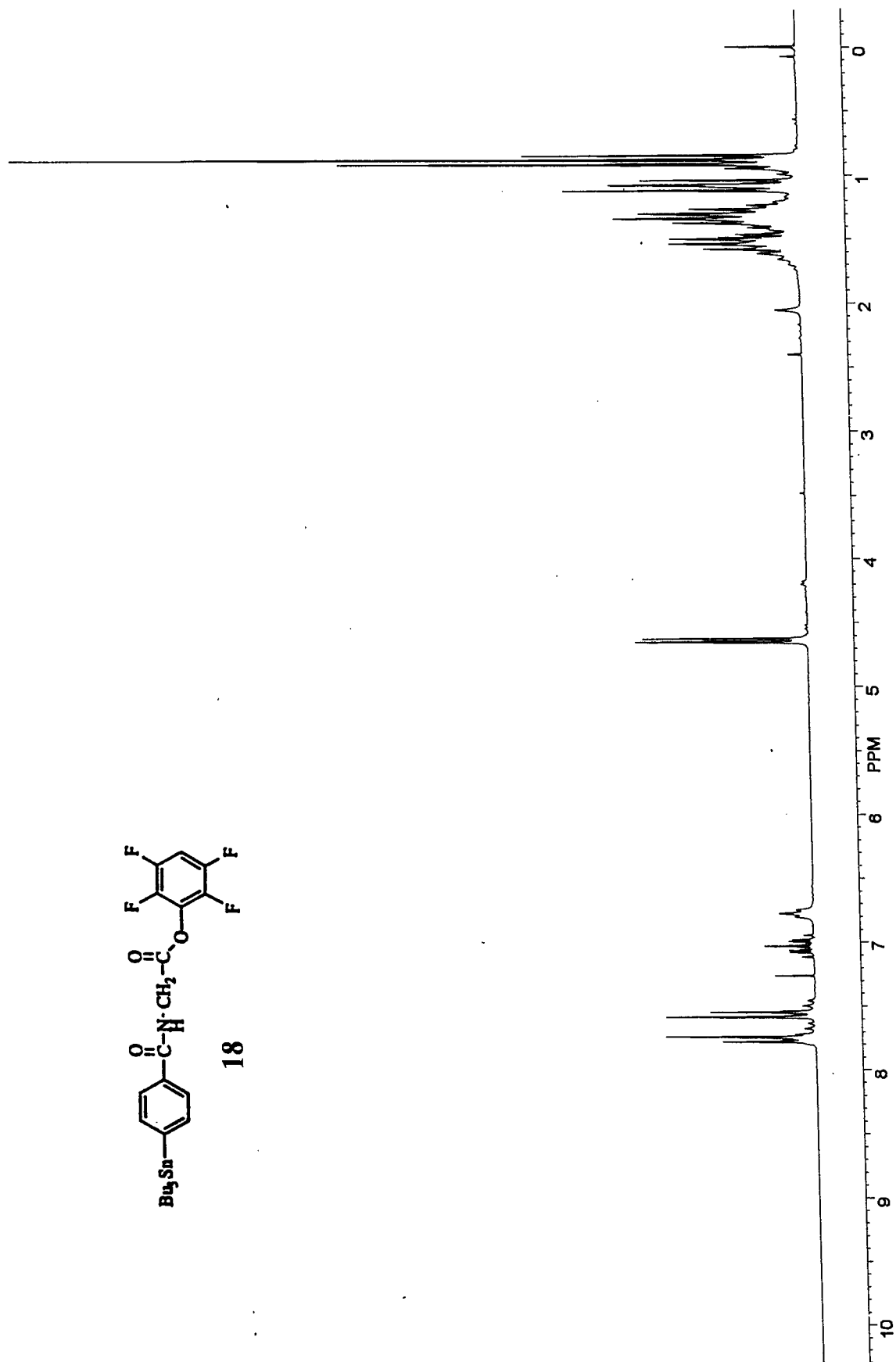
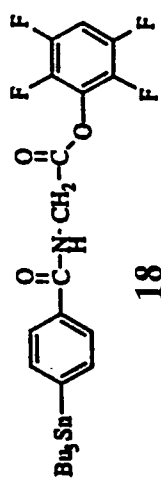




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Characterization of Protein–Hapten Conjugates. 2. Electrospray Mass Spectrometry of Bovine Serum Albumin–Hapten Conjugates[†]

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Fifteen hapten–bovine serum albumin (BSA) conjugates were prepared from five commercially available activated haptens. Each hapten was coupled to BSA at three different ratios. The conjugates were characterized by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) and two mass spectrometry (MS) methods: matrix-assisted laser desorption ionization (MALDI) and liquid chromatography–electrospray ionization (LC–ESI). SDS–PAGE was useful in detecting protein cross-linking, but not in assessing hapten density. MALDI-MS and LC–ESI-MS gave comparable qualitative results, but LC–ESI-MS provided a clearer representation of the distribution of hapten–protein species present in the conjugates. Conjugate species substituted with up to 25 haptens per BSA were recorded by LC–ESI-MS.

INTRODUCTION¹

The chemical derivatization of proteins to form bioconjugates is an extremely important process in the field of biotechnology. Still, the characterization of such bioconjugates is a challenging problem. Several methods have been used to estimate the degree of protein derivatization. Direct UV absorbance measurements have been used when the ϵ_{max} of the introduced chromophore is distinct from that of the protein (1). UV difference spectroscopy provided an alternative method when there was spectral overlap (2). When the derivatizing agent is doped with a radiolabeled analog, the extent of modification can be followed directly by measuring the associated radioactivity (3). Assay for the unreacted amino groups on the protein either indirectly, by TNBS titration (4–6), or directly, by amino acid analysis (7–10), is an additional method of bioconjugate characterization.

Another means of bioconjugate characterization relies on the measurement of the molecular weight of the modified protein. Until recently, only low-resolution methods such as SDS–PAGE (11) or isoelectric focusing (1, 12) were available. Now, mass spectrometry techniques (FAB, MALDI, ESI) have emerged as powerful tools for directly observing the mass of modified proteins (13, 14) and thereby offer a means of quantifying the extent and type of modification (15, 16). Recently, we compared MALDI-MS with other more traditional methods for the characterization of a series of conjugates (17). At that time, MALDI-MS appeared to be the method of choice for the direct observation of the average molecular weight of the derivatized protein. In some cases, sub-

populations of the conjugate which differed in molecular weight were observed, but the limited resolution of MALDI-MS precluded more detailed analysis.

Electrospray ionization mass spectrometry (ESI-MS) is a soft ionization technique which typically gives only the parent ion with little or no fragmentation under normal conditions (14, 18). Unlike MALDI-MS, samples are introduced as solutions in water, organic solvents, or a combination of the two. This is particularly convenient for the analysis of proteins. In addition to direct sample introduction, the use of solutions further allows HPLC or CZE to be interfaced with the mass spectrometer. Regardless of the interface, samples are sprayed into the first chamber of the ESI-MS instrument as a fine mist at atmospheric pressure through a metal needle. Variable ionization potential is applied to the needle, and the charge is transferred to the exiting fine droplets. As the droplets evaporate, the charge is transferred from the solvent to the analyte. The charged analyte enters the high vacuum compartment through an orifice located at the end of the first chamber and is subsequently mass analyzed. Modern instruments are capable of mass determination of molecules ranging from small, simple organics (30 Da) to proteins (up to 200 kDa) with an accuracy of greater than 0.01% (19). Thus, ESI-MS is becoming a common tool for molecular weight determination of proteins, DNA/RNA (20–22), peptides, and other macromolecules. However, there have been limited reports on characterization of conjugates using ESI-MS. A recent report detailed the characterization of hapten–enzyme conjugates and concluded that for proteins in the mass range of 55 kDa, only conjugates with a hapten density of eight or less could be analyzed (23).

In the present work we have prepared a series of BSA conjugates using five structurally different haptens. Three molar ratios of each activated hapten to BSA were used to achieve different levels of modification in order to study the scope and limitations of ESI-MS as compared with MALDI-MS and SDS–PAGE methods.

MATERIALS AND METHODS

The *N*-hydroxysuccinimide (NHS) esters of 5-carboxy-fluorescein (**1a**), 7-(dimethylamino)-4-coumarinacetic acid (**2a**), and 3-aminodigoxigenin hemisuccinimide (**4a**) were obtained from Molecular Probes (Eugene, OR). Dansyl chloride (**3a**) was purchased from Fluka Chemical

[†] In memory of Dr. Roger L. Boeckx, 1946–1995.

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¹ Abbreviations: BSA, bovine serum albumin; CZE, capillary zone electrophoresis; DCC, *N,N*-dicyclohexylcarbodiimide; DMF, dimethylformamide; EDAC, *N*-ethyl-*N*-(3-(dimethylamino)propyl)carbodiimide hydrochloride; ESI, electrospray ionization; FAB, fast atom bombardment; HAP, hapten; HPLC, high-performance liquid chromatography; LC, liquid chromatography; MALDI, matrix-assisted laser desorption ionization; NHS, *N*-hydroxysuccinimide; SDS–PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TNBS, trinitrobenzenesulfonic acid.

(Ronkokoma, NY), and biotin NHS (**5a**) was obtained from Pierce Chemical (Rockford, IL). BSA (fraction V powder) and Sephadex G-25 Fine were purchased from Sigma Chemical Co. (St. Louis, MO). Anhydrous dimethylformamide (Aldrich Chemical Co., Milwaukee, WI) was vacuum degassed and then used immediately. All other chemicals were of the purest grades commercially available. Solutions were prepared using water from a Millipore water system (Marlborough, MA). Dialysis was performed using the Pierce Slide-A-Lyzer with a 10 kDa molecular weight cutoff and Pierce BupH phosphate-buffered saline (pH 7.2). Analysis by electrospray ionization mass spectrometry was carried out on a Perkin-Elmer Sciex API 100 Benchtop LC/MS system (Norwalk, CT) employing the Turbo IonSpray interface, interfaced with an binary microbore HPLC system (Ultra Plus, Microtech Scientific, Sunnyvale, CA). Analysis by matrix-assisted laser desorption ionization mass spectrometry employed a Bruker Reflex (Bruker Instruments, Billerica, MA) time-of-flight mass spectrometer equipped with a nitrogen laser (337 nm). Gel electrophoresis was performed on a Pharmacia PhastSystem (Piscataway, NJ) utilizing 12.5% polyacrylamide SDS-PAGE precast gels (PhastGel). Gel permeation chromatography was carried out on a Pharmacia P-50 system (Piscataway, NJ) with UV detection at 280 nm. The theoretical average molecular weight of BSA was calculated from the published sequence (24) with the Protein Analysis Worksheet (PAWS) version 5.1 by R. Beavis [mcphar04.med.nyu.edu].

Preparation of Conjugates. Conjugates were prepared at three different ratios of hapten to BSA (5:1, 15:1, and 30:1). The BSA concentration, the organic solvent content, and the reaction volume were held constant for each preparation. Thus, a stock solution of BSA (300 μ M) was prepared by dissolving BSA (40 mg, 0.6 μ mol) in phosphate buffer (2 mL, pH 8.0, 50 mM, Fisher Chemical, Fairlawn, NJ). Stock solutions (21 mM) for each of the activated haptens (**1a–5a**) were prepared by dissolving each hapten (21 μ mol) in anhydrous dimethylformamide (1 mL). For each conjugate, the BSA stock solution (0.5 mL, 0.15 μ mol) was placed in a vial and treated as follows: (A) For the 5:1 coupling ratio, DMF (179 μ L) and the activated hapten (35.8 μ L, 0.75 μ mol, 500 mol %) were added; (B) For the 15:1 coupling ratio, DMF (107 μ L) and the activated hapten (107 μ L, 2.27 μ mol, 1500 mol %) were added; (C) For the 30:1 coupling ratio, DMF (215 μ L) and the activated hapten (215 μ L, 4.55 μ mol, 3000 mol %) were added. After 4 h, an aliquot (200 μ L) from each vial was removed and dialyzed against phosphate buffer with 10% ethanol (500 mL, 2 $\times$, 24 h each), then against 10% aqueous ethanol (500 mL, 2 $\times$, 4h each), and finally against water (500 mL, 4 h). The remaining vial contents were passed through G-25 column (1 $\times$ 20 cm) eluting at 1 mL/min with ammonium acetate buffer (100 mM, 10% methanol). Fractions (3 mL each) were collected, and those for the conjugate peak were pooled. An aliquot (100 μ L) of each of the pooled fractions was retained, and the remainder was lyophilized (48 h).

Analysis of Conjugates. Each lyophilized conjugate was analyzed by SDS-PAGE, MALDI-MS, and LC-ESI-MS. In addition, a LC-ESI-MS spectrum was recorded for each conjugate before purification, after dialysis, and after G-25 gel chromatography.

Gel Electrophoresis. Conjugates were dissolved in SDS-PAGE sample buffer (10 mM TRIS/HCl, 1 mM EDTA, 2.5% SDS, and 5.0% β -mercaptoethanol, pH 8.0) to 1 mg/mL and heated to 100 $^{\circ}$ C for 5 min. Gels were loaded with 2–3 μ L of sample and run under denaturing conditions using the manufacturer's protocol. Upon completion, the gels were stained with Coomassie Blue

using a standard developing routine from the manufacturer. The resulting gels were evaluated first by scanning (Apple OneScanner, Apple Computers, CA) and second by analyzing the image with the gel-plotting macros of NIH-Image (v 1.58, public domain image processing and analysis program for the Apple Macintosh PowerPC from NIH, Bethesda, MD, downloaded from zippy.nimh.nih.gov).

Mass Spectrometry: (A) MALDI-MS. Analysis by matrix-assisted laser desorption ionization mass spectrometry employed a Bruker Reflex (Bruker Instruments, Billerica, MA) time-of-flight mass spectrometer equipped with a nitrogen laser (337 nm). The crystal matrix, sinapinic acid (Aldrich Chemical Co., Milwaukee, WI), was prepared at a concentration of 15 mg/mL in acetonitrile. Protein samples were typically 10–50 pmol/ μ L in a 2:1 water/acetonitrile solution. Sample and matrix solutions were mixed in equal volumes (typically 1.5 μ L each) directly on the stainless steel probe tip (target) and allowed to dry ($\sim$ 10 min) in a fume hood at room temperature. The crystallized analyte–matrix sample was then rinsed with 0.1% TFA solution by placing approximately 2 μ L of the solution on the probe sample at room temperature, allowing it to stand for about 5 s, and then gently drying the crystals with a stream of nitrogen. Spectra were recorded at a threshold laser irradiance for 50–150 shots in the linear mode at 30 kV. The resulting data were analyzed using XMASS, the postacquisition software supplied with the spectrometer (17).

(B) LC-ESI-MS. Protein conjugates samples were dissolved in 0.2% formic acid (1 mg/mL) and introduced onto the ESI-MS by HPLC employing a PLRP-S column (2.1 $\times$ 50 mm, Polymer Laboratories Ltd., Amherst, MA) using the following linear gradient: 90:10 0.2% formic acid/methanol to 100% methanol over 20 min at a flow of 100 μ L/min. Ion source conditions: ion spray (IS) 4800 V, orifice (OR) 30 V, nebulizing gas 1.46 L/min, and TurboProbe jet 350 $^{\circ}$ C with air flow of 4 L/min. The samples were scanned over two m/z ranges (150–700 and 1200–2400) with a total acquisition time of 7.2 s. Small molecule impurities were detected by reading their molecular weights directly from the low m/z spectra. Molecular weights of the conjugates were determined from the high m/z scans after converting the complex spectra of multicharged protein species to the mass domain using the manufacturer's deconvolution software (BioReconstruct).

RESULTS

Conjugation of Haptens. Commercially available, activated haptens (**1a–5a**) reacted with BSA in pH 8 buffer at three molar ratios (5:1, 15:1, and 30:1) to give the corresponding series of conjugates **1c–5c** along with the hydrolyzed haptens **1b–5b** (Scheme 1). On the basis of the MS analysis, purification by either gel permeation chromatography or dialysis separated the conjugate from the low molecular weight components. Covalent attachment of each hapten proceeded through the amino groups (59, lysine ϵ -amino groups, plus the amino terminal) on the protein with a detectable increase in the molecular weight of the conjugate. The incremental change in molecular weight due to incorporation of the hapten radicals ranged from 227 to 473 (fluorescein **1**, Δ MW 359; coumarin **2**, Δ MW 230; dansyl **3**, Δ MW 234; 3-amino-digoxigenin **4**, Δ MW 473; and biotin **5**, Δ MW 227). Apparent coupling efficiency to BSA was similar for all the haptens but coumarin **2a**, which coupled poorly. All

Table 1. Calculated Molecular Weights of BSA Conjugates

no.	ΔMW^a	equiv ^b	SDS-PAGE ^c	MALDI ^d	conjugate molecular weight	
					ES/MS ^e	
1c	359	5	69 732	68 957	67 521 ($M_{BSA} + 3HAP$, 60), 67 870 ($M_{BSA} + 4HAP$, 100), 68 227 ($M_{BSA} + 5HAP$, 95), 68 587 ($M_{BSA} + 6HAP$, 72), 68 944 ($M_{BSA} + 7HAP$, 20), 69 291 ($M_{BSA} + 8HAP$, 15)	
		15	71 402	71 075	68 962 ($M_{BSA} + 7HAP$, 26), 69 306 ($M_{BSA} + 8HAP$, 53), 69 662 ($M_{BSA} + 9HAP$, 92), 70 020 ($M_{BSA} + 10HAP$, 100), 70 381 ($M_{BSA} + 11HAP$, 92), 70 732 ($M_{BSA} + 12HAP$, 46), 71 088 ($M_{BSA} + 13HAP$, 16)	
		30	73 749	72 274	70 379 ($M_{BSA} + 11HAP$, 10), 70 735 ($M_{BSA} + 12HAP$, 21), 71 097 ($M_{BSA} + 13HAP$, 38), 71 452 ($M_{BSA} + 14HAP$, 72), 71 812 ($M_{BSA} + 15HAP$, 73), 72 168 ($M_{BSA} + 16HAP$, 66), 72 524 ($M_{BSA} + 17HAP$, 100), 72 904 ($M_{BSA} + 18HAP$, 51)	
2c	230	5	63 729	67 037	66 437 ($M_{BSA} + 0HAP$, 100)	
		15	63 127	67 381	66 437 ($M_{BSA} + 0HAP$, 100), 66 670 ($M_{BSA} + 1HAP$, 32), 66 896 ($M_{BSA} + 2HAP$, 5)	
		30	63 524	67 488	66 437 ($M_{BSA} + 0HAP$, 100), 66 665 ($M_{BSA} + 1HAP$, 79), 66 895 ($M_{BSA} + 2HAP$, 44), 67 120 ($M_{BSA} + 3HAP$, 10)	
3c	234	5	61 728	67 697	66 463 ($M_{BSA} + 0HAP$, 51), 66 693 ($M_{BSA} + 1HAP$, 86), 66 932 ($M_{BSA} + 2HAP$, 100), 67 158 ($M_{BSA} + 3HAP$, 53), 67 401 ($M_{BSA} + 4HAP$, 28)	
		15	63 009	68 921	67 396 ($M_{BSA} + 4HAP$, 1), 67 633 ($M_{BSA} + 5HAP$, 37), 67 866 ($M_{BSA} + 6HAP$, 94), 68 106 ($M_{BSA} + 7HAP$, 100), 68 325 ($M_{BSA} + 8HAP$, 57), 68 560 ($M_{BSA} + 9HAP$, 14)	
		30	63 193	69 781	69 280 ($M_{BSA} + 11HAP$, 17), 69 491 ($M_{BSA} + 12HAP$, 45), 69 741 ($M_{BSA} + 13HAP$, 91), 69 962 ($M_{BSA} + 14HAP$, 100)	
4c	473	5	64 321	68 236	66 911 ($M_{BSA} + 1HAP$, 38), 67 381 ($M_{BSA} + 2HAP$, 92), 67 852 ($M_{BSA} + 3HAP$, 100), 68 327 ($M_{BSA} + 4HAP$, 87), 68 793 ($M_{BSA} + 5HAP$, 58), 69 264 ($M_{BSA} + 6HAP$, 36), 69 737 ($M_{BSA} + 7HAP$, 22)	
		15	66 556	71 416	69 268 ($M_{BSA} + 6HAP$, 35), 69 737 ($M_{BSA} + 7HAP$, 73), 70 212 ($M_{BSA} + 8HAP$, 80), 70 684 ($M_{BSA} + 9HAP$, 86), 71 153 ($M_{BSA} + 10HAP$, 100), 71 622 ($M_{BSA} + 11HAP$, 78), 72 095 ($M_{BSA} + 12HAP$, 53)	
		30	69 843	76 618	72 100 ($M_{BSA} + 12HAP$, 28), 72 568 ($M_{BSA} + 13HAP$, 49), 73 046 ($M_{BSA} + 14HAP$, 70), 73 519 ($M_{BSA} + 15HAP$, 63), 73 987 ($M_{BSA} + 16HAP$, 75), 74 456 ($M_{BSA} + 17HAP$, 60), 74 933 ($M_{BSA} + 18HAP$, 83), 75 400 ($M_{BSA} + 19HAP$, 100)	
5c	227	5	68 493	68 143	66 659 ($M_{BSA} + 1HAP$, 3), 66 891 ($M_{BSA} + 2HAP$, 29), 67 118 ($M_{BSA} + 3HAP$, 77), 67 343 ($M_{BSA} + 4HAP$, 100), 67 569 ($M_{BSA} + 5HAP$, 93), 67 797 ($M_{BSA} + 6HAP$, 59), 68 022 ($M_{BSA} + 7HAP$, 36), 68 249 ($M_{BSA} + 8HAP$, 19)	
		15	69 134	70 618	68 250 ($M_{BSA} + 8HAP$, 8), 68 482 ($M_{BSA} + 9HAP$, 22), 68 702 ($M_{BSA} + 10HAP$, 47), 68 936 ($M_{BSA} + 11HAP$, 69), 69 153 ($M_{BSA} + 12HAP$, 81), 69 379 ($M_{BSA} + 13HAP$, 93), 69 607 ($M_{BSA} + 14HAP$, 100), 69 837 ($M_{BSA} + 15HAP$, 97)	
		30	70 345	72 234	70 526 ($M_{BSA} + 18HAP$, 15), 70 736 ($M_{BSA} + 19HAP$, 24), 70 964 ($M_{BSA} + 20HAP$, 47), 71 195 ($M_{BSA} + 21HAP$, 77), 71 420 ($M_{BSA} + 22HAP$, 100), 71 644 ($M_{BSA} + 23HAP$, 81), 71 872 ($M_{BSA} + 24HAP$, 83), 72 098 ($M_{BSA} + 25HAP$, 80)	

^a Molecular weight increase due to each hapten conjugated to BSA. ^b Equiv is the molar equivalents of hapten to BSA used in conjugate preparation. ^c Electrophoresis values are listed as apparent mass determinations. ^d MALDI values were calculated from the peak centroid. ^e m/z (M + no. of haptens, relative intensity).

spectively). However, after dialysis or gel chromatography, none of the impurities were observed. In the high mass range, LC-ESI-MS of each conjugate provided a complex spectrum consisting of multiple protein species in multicharged states. The molecular weights of the individual modified protein species in each conjugate were recorded after deconvolution of the data. No significant difference in spectral quality was observed between spectra recorded from the purified or unpurified conjugates. Figure 3 illustrates a typical spectrum for BSA before and after conjugation with activated hapten **5a**. The corresponding deconvoluted spectra for the entire **5c** conjugate series are shown in Figure 4.

No free BSA was detected in the conjugates of series **1c**, **4c**, and **5c** by LC-ESI. Unmodified BSA was the dominant species present in all the conjugates of **2c** series and the **3c** (5:1) conjugate.

Comparison of Methods. Table 2 compares the number of hapten incorporated into BSA as measured by SDS-PAGE, MALDI-MS and LC-ESI. Values for SDS-PAGE and MALDI-MS were determined using the equation:

$$\text{number of hapten} = \frac{(\text{conjugate MW}) - (\text{BSA MW})}{(\text{hapten } \Delta MW)}$$

The values reported for LC-ESI were calculated using the base peak from the respective spectra listed in Table 1. MALDI-MS, and LC-ESI-MS results indicated hapten density increased in parallel with the hapten to

protein coupling ratio for all but the coumarin conjugate series **2c**. SDS-PAGE also showed the same trend for conjugates **1c** and **5c**, while the data for the other conjugates was in conflict. LC-ESI-MS for conjugate **2c** showed that the most abundant species was unmodified BSA at all coupling ratios, while MALDI-MS showed a progression from 2.6 to 4.6 haptens per BSA. LC-ESI-MS allowed detection of less abundant species with a hapten density of 1–3. For all the conjugates, LC-ESI-MS displayed the range of less prominent individual species of varying hapten density present in each conjugate.

DISCUSSION

In light of ever increasing quality and regulatory demands, the characterization of bioconjugates has become critically important. The hapten density of the conjugate is often cited as an important property (25, 26). This is well illustrated in the art of immunogen preparation, where the relationship between the immunoconjugate's characteristics and the quality of the produced antibody has been debated for some time. The accepted practice is based on the assumption that the highest antibody (IgG) titer is obtained with an hapten density of 15–30 molecules per carrier protein. Higher substitution can result in an IgM response which exceeds that of IgG and produces antibodies of lower affinity. A lower hapten density induces a slower immune response, but often leads to an antibody with higher affinity (25, 26).

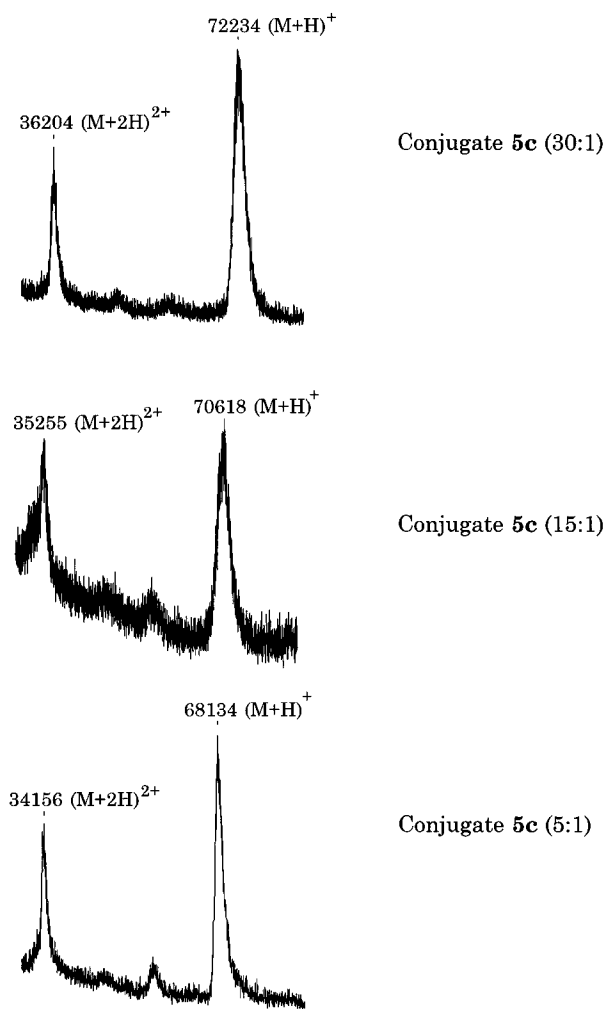


Figure 2. MALDI mass spectra of **5c** conjugate series.

While great emphasis has been placed on measuring hapten density on bioconjugates, relying solely on hapten density for characterization may be misleading and insufficient. Other factors such as protein cross-linking, coupling with species other than hapten, hapten degradation, and noncovalently bound hapten affect both the measurement of hapten density and the performance of the conjugate. The common method of directly conjugating haptens to proteins with carbodiimides (27) ensures that the cross-linking will occur along with *N*-acylurea formation on the protein (28–30). Use of pure, isolated activated haptens, or those prepared such that they are free from excess coupling reagent (31), can obviate some of these problems. Still, the methods used to characterize bioconjugates must address these variables before any definitive conclusions can be drawn.

Several methods have been used to characterize conjugates in the past (e.g. TNBS titration, UV–difference spectroscopy, gel electrophoresis). Each of these methods have their own limitations (17). Classical biochemical methodologies, however, can be greatly augmented or replaced by bioanalytical mass spectrometry. The two most popular methods for bioconjugate characterization are MALDI-MS and ESI-MS (14). In this paper we evaluated both methods in addition to SDS–PAGE for characterization of five structurally diverse BSA conjugates.

The use of pure activated haptens and a high grade of BSA resulted in high yields of soluble conjugates. Solubility of the conjugates allowed for the successful SDS–PAGE analysis of each sample. Each sample generated

well defined bands. The increase in the apparent molecular weight of the conjugates paralleled the increase hapten to protein coupling ratio in each series. Conjugates of the **2c**, **3c**, and **4c** series gave apparent molecular weights that were below or equal to that of unmodified BSA, but within the series, higher coupling ratios produced higher molecular weights. In these cases SDS–PAGE was sensitive to the polarity of the hapten. We concluded that the unmodified protein molecular weight standards were not adequate for assessing the molecular weight of these conjugates. More polar haptens **1a** and **5a** gave results that paralleled MALDI-MS and ESI-MS.

Thus, SDS–PAGE does not predictably reflect the degree of hapten incorporation. Its main advantage is in detecting cross-linked conjugates. In earlier work (17), where the conjugation procedure produced conjugates with poor solubility, SDS–PAGE showed smeared bands along with bands for polymerized BSA. An additional drawback to SDS–PAGE is its inherent insensitivity to small changes in molecular weight. The accuracy of SDS–PAGE is poor (<5%) (32), so differentiating between bound hapten and other coreacting impurities is not possible. Also, it cannot detect free hapten or low molecular weight impurities in the conjugate.

MALDI-MS is an improvement over SDS–PAGE. MALDI-MS allows a more accurate determination of average molecular weight. Unmodified BSA had a molecular weight of 66 531, while the molecular weight based on the published sequence (24) is 66 432.9, corresponding to an accuracy of 0.13%. Theoretically, this means that a difference of one hapten (ΔMW 81) on BSA can be detected. For the coumarin conjugate series **2c**, the average molecular weight derived by MALDI-MS clearly indicated a difference between the lightly loaded conjugates (2.6–4.6 hapten/BSA). For the other conjugate series, differences in the MALDI-MS average molecular weights indicated hapten densities between 3.8–25.5. MALDI-MS results were not sensitive to the polar or nonpolar nature of the hapten.

In light of the LC–ESI-MS results discussed below, the meaning of MALDI-MS results is not completely clear. The low mass resolution of MALDI-MS precludes stating that the increase in molecular weight is due solely to hapten incorporation. While MALDI-MS has a high tolerance to the buffer salts (Na^+ , K^+) used in conjugate preparation, their presence can be reflected in an overestimation of the average molecular weight of the conjugate. Unlike the conjugates used in this study, partially soluble conjugates can be analyzed by MALDI-MS. However, care must be taken when very insoluble proteins are analyzed. We have noted that in some cases where conjugates have limited solubility, a MALDI-MS spectrum can be recorded, but it is not representative of the entire sample, i.e. in comparing a conjugate prepared with a high coupling ratio to a low ratio, the low ratio gave higher average molecular weight (33). Recent studies have addressed MALDI-MS solubility issues and the analysis of peptides and small proteins (10 kDa) (34). We found that MALDI-MS was not useful in the determination of low molecular weight impurities, or following the progress of their removal during conjugate purification.

Initially we attempted to analyze our conjugate samples directly by ESI-MS, but the normal buffer salts employed in the conjugation procedure prevented successful deconvolution of the raw spectral data. Only on analysis with LC–ESI-MS were the interfering salts removed and the raw data deconvoluted to give satisfactory molecular

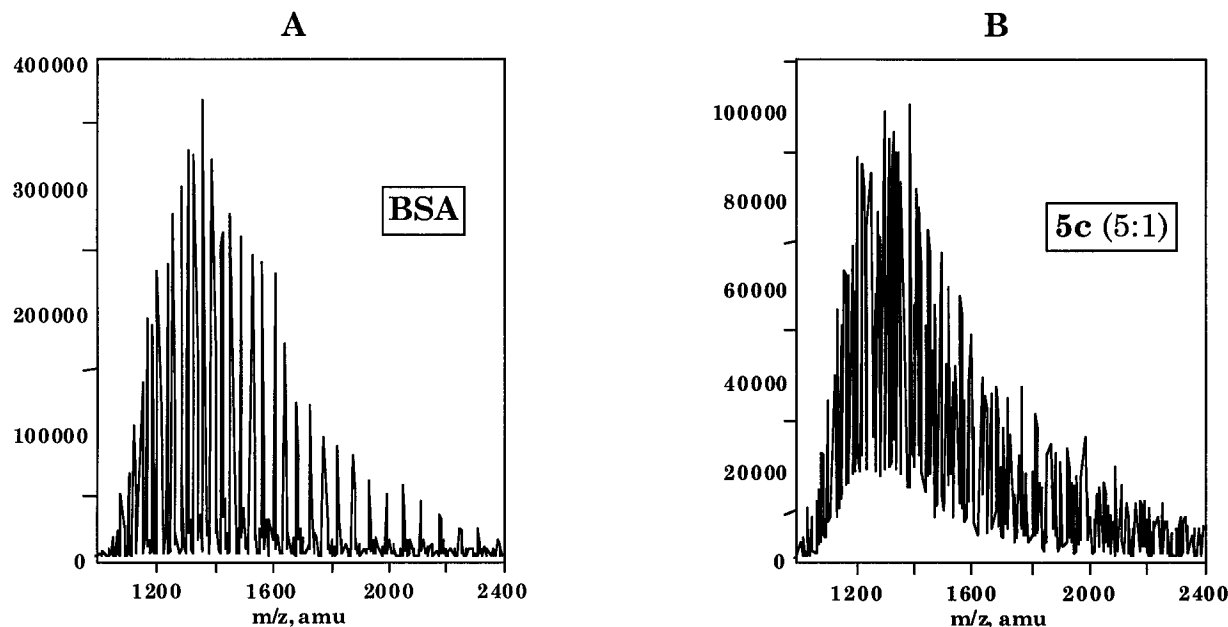


Figure 3. (A) ESI mass spectrum of BSA. (B) ESI mass spectrum of conjugate **5c** (5:1).

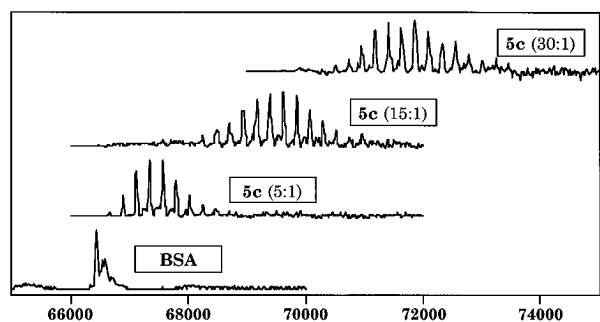


Figure 4. Deconvoluted spectra of conjugate series **5c** and BSA.

Table 2. Comparison of Hapten Density by Three Methods

no.	equiv ^a	average hapten incorporation		
		SDS-PAGE ^b	MALDI ^b	LC-ESI ^c
1c	5	4.2	7.0	4 (3–8)
	15	13.8	12.9	10 (7–13)
	30	20.4	16.3	17 (11–18)
2c	5	<i>d</i>	2.6	0 (0)
	15	<i>d</i>	4	0 (0–2)
	30	<i>d</i>	4.6	0 (0–3)
3c	5	<i>d</i>	5.4	2 (1–4)
	15	<i>d</i>	10.6	7 (1–9)
	30	<i>d</i>	14.3	14 (11–14)
4c	5	<i>d</i>	3.8	3 (1–7)
	15	<i>e</i>	10.5	10 (6–12)
	30	7.2	21.5	19 (12–19)
5c	5	9.1	7.5	4 (1–8)
	15	11.9	18.4	14 (8–15)
	30	17.2	25.5	22 (18–25)

^a Equiv is the molar equivalents of hapten to BSA used in conjugate preparation. ^b Calculated average number of haptens.

^c Most abundant species (range). ^d Negative calculated values.

^e Less than one hapten.

weight information. Using LC-ESI-MS the conjugation could be monitored for both appearance of the modified protein and the disappearance of the activated hapten. During the purification, the removing of coupling byproducts, i.e., hapten and NHS, could be followed, ensuring that the final conjugate was free from potential interferences. Of the three methods explored, LC-ESI-MS has

the greatest resolution; therefore, the stoichiometry of the protein modification can be analyzed more in-depth. With a reported accuracy of 0.01% (19), changes in molecular weight of the modified BSA as small as 7 mass units can be detected by ESI-MS. We recorded a molecular weight for BSA of 66 437, while the molecular weight based on the published sequence (24) is 66 432.9, corresponding to an accuracy of 0.006%. The accuracy of the method is such that even small changes in conjugate molecular weight are observable. Thus, it is possible to differentiate between conjugation of hapten, secondary reactions of the hapten during conjugation, and other covalent or noncovalent modifications of the protein. In the present study, the finer resolution readily allowed observation of individual conjugate species that differed by a single hapten unit. The quality of information for hapten density of our conjugates was unaffected by the hapten structure; both polar and nonpolar haptens produced well-resolved spectra. Hapten densities from 0 to 25 were easily measured. In the case of coumarin conjugate series **2c**, LC-ESI-MS showed that the principal species at all coupling ratios was unmodified BSA. At the highest coupling ratio, species with up to three coumarins were detected in lesser abundance. This is in contrast to the MALDI-MS results which indicated higher hapten densities at all coupling ratios. In general, MALDI-MS appeared to overestimate hapten density for all the conjugates tested in this study. The overestimation of hapten density by MALDI-MS may be attributed to adducts formed with alkali metals, small molecules, or the hapten-sized matrix molecule, sinapinic acid (MW 224) (35).

In summary of this study, LC-ESI-MS appears to be the best single method for characterizing BSA bioconjugates. LC-ESI-MS was able to detect BSA conjugate species covalently labeled with up to 25 haptens. The method proved useful for monitoring the course of the conjugation and purification procedures, following both the fate of the low and high molecular weight components in a single analysis. MALDI-MS provided more qualitative information about the modified protein, but not with enough detail to differentiate between small changes. The utility of SDS-PAGE was limited to detecting crosslinked protein.

LITERATURE CITED

- (1) Barbarkis, M. S., and Bachas, L. G. (1991) Isoelectric focusing of protein-ligand conjugates. Effect of the degree of substitution. *Clin. Chem.* 37, 87-90.
- (2) Metelitz, D. I., Eryomin, A. N., Karasyova, E. I., and Markina, V. L. (1991) Catalytic activity and thermostability of dehydrogenase conjugates with cortisol and progesterone. *Bioconjugate Chem.* 2, 309-316.
- (3) Feng, P. C. C., Horton, S. R., and Sharp, C. R. (1992) A general method for developing immunoassays to chloroacetanilide herbicides. *J. Agric. Food Chem.* 40, 211-214.
- (4) Habeeb, A. F. S. A. (1966) Determination of free amino groups in proteins by trinitrobenzenesulfonic acid. *Anal. Biochem.* 14, 328-336.
- (5) Shinoda, T., and Tsuzukida, Y. (1974) Identification of readily trinitrophenylating amino groups of human Bence-Jones proteins modified by incubating for 45 min at 37 °C before measuring UV absorbance. *J. Biochem.* 75, 23-44.
- (6) Sashidar, R. B., Capoor, A. K., and Ramana, D. (1994) Quantitation of ϵ -amino group using amino acids as reference standards by trinitrobenzene sulfonic acid. A simple spectrophotometric method for the estimation of hapten to carrier protein ratio. *J. Immunol. Methods* 167, 121-127.
- (7) Antoni, G., and Presentini, R. (1989) A least-squares computer method for the determination of the molecular ratio of conjugates between two different proteins from the results of the amino acid analysis. *Anal. Biochem.* 179, 158-61.
- (8) Tsao, J., Lin, X., Lackland, H., Tous, G., Wu, Y., and Stein, S. (1991) Internally standardized amino acid ratioanalysis for determining peptide/carrier protein coupling ratio. *Anal. Biochem.* 197, 137-42.
- (9) Shuler, K. R., Dunham, R. G., and Kanda, P. (1992) A simplified method for determination of peptide-protein molar ratios using amino acid analysis. *J. Immunol. Methods* 156, 137-49.
- (10) Zhu, T., Peng, Y., Lackland, H., and Stein, S. (1993) Confirmation of oligonucleotide-peptide structure by amino acid analysis. *Anal. Biochem.* 214, 585-7.
- (11) Wetzel, R., Halualani, R., Stults, J. T., and Quan, C. (1990) A general method for highly selective cross-linking of unprotected polypeptides via pH-controlled modification of N-terminal α -amino groups. *Bioconjugate Chem.* 1, 114-122.
- (12) Kamps-Holtzapfel, C., Carlin, R. J., Sheffield, C., Kubena, L., Stanker, L., and DeLoach, J. F. (1993) Analysis of hapten-carrier protein conjugates by nondenaturing gel electrophoresis. *J. Immunol. Methods* 164, 245-253.
- (13) Nguyen, D. N., Becker, G. W., and Riggan, R. M. (1995) Protein mass spectrometry: application to analytical biotechnology. *J. Chromatogr. A*, 705, 21-45.
- (14) Loo, J. A. (1995) Bioanalytical mass spectrometry: many flavors to choose. *Bioconjugate Chem.* 6, 644-665.
- (15) Stults, J. T., Henzel, W. J., Bourell, J. H., and Griffin, P. R. (1990) Post-translational modifications of proteins: the necessity for mass spectrometry. In *Biological Mass Spectrometry* (A. L. Burlingame and J. A. McCloskey, Eds.) pp 599-620, Elsevier, Amsterdam.
- (16) Biemann, K., and Martin, S. A. (1987) Mass spectrometric determination of the amino acid sequence of peptides and proteins. *Mass Spectrom. Rev.* 6, 1-76.
- (17) Adamczyk, M., Buko, A., Chen, Y.-Y., Fishpough, J. R., Gebler, J. C., and Johnson, D. D. (1994) Characterization of protein-hapten conjugates. 1. Matrix-assisted laser desorption ionization mass spectroscopy of immuno BSA-hapten conjugates and comparison with other characterization methods. *Bioconjugate Chem.* 5, 631-635.
- (18) Siuzdak, G. (1994) The emergence of mass spectrometry in biochemical research. *Proc. Natl. Acad. Sci. U.S.A.* 91, 11290-11297.
- (19) Loo, J. A., Edmonds, C. G., and Smith, R. D. (1991) Tandem mass spectrometry of very large molecules: serum albumin sequence information from multiply charged ions formed by electrospray ionization. *Anal. Chem.* 63, 2488-2499.
- (20) Ganem, B., Li, Y. T., and Henion, J. D. (1993) Detection of oligonucleotide duplex forms by ion-spray mass spectrometry. *Tetrahedron Lett.* 34, 1445-1448.
- (21) Little, D. P., and McLafferty, F. W. (1995) Sequencing 50-mer DNAs using electrospray tandem mass spectrometry and complementary fragmentation methods. *J. Am. Chem. Soc.* 117, 6783-6784.
- (22) Stults, J. T., and Marsters, J. C. (1991) Improved electrospray ionization of synthetic oligonucleotides. *Rapid Commun. Mass Spectrom.* 5, 359-363.
- (23) Straub, K. E., and Levy, M. K. (1994) Characterization of hapten binding to immunoconjugates by electrospray ionization mass spectrometry. *Bioconjugate Chem.* 5, 194-198.
- (24) Jirayama, K., Akashi, S., Furuya, M., and Kukuvara, D. (1990) Rapid confirmation and revision of the primary structure of bovine serum albumin by ESIMS and FRIT LC/MS. *Biochem. Biophys. Res. Commun.* 173, 639-646.
- (25) Klaus, G. G. B., and Cross, A. M. (1974) The influence of epitope density on the immunological properties of hapten-protein conjugates. *Cell. Immunol.* 14, 226-241.
- (26) Malaitsev, V. V., and Azhipa, O. Y. (1993) Influence of epitope density on immunogenic properties of hapten-protein conjugates. *Bull. Exp. Biol. Med.* 115, 726-728.
- (27) Erlanger, B. F. (1980) The preparation of antigenic hapten-carrier conjugates: a survey. *Methods Enzymol.* 70, 84-104.
- (28) Quesniaux, V., Himmelsbach, K., and Regenmortel, M. H. V. (1985) An enzyme immunoassay for the screening of monoclonal antibodies to cyclosporin. *Immunol. Lett.* 9, 99-104.
- (29) Quesniaux, V. F. J., Tees, R., Schreier, M. H., Wenger, R. M., and Regenmortel, M. H. V. (1987) Fine specificity and cross-reactivity of monoclonal antibodies to cyclosporine. *Mol. Immunol.* 24, 1159-1168.
- (30) Bodansky, M., and Martinez, J. (1981) Side reactions in peptide synthesis. *Synthesis*, 333-356.
- (31) Adamczyk, M., Fishpough, J. R., and Mattingly, P. G. (1995) An easy preparation of hapten active esters via solid supported EDAC. *Tetrahedron Lett.* 35, 8345-8346.
- (32) Barinaga, M. (1989) Protein chemists gain a new analytical tool. *Science* 246, 32-33.
- (33) Adamczyk, M., Gebler, J., Mattingly, P. G., and Pan, Y. Unpublished observations.
- (34) Cohen, S. L., and Chait, B. T. (1996) Influence of matrix solution conditions on the MALDI-MS analysis of peptides and proteins. *Anal. Chem.* 68, 31-37.
- (35) Roberts, G. D., Johnson, W. P., Burman, S., Anumula, K. R., and Carr, S. A. (1995) An integrated strategy of structural characterization of the protein and carbohydrate components of monoclonal antibodies: application to anti-respiratory syncytial virus MAb. *Anal. Chem.* 67, 3613-3625.

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Texas Red-X and Rhodamine Red-X, New Derivatives of Sulforhodamine 101 and Lissamine Rhodamine B with Improved Labeling and Fluorescence Properties

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Texas Red sulfonyl chloride (TR-SC) and Lissamine rhodamine B sulfonyl chloride (LRB-SC) are popular dyes often used to prepare red fluorescent conjugates that are useful second labels in combination with fluorescein. Unfortunately, being sulfonyl chloride derivatives, both are unstable to moisture during storage and prone to hydrolysis in the conjugation reaction. Their instability causes the percentage of reactive dye to vary from lot to lot and requires use of low temperatures and a relatively high pH to optimize conjugation efficiency. Succinimidyl esters of the aminohexanoic acid sulfonamides of both dyes have been prepared, which are designated Texas Red-X succinimidyl ester (TR-X-SE) and Rhodamine Red-X succinimidyl ester, respectively. Their spectral properties are similar to those of their sulfonyl chloride analogs; moreover, incorporation of the succinimidyl ester at the end of the aliphatic chain spacer facilitates conjugation, decreases precipitation of proteins during conjugation and storage, and usually increases the fluorescence yield of the conjugate. Comparison of the rate of hydrolysis of TR-SC with that of TR-X-SE shows that, while the former was completely hydrolyzed within 5 min by exposure to water, TR-X-SE retains most of its reactivity for more than an hour. The reactivity of both new derivatives is high between pH 7.5 and 8.5, allowing conjugation of proteins that do not tolerate the high pH required for reaction with sulfonyl chlorides. In addition, Texas Red maleimides and haloacetamides containing spacer groups were prepared for labeling sulfhydryl groups. A Texas Red-X derivative of phalloidin has also been prepared, and its use for labeling F-actin has been characterized.

INTRODUCTION

Texas Red sulfonyl chloride (TR-SC) and Lissamine rhodamine B sulfonyl chloride (LRB-SC) are derivatives of sulforhodamine 101 and sulforhodamine B, respectively. Both dyes have strong red fluorescence and are frequently used in combination with fluorescein for dual or multiparameter fluorescence analysis (Titus et al., 1982; Wessendorf et al., 1990; Staines et al., 1988). The absorption and emission spectra of their conjugates are sufficiently shifted to the red so that they encounter little interference from the fluorescence of fluorescein conjugates when used for multicolor labeling experiments or the intrinsic fluorescence in biological samples. Texas Red conjugates absorb further in the red than Lissamine rhodamine B conjugates, and Texas Red is generally considered the more useful of the two dyes. However, being sulfonyl chlorides, both reactive dyes hydrolyze rapidly in aqueous environments and their conjugation to biomolecules usually requires a pH of 9–9.5, a pH value at which some proteins denature. In addition, sulfonyl chlorides form conjugates with tyrosine, serine, and histidine residues that may be unstable (Wong, 1991). Conjugations with TR-SC and LRB-SC must be performed at a low temperature to reduce the rate of dye hydrolysis. The low temperature and basic pH serve to maximize the reaction efficiency, though it often remains low. Furthermore, sensitivity of the reactive dyes to traces of moisture during storage affects the reactive dye's purity and, consequently, the percentage of reactive sulfonyl chlorides fluctuates from batch to batch. Un-

certainly regarding dye purity, combined with variations in the time that elapses between dissolving the dye and initiating the reaction, results in difficulty in reproducing conjugation reactions.

In an effort to facilitate and improve conjugations, we have added a 6-aminohexanoic acid ("X") spacer between the fluorophore and the reactive group. This allows us to prepare succinimidyl ester derivatives, the most amine-selective and stable reactive group for labeling amines (Drexage, 1990). We have named these derivatives Texas Red-X succinimidyl ester (TR-X-SE) and Rhodamine Red-X succinimidyl ester (RhR-X-SE). Their spectral properties and extinction coefficients are similar to those of their sulfonyl chloride analogs. However, they can be conjugated to proteins at a lower pH, at room temperature, and with greater reproducibility. In many cases, less precipitation of proteins occurs during conjugation and storage, and the products have increased fluorescence yields. Also, the greater stability of the reactive dyes permits use of less dye when using the succinimidyl esters to achieve the same degree of dye conjugation. In addition, we have prepared iodoacetamide, bromoacetamide, and maleimide derivatives of the Texas Red chromophore with similar aliphatic spacers, thus obtaining previously unavailable red fluorescence-emitting reagents that are useful for the labeling of thiol groups. Use of these reagents allows preparation of conjugates that are otherwise difficult to make.

MATERIALS AND METHODS

TR-SC, Texas Red cadaverine, and LRB-SC were obtained from Molecular Probes, Inc., Eugene, OR. Methyl 6-aminohexanoate, *O*-(*N*-succinimidyl)-*N,N,N',N'*-tetramethyluronium tetrafluoroborate, iodoacetic anhy-

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dride, and bromoacetyl bromide were purchased from Fluka Chemical Corp., Ronkonkoma, NY. All other common reagents were purchased from Aldrich Chemical Co., Milwaukee, WI. Thin-layer chromatography was performed on silica gel 60 F-254 sheets and column chromatography on silica gel 60 (230–400 mesh, E. Merck, No. 9385, Gibbstown, NJ). ^1H NMR spectra were recorded on a Nicolet QE-400 MHz (General Electric) spectrometer. Absorption spectra were obtained using an IBM 9429 UV–visible spectrophotometer. Emission spectra were obtained using a Perkin-Elmer Model 650-40 fluorescence spectrophotometer. Mass spectra were recorded on a Kratos MS-50 mass spectrometer (Manchester, England).

9-[2(or 4)-[[[5-[N-(Succinimidyl)oxy]carbonylpentyl]amino]sulfonyl]-4(or 2)-sulfophenyl]-2,3,6,7,12,13,16,17-octahydro-1H,5H,11H,15H-xantheno[2,3,4-ij:5,6,7-i'j']diquinolizin-18-ium, Inner Salt (3). To a solution of methyl 6-aminohexanoate hydrochloride (0.40 g, 2.20 mmol) and 0.6 mL of triethylamine in 40 mL of chloroform was added TR-SC (**1**) (1.00 g, 1.60 mmol) in small portions over a period of 10 min while the reaction mixture was stirred at 0 °C. After the reaction mixture was stirred at room temperature for 15 h, it was washed with three 50 mL portions of water. The organic layer was separated, dried over anhydrous sodium sulfate, and concentrated under reduced pressure to give a dark purple solid. This crude methyl ester derivative was purified by silica gel column chromatography with 2.5% methanol in chloroform as eluant to give 0.80 g (68%) of the methyl ester derivative. To a suspension of the above methyl ester (0.80 g, 1.09 mmol) in 15 mL of dioxane was added 25 mL of 6 M HCl dropwise over a period of 5 min. After the reaction mixture was stirred at room temperature for 18 h, it was poured into 200 mL of water. The resulting solid was collected by filtration and purified by column chromatography on silica gel with 15% methanol in chloroform as eluant to give 0.68 g (87%) of **2** (TR-X) as a dark purple solid. Compound **2** had an absorption maximum at 591 nm with a molar absorptivity of 85 400 $\text{cm}^{-1} \text{M}^{-1}$ and an emission maximum of 610 nm in pH 7.5 phosphate buffer solution. To a solution of compound **2** (300 mg, 0.42 mmol) in 3 mL of DMF was added *O*-(*N*-succinimidyl)-*N,N,N',N'*-tetramethyluronium tetrafluoroborate (150 mg, 0.50 mmol). After the reaction mixture was stirred at room temperature for 1 h, it was diluted with 50 mL of chloroform, washed with water, dried over anhydrous sodium sulfate, and concentrated under reduced pressure to a volume of 10 mL. The resulting solution was added dropwise into 100 mL of ether while being stirred vigorously at room temperature. The resulting precipitate was collected by filtration and dried under vacuum to give 295 mg (87%) of **3** as a dark purple solid; one spot on TLC ($R_f = 0.20$, 10% methanol in chloroform); ^1H NMR (DMSO- d_6) δ 8.39 (d, 1H, ArH), 7.92–7.84 (m, 1H, ArH), 7.48–7.33 (m, 1H, ArH), 6.53 (d, 2H, 2 $\times$ ArH), 3.54–3.40 (m, 8H, 4 $\times$ CH₂), 3.13–2.57 (m, 8H, 4 $\times$ CH₂), 2.80 (s, 4H, 2 $\times$ CH₂), 2.08–1.74 (m, 8H, 4 $\times$ CH₂), 1.65–1.12 (m, 6H, 3 $\times$ CH₂); absorption maximum, 583 nm (ϵ 116 300 $\text{cm}^{-1} \text{M}^{-1}$), emission maximum, 603 nm in methanol; high-resolution FAB-MS, m/e ($M + H$) calcd 817.2580, found 817.2580. Anal. Calcd for C₄₁H₄₄N₄O₁₀S₂·2.5H₂O: C, 57.13; H, 5.73; N, 6.50. Found: C, 57.38; H, 5.87; N, 7.14.

3,6-Bis(diethylamino)-9-[2(or 4)-[[[5-[N-(succinimidyl)oxy]carbonylpentyl]amino]sulfonyl]-4(or 2)-sulfophenyl]xanthylum, Inner Salt (6). Compound **6** was prepared in a similar manner as described for the synthesis of **3** starting with LRB-SC instead of TR-SC. Compound **5** (RhR-X) was obtained as a dark red solid;

yield, 62%; one spot on TLC ($R_f = 0.37$, 20% methanol in chloroform); ^1H NMR (DMSO- d_6) δ 8.40 (s, 1H, ArH), 7.96–7.85 (m, 2H, 2 $\times$ ArH), 7.47 (d, 1H, ArH), 7.08–7.02 (m, 2H, 2 $\times$ ArH), 6.98 (d, 1H, ArH), 6.93 (s, 2H, 2 $\times$ ArH), 3.73–3.60 (q, 8H, 4 $\times$ CH₂), 2.90–2.83 (m, 2H, CH₂), 2.19–2.11 (m, 2H, CH₂), 1.50–1.40 (m, 4H, 2 $\times$ CH₂), 1.30–1.26 (m, 2H, CH₂), 1.25 (t, 12H, 4 $\times$ CH₃); absorption maximum, 570 nm (ϵ 114 850 $\text{cm}^{-1} \text{M}^{-1}$), emission maximum, 590 nm in pH 7.5 phosphate buffer; high-resolution FAB-MS, m/e ($M + H$) calcd 672.2416, found 672.2417. Compound **6** was obtained as a dark red solid; yield, 83%; one spot on TLC ($R_f = 0.36$, 15% methanol in chloroform); ^1H NMR (DMSO- d_6) δ 8.41 (s, 1H, ArH), 7.96–7.88 (m, 2H, 2 $\times$ ArH), 7.48 (d, 1H, ArH), 7.07–7.01 (m, 2H, 2 $\times$ ArH), 6.99 (d, 1H, ArH), 6.96 (s, 2H, 2 $\times$ ArH), 3.70–3.57 (q, 8H, 4 $\times$ CH₂), 2.89–2.81 (m, 2H, CH₂), 2.80 (s, 4H, 2 $\times$ CH₂), 2.69–2.63 (m, 2H, CH₂), 1.70–1.49 (m, 2H, CH₂), 1.52–1.35 (m, 4H, 2 $\times$ CH₂), 1.20 (t, 12H, 4 $\times$ CH₃); high-resolution FAB-MS, m/e ($M + H$) calcd 769.2580, found 769.2579. Anal. Calcd for C₃₇H₄₄N₄O₁₀S₂·0.5H₂O: C, 57.14; H, 5.83; N, 7.21. Found: C, 57.08; H, 5.80; N, 7.20.

9-[2(or 4)-[[[5-(*N*-Iodoacetyl)aminopentyl]amino]sulfonyl]-4(or 2)-sulfophenyl]-2,3,6,7,12,13,16,17-octahydro-1H,5H,11H,15H-xantheno[2,3,4-ij:5,6,7-i'j']diquinolizin-18-ium, Inner Salt (8). To a suspension of Texas Red cadaverine (**7**) (50 mg, 0.07 mmol) in 10 mL of chloroform was added *N,N*-diisopropylethylamine (15 μL , 0.09 mmol), followed by addition of iodoacetic anhydride (25 mg, 0.07 mmol), and the mixture was stirred at room temperature in the dark for 5 h. The reaction mixture was then diluted with 50 mL of chloroform, washed with water (3 $\times$ 50 mL), dried over anhydrous sodium sulfate, and concentrated under reduced pressure to give a crude product. The product was purified by column chromatography on silica gel with 5% methanol in chloroform as eluant to give 14 mg (88%) of **8** as a dark purple solid; one spot on TLC ($R_f = 0.21$, 10% methanol in chloroform); ^1H NMR (DMSO- d_6) δ 8.38 (d, 1H, ArH), 7.90–7.80 (m, 1H, ArH), 7.38–7.35 (m, 1H, ArH), 6.50 (d, 2H, 2 $\times$ ArH), 3.60 (s, 2H, CH₂I), 3.58–3.40 (m, 8H, 4 $\times$ CH₂), 3.05 (m, 6H, 3 $\times$ CH₂), 2.68–2.54 (m, 6H, 3 $\times$ CH₂), 2.08–1.98 (m, 4H, 2 $\times$ CH₂), 1.90–1.78 (m, 4H, 2 $\times$ CH₂), 1.45–1.12 (m, 6H, 3 $\times$ CH₂); absorption maximum, 591 nm, emission maximum, 613 nm in pH 7.5 phosphate buffer; high-resolution FAB-MS, m/e ($M + H$) calcd 859.1699; found 859.1700.

9-[2(or 4)-[[[5-(*N*-Bromoacetyl)aminopentyl]amino]sulfonyl]-4(or 2)-sulfophenyl]-2,3,6,7,12,13,16,17-octahydro-1H,5H,11H,15H-xantheno[2,3,4-ij:5,6,7-i'j']diquinolizin-18-ium, Inner Salt (9). To a suspension of Texas Red cadaverine (50 mg, 0.07 mmol) in 10 mL of chloroform was added *N,N*-diisopropylethylamine (15 μL , 0.09 mmol), followed by addition of bromoacetyl bromide (7 μL , 0.08 mmol), and the reaction mixture was stirred at room temperature in the dark for 5 h. It was then diluted with 50 mL of chloroform, washed with water (3 $\times$ 50 mL), dried over anhydrous sodium sulfate, and concentrated under reduced pressure to give a crude product. It was purified by column chromatography on silica gel with 5% methanol in chloroform as eluant to give 41 mg (73%) of **9** as a dark purple solid; one spot on TLC ($R_f = 0.20$, 10% methanol in chloroform); ^1H NMR (DMSO- d_6) δ 8.38 (d, 1H, ArH), 7.91–7.80 (m, 1H, ArH), 7.40–7.35 (m, 1H, ArH), 6.50 (d, 2H, 2 $\times$ ArH), 3.80 (s, 2H, CH₂Br), 3.60–3.40 (m, 8H, 4 $\times$ CH₂), 3.06–2.83 (m, 6H, 3 $\times$ CH₂), 2.67–2.53 (m, 6H, 3 $\times$ CH₂), 2.09–1.97 (m, 4H, 2 $\times$ CH₂), 1.90–1.78 (m, 4H, 2 $\times$ CH₂), 1.44–1.12 (m, 6H, 3 $\times$ CH₂); absorption maximum, 583 nm (ϵ 112 700 $\text{cm}^{-1} \text{M}^{-1}$), emission

maximum, 603 nm in methanol; high-resolution FAB-MS, m/e ($M + H$) calcd 811.1837, found 811.1840.

9-[2(or 4)-[[[(5-Maleimidylpentyl)-*N*-acetyl]amino]sulfonyl]-4(or 2)-sulfophenyl]-2,3,6,7,12,13,16,17-octahydro-1*H*,5*H*,11*H*,15*H*-xantheno[2,3,4-*ij*:5,6,7-*i'*']diquinolizin-18-ium, Inner Salt (10**).** To a solution of Texas Red cadaverine (50 mg, 0.07 mmol) and *N,N*-diisopropylethylamine (25 μ L, 0.14 mmol) in 5 mL of chloroform was added maleic anhydride (10 mg, 0.10 mmol). After the reaction mixture was stirred at room temperature for 5 h, it was concentrated to a volume of 1 mL under reduced pressure. The residual solution was poured into 10 mL of ether. The resulting precipitate was collected by filtration to give 43 mg of the maleamic acid intermediate as a dark purple solid. A suspension of this intermediate and 10 mg of sodium acetate in 2 mL of acetic anhydride was heated at 80 °C. After 0.5 h, the reaction mixture was concentrated under reduced pressure. The residue was diluted with 50 mL of chloroform, washed with water (3 $\times$ 50 mL), dried over anhydrous sodium sulfate, and concentrated under reduced pressure to give a crude product. It was purified by chromatography on silica gel with 5% methanol in chloroform as eluant to give 14 mg (25%) of **10** as a dark purple solid; one spot on TLC (R_f = 0.13, 10% methanol in chloroform); ^1H NMR ($\text{DMSO}-d_6$) δ 8.41 (s, 1H, ArH), 8.08 (d, 1H, ArH), 7.44 (d, 1H, ArH), 7.01 (d, 2H, $-\text{CH}=\text{CH}-$), 6.44 (s, 2H, 2 $\times$ ArH), 3.58–1.19 (m, 34H, 17 $\times$ CH_2), 1.97 (s, 3H, CH_3); absorption maximum, 601 nm, emission maximum, 619 nm, in pH 7.5 phosphate buffer; high-resolution FAB-MS, m/e ($M + 1$) calcd 813.2631, found 813.2633.

9-[2(or 4)-[(2-Maleimidylethyl)amino]sulfonyl]-4(or 2)-sulfophenyl]-2,3,6,7,12,13,16,17-octahydro-1*H*,5*H*,11*H*,15*H*-xantheno[2,3,4-*ij*:5,6,7-*i'*']diquinolizin-18-ium, Inner Salt (11**).** To a solution of *N*-(2-aminoethyl)maleimide trifluoroacetic acid salt (100 mg, 0.39 mmol) and *N,N*-diisopropylethylamine (135 μ L, 0.78 mmol) in 30 mL of dichloromethane was added TR-SC (245 mg, 0.39 mmol) in small portions over a period of 5 min. After the reaction mixture was stirred at room temperature overnight, it was washed with water (2 $\times$ 30 mL). The organic layer was separated, dried over anhydrous sodium sulfate, and concentrated under reduced pressure to give a crude product. It was purified by column chromatography on silica gel with 5% methanol in chloroform to give 185 mg (65%) of **11** as a dark purple solid; one spot on TLC (R_f = 0.45, 15% methanol in chloroform); ^1H NMR (CDCl_3) δ 8.51 (s, 1H, ArH), 7.79 (d, 1H, ArH), 7.13 (d, 1H, ArH), 6.70 (s, 2H, $-\text{CH}=\text{CH}-$), 6.68 (s, 2H, 2 $\times$ ArH), 3.60 (t, 2H, CH_2), 3.50–3.34 (m, 8H, 4 $\times$ CH_2), 3.20 (t, 2H, CH_2), 3.04–2.90 (m, 4H, 2 $\times$ CH_2), 2.72–2.52 (m, 4H, 2 $\times$ CH_2), 2.10–1.90 (m, 8H, 4 $\times$ CH_2); absorption maximum, 582 nm (ϵ 108 100 $\text{cm}^{-1} \text{M}^{-1}$), emission maximum, 600 nm in methanol; high-resolution FAB-MS, m/e ($M + 1$) calcd 729.2055, found 729.2050.

Spectral Properties of Texas Red-X and Rhodamine Red-X Derivatives. Figures 4 and 5 show the similarity of the normalized absorption and fluorescence emission spectra of TR-X and RhR-X with those of sulforhodamine 101 and sulforhodamine B, dissolved in phosphate-buffered saline (PBS). The "X" derivatives exhibit a very small shift (~ 5 nm) toward the red portion of the spectrum. RhR-X conjugates can be efficiently excited by the 568 nm line of the krypton ion laser. Both dyes are well suited for multiple labeling of biopolymers, as their fluorescence emission spectrum is further separated from fluorescein than that of tetramethylrhodamine.

Conjugations of TR-X and RhR-X Succinimidyl

Table 1. Labeling Efficiency of Texas Red Sulfonyl Chloride versus Texas Red-X Succinimidyl Ester, and Lissamine Rhodamine B Sulfonyl Chloride versus Rhodamine Red-X Succinimidyl Ester As Determined by Preparing Conjugates of Selected Proteins with Different Dye-to-Protein Molar Ratios^a

protein	dye:protein ratio	fluorophores/protein (mol/mol)			
		TR-SC	LRB-SC	TR-X-SE	RhR-X-SE
BSA	15.0	3.2		4.5	
GAM	2.5	0.6		1.3	
GAM	5.5	1.0		2.5	
GAM	8.0	1.3		3.3	
streptavidin	1.0	0.1		0.8	
streptavidin	2.5	1.2		1.6	
streptavidin	5.5	2.4		4.4	
streptavidin	8.0	3.0		5.6	
streptavidin	10.0	3.9		6.0	
streptavidin	3		0.8		2.4
streptavidin	5		1.2		3.5
streptavidin	7		1.4		4.9
streptavidin	10		1.8		5.7
streptavidin	15		2.2		5.8

^a Conjugations with TR-SC and LRB-SC were performed at 4 °C and pH 9. TR-X-SE and RhR-X-SE conjugates were prepared at room temperature and pH 8.3. The results demonstrate that TR-X-SE and RhR-X-SE consistently yield conjugates with more dyes per protein.

Esters to Proteins. To compare the reactivity of TR-X-SE and RhR-X-SE with that of the respective sulfonyl chloride analogs, the dyes were reacted with different proteins at the molar ratios of dye-to-protein of 1:1 to 1:15, as reported in Table 1. Proteins were either dissolved at 10 mg/mL in 0.1 M NaHCO_3 , pH 8.3, or, when already solubilized in PBS, diluted with $1/10$ volume of 1 M NaHCO_3 , pH 8.3. The dye was then weighed and dissolved at 10 mg/mL in dimethylformamide (DMF). Aliquots of the dye solution were immediately added to the protein at the desired molar ratios. Reactions were run for 1 h at room temperature, at which point $1/10$ volume of 1.5 M hydroxylamine (adjusted to pH 8) was added and the reaction was left for an additional 30 min. Hydroxylamine serves to stop the reaction and to dissociate the dye from unstable conjugates formed with alcohols (Brinkley, 1992). Any visible precipitate was removed by microcentrifugation, and the conjugate that remained in solution was then purified on Bio-Gel P-30, fine grade, spin columns (Bio-Rad, Hercules, CA).

Conjugation of TR-SC and LRB-SC to Proteins. These conjugations were performed in parallel with the conjugations of TR-X-SE and RhR-X-SE (see above), except that the buffer pH was adjusted to pH 9.0 rather than pH 8.3. Conjugations with TR-SC were performed in an ice bath. Low temperatures and pH 9.0 are the optimal conditions for conjugations with TR-SC, as reported by Titus et al. (1982). The dyes were dissolved in DMF at 10 mg/mL with brief sonication, and aliquots were immediately added to the protein at the calculated molar ratios. The reactions were allowed to proceed for 1 h and then stopped by the addition of $1/10$ volume of 1.5 M hydroxylamine, pH 8.0. Any visible precipitate was spun down, and the conjugates were purified on Bio-Gel P-30 spin columns. The absence of free dye in the conjugate solution was assayed by thin-layer chromatography in chloroform/methanol (70:30) and, when necessary, the column purification repeated until no free dye was detected.

Dye/Protein Degree of Labeling. Molarities of dye and protein in the conjugates were calculated from the absorbances of the conjugates at 280 nm and at the absorbance peaks of the different dyes. Since both fluorophores also absorb at 280 nm, their contribution

to the absorbance at that wavelength was subtracted to determine the absorbance due to protein. The absorbance contributions of conjugates of TR-X-SE and TR-SC at 280 nm are both about 20% that of their peak absorbance at 595 nm, based on the spectra of the acid forms of the dyes, while absorbance at 280 nm of both LRB-SC and RhR-X-SE is about 17% that of the dyes at their absorbance peaks. The degree of labeling was calculated as the molarity of dye divided by the molarity of protein, as described by Haugland (1995).

Cross-Binding of Anti-Texas Red and Anti-Tetramethylrhodamine Antibodies to TR-X and RhR-X. Similarity between TR-X or RhR-X and sulforhodamine 101 or sulforhodamine B was investigated by measuring the fluorescence quenching capacity of rabbit polyclonal antibodies to these fluorophores. These antibodies were raised against KLH labeled with TR-SC or with tetramethylrhodamine isothiocyanate, respectively (Molecular Probes). Identical aliquots of antibody solutions at the concentration of 1 mg/mL were added to 2 mL of 5×10^{-8} M TR-X (2) or RhR-X (5). The fluorescence output was measured after each addition until no further quenching of the fluorescence was obtained (saturation).

Time Course of the Hydrolysis of TR-SC and TR-X-SE. The ability of TR-X-SE and TR-SC to form conjugates after exposure to water was investigated by dissolving the reactive dyes at 10 mg/mL in DMF and diluting 4-fold in water to bring the final composition of the solution to 25% DMF. Following incubation at room temperature for 1, 5, 10, 20, 30, and 60 min after dilution with water, aliquots of the dye solutions were used to label 2 mg of goat IgG dissolved in 0.2 mL of buffer that had been adjusted with a solution of sodium bicarbonate to pH 8.3 for TR-X-SE and to pH 9.0 for TR-SC. The labeling reactions were left for 1 h at room temperature, and then the conjugates were purified on spin columns as previously described.

Phalloidin Labeling with TR-X-SE. To a solution of 3 mg of aminophalloidin *p*-toluenesulfonate (Wieland et al., 1983) and 4 mg of TR-X-SE in 300 μ L of DMF was added 5 μ L of triethylamine, and the mixture was stirred at room temperature for 1 h. To the reaction mixture was added 7 mL of ether, and the resulting precipitate was collected by centrifugation. The crude product was purified by chromatography over lipophilic Sephadex LH-20 using water for elution. The desired fractions were combined and lyophilized to give 3 mg of a dark purple solid of the TR-X phalloidin conjugate; one spot on TLC (R_f = 0.53, 70 chloroform/27 methanol/3 water).

F-Actin Staining by TR-X Phalloidin. F-Actin in 2-day-old bovine aorta endothelial cell (BAEC) cultures was stained with TR-X phalloidin according to the standard method described by Haugland et al. (1994).

Labeling of an Oligonucleotide Primer with TR-X-SE. To a solution of 500 μ g of a 5'-amine-modified, 24-base M13 primer sequence in 220 μ L of sodium bicarbonate, pH 8.5, was added a solution of 1 mg of TR-SC or TR-X-SE, each dissolved in 35 μ L of DMF. After 16 h at room temperature, the conjugates were precipitated by the addition of salt and cold ethanol. The labeled oligonucleotides were purified by HPLC on a C_8 reversed phase column (Rainin Instrument Co., Woburn, MA) with a gradient of acetonitrile in 0.1 M triethylamine, pH 7.0. Detection was accomplished using a Waters 490 detector, monitoring at 254 and 590 nm.

Cell Staining and Photobleaching Studies Using Streptavidin Conjugates of TR-SC and TR-X-SE. Cell staining was performed on commercially prepared HEP-2 ANA slides (INOVA Diagnostics Inc., San Diego,

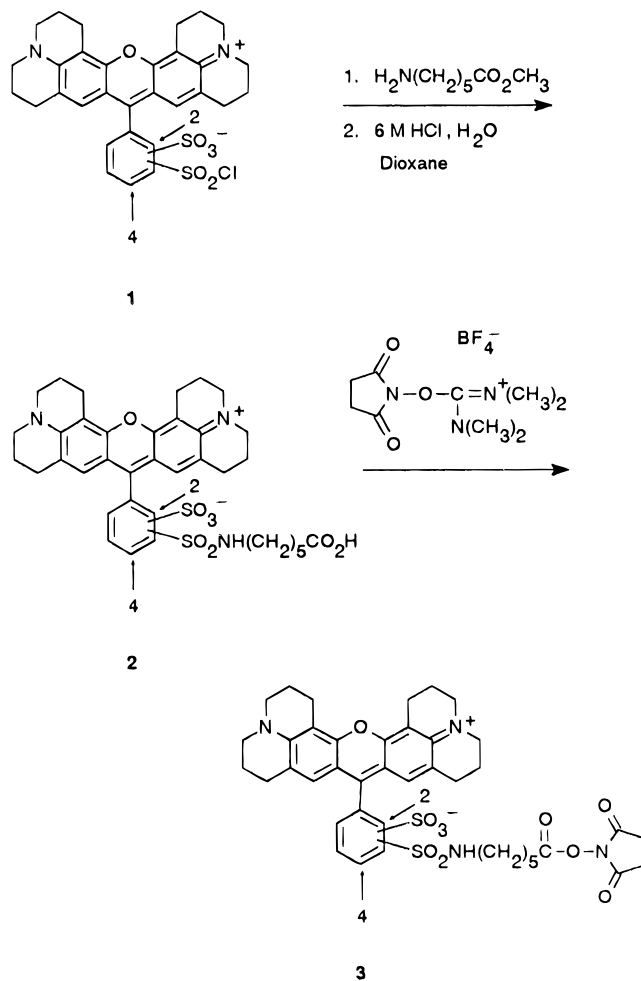


Figure 1. Reaction scheme for the synthesis of TR-X-SE (3) from TR-SC (1).

CA). Human anti-nuclear autoantibody (INOVA Diagnostics Inc.) was applied to the positive wells, while negative control wells were incubated in PBS. All of the wells were then treated with mouse anti-human IgG (Zymed Laboratories, S. San Francisco, CA) followed by biotinylated protein A. Conjugates of streptavidin with either TR-SC or TR-X-SE were added at 1, 5, and 10 μ g/mL in 1% BSA/PBS. The slides were then viewed through a longpass filter optimized for Texas Red conjugates (Omega Optical Inc., Brattleboro, VT).

The above HEP-2 cells, labeled with the Texas Red-streptavidin conjugates, were illuminated with a 100 W mercury arc lamp using a 40×0.65 NA E PLAN lens (Nikon, Inc., Melville, NY). Images were acquired using a Star-1 CCD camera (Photometrics, Tucson, AZ) at 5 s intervals over a period of continuous illumination totaling 100 s. The fluorescence intensity at each time point was integrated over the frame using an Image 1 processor (Universal Imaging, West Chester, PA) with the signal threshold set above background.

Photobleaching in solution was conducted by illuminating streptavidin conjugates that had been adjusted to the same optical density at their excitation maxima while their emission intensity over time was measured. Fluorescence spectra were measured using an SPF-500C spectrofluorometer (SLM Instruments Inc., Urbana, IL).

Conjugation of the Thiol-Reactive Texas Red Derivatives to β -Galactosidase. As an example of the possibility of obtaining thiol derivatives with the spectral properties of TR, β -galactosidase was conjugated with Texas Red iodoacetamide, bromoacetamide, or maleim-

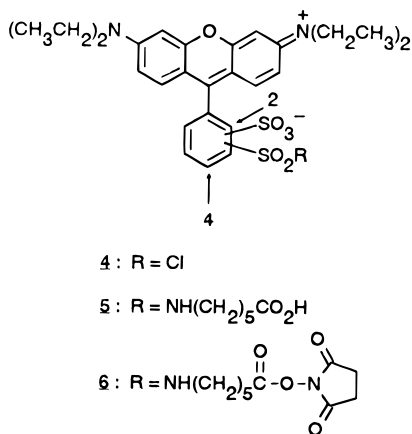


Figure 2. Structures of LRB-SC (**4**), RhR-X (**5**), and RhR-X-SE (**6**).

ide. The protein was dissolved at 10 mg/mL in 0.1 M sodium phosphate, 0.1 M NaCl, and 2 mM EDTA, pH 7.5, and the dyes were dissolved at 10 mg/mL in DMF using a molar ratio of dye-to-protein of 15. The reactions were performed under argon for 1 h at room temperature. Because iodoacetamide derivatives are light sensitive, exposure to light was strictly limited throughout the conjugation procedure. The conjugates were purified by size exclusion chromatography on Cellufine GH-25 (Amicon, Beverly, MA) columns equilibrated in the same pH 7.5 buffer.

RESULTS AND DISCUSSION

Synthesis of TR-X and RhR-X Reactive Forms.

The synthesis of TR-X-SE, (**3**), which involved three

steps, is outlined in Figure 1. The aminohexanoic acid derivative, TR-X (**2**), was prepared using modifications of a published procedure (Abuelyaman et al., 1994). Use of acidic hydrolysis went more smoothly than the published method. In a similar manner, RhR-X-SE (**6**) was prepared in three steps starting from LRB-SC (**4**) (Figure 2). Thiol-reactive Texas Red haloacetamide and maleimide derivatives were prepared as shown in Figure 3. To make a Texas Red maleimide derivative, Texas Red cadaverine (**7**) was converted to an intermediate maleamic acid, which was then cyclized under dehydrative conditions using acetic anhydride/sodium acetate (Mehta et al., 1960). Cyclization of the maleamic acid derivative produced compound **10**, which contains an *N*-acetyl group in addition to the maleimide, as a major product. Its structure was confirmed by high-resolution mass spectrometry and ¹H NMR analysis.

Spectral Properties of TR-X-SE and RhR-X-SE.

As illustrated in Figures 4 and 5, the spectral properties of TR-X-SE and RhR-X-SE are very similar to those of their parent molecules. The absorption and fluorescence emission spectra of both dyes shift only a few nanometers.

Cross-Binding of Anti-Texas Red and Anti-Tetramethylrhodamine Antibodies. Similarity of TR-X and RhR-X to sulforhodamine 101 and sulforhodamine B was confirmed by comparing the fluorescence quenching ability of anti-Texas Red and anti-tetramethylrhodamine antibodies when added to the respective solutions of the above dyes. We determined that, by coincidence, the same amount of antibody (90 μg) was needed to maximally quench either form of dye, when either dye was dissolved at a concentration of 5 × 10⁻⁸ M.

TR-X and RhR-X Conjugates. The fluorescence yields of protein conjugates with TR-X-SE and RhR-X-

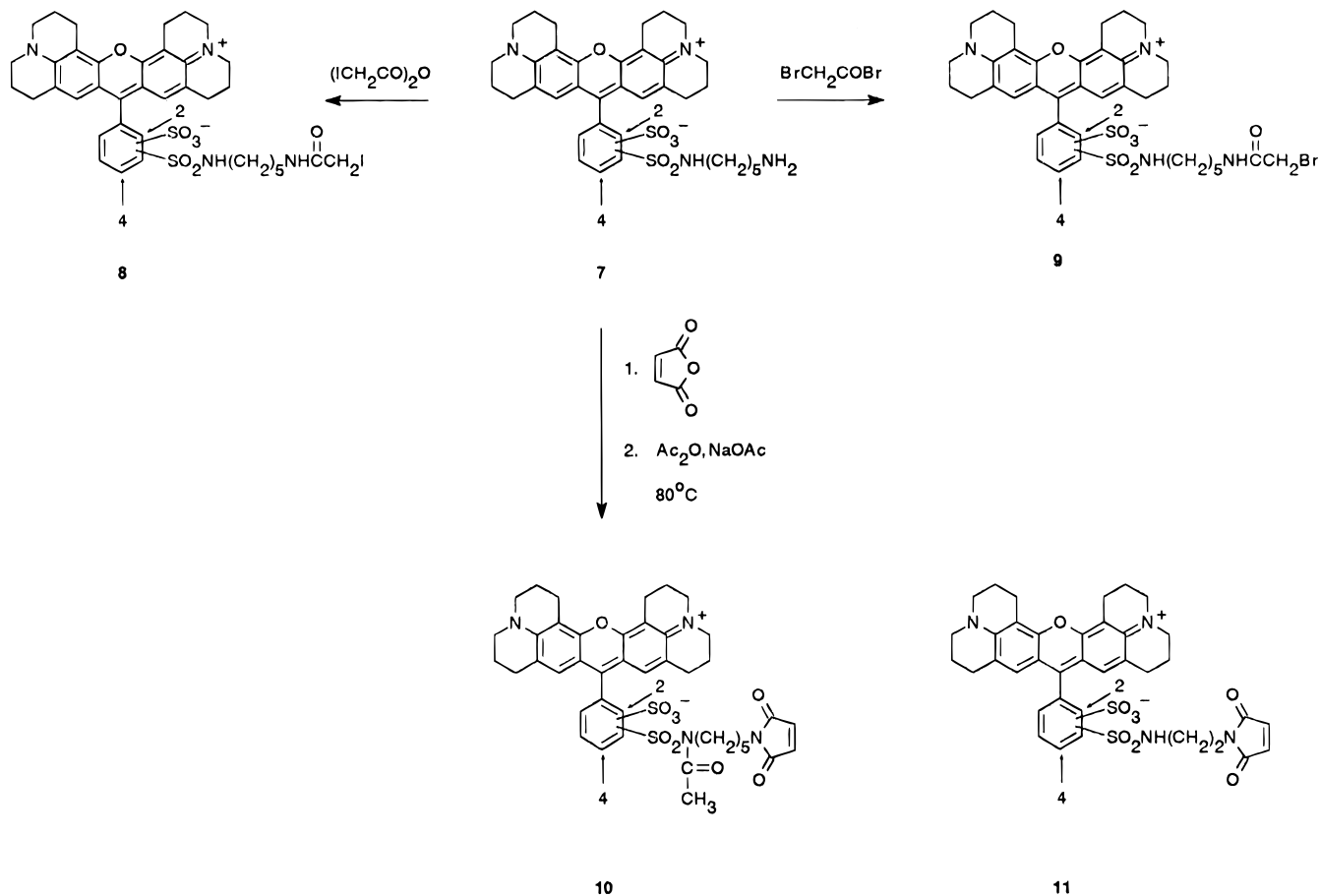


Figure 3. Reaction scheme for the synthesis of haloacetamide and maleimide derivatives of Texas Red from Texas Red cadaverine (**7**).

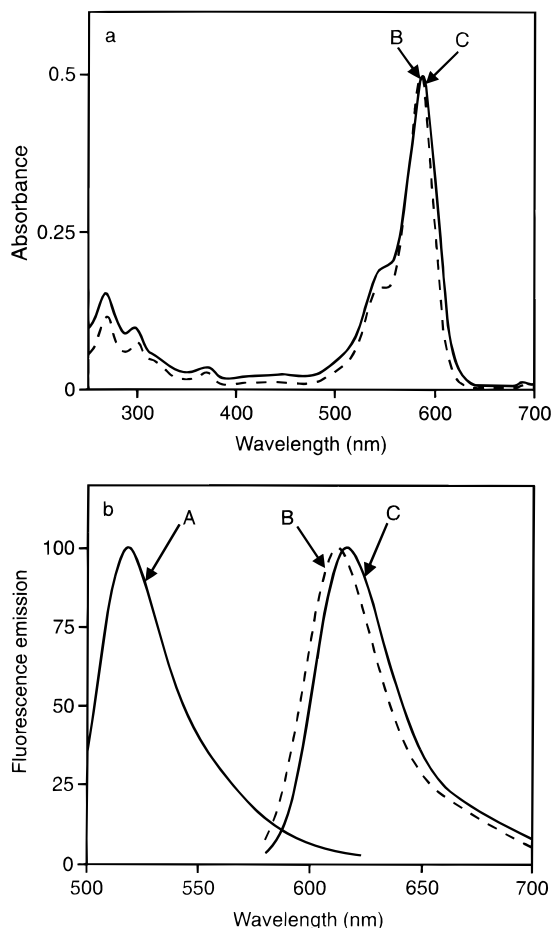


Figure 4. Normalized absorption of sulforhodamine 101 (B) and TR-X (C) (panel a) and normalized emission spectra (panel b) of fluorescein (A), sulforhodamine 101 (B), and TR-X (C) in PBS. The patterns of the absorption and emission of both Texas Red dyes are very similar and well distinguished from that of fluorescein.

SE are shown in Figures 6 and 7, respectively. Both new dyes produced conjugates of streptavidin and goat anti-mouse (GAM) that were more fluorescent than the conjugates prepared from their sulfonyl chloride analogs. We also observed less precipitation of protein conjugates prepared from TR-X-SE and RhR-X-SE than of conjugates prepared from the sulfonyl chloride derivatives. TR-X-SE produced brighter conjugates with goat IgG, goat anti-rabbit IgG, streptavidin, wheat germ agglutinin, and concanavalin A than did TR-SC (part of the data not shown). All of the conjugates prepared from TR-X-SE that we have tried have higher fluorescence than conjugates of TR-SC, with the exception of bovine serum albumin, DNase I, human transferrin, and dextrans. However, TR-X-SE and RhR-X-SE clearly react with greater efficiency with biopolymers than TR-SC and LRB-SC. Table 1 shows that much lower ratios of TR-X-SE and RhR-X-SE are required to achieve the same given degree of labeling. Banks and Paquette (1995) reported that, when compared to the isothiocyanate and dichlorotriazine derivatives, the succinimidyl ester derivatives of fluorophores are more reactive and yield more stable conjugates.

Figure 6 shows that the total fluorescence of TR-X-SE GAM conjugate, determined as described in the legend, is about 65% higher than the fluorescence of TR-SC conjugate, while the total fluorescence of TR-X-SE-streptavidin is about double the fluorescence of TR-SC-streptavidin. The curves have a broad bell shape, because the fluorescence of the conjugates is being

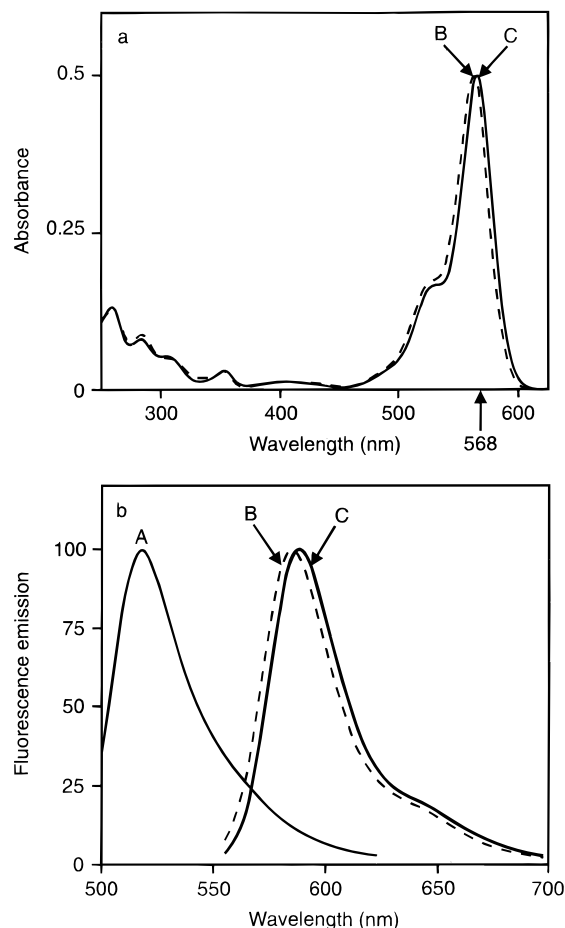


Figure 5. Normalized absorption of sulforhodamine B (B) and RhR-X (C) (panel a) and overlap of the normalized emission spectrum of fluorescein (A) with the normalized emission spectra of sulforhodamine B (B) and RhR-X (C) (panel b). The arrow at 568 nm of the absorption spectrum shows that this probe is suitable for excitation by the krypton ion laser.

quenched after the optimal number of fluorophores per protein is reached. The difference in the number of dyes that can be conjugated to the protein before quenching occurs is more similar for GAM (2–3) than for streptavidin. The latter can be labeled with 4 TR-X-SE/protein before the fluorescence is quenched, while the fluorescence of the TR-SC conjugate is already quenched by 2.5–3 TR-SC/streptavidin. Similar results are shown in Figure 7 for a comparison of conjugates of GAM or streptavidin with LRB-SC and RhR-X-SE. Thus, it appears that, especially for streptavidin, not only are the conjugates with TR-X-SE or RhR-X-SE more fluorescent, but they can be labeled with a larger number of fluorophores before their fluorescence is quenched.

Stability of TR-X-SE. The resistance of TR-X-SE to hydrolysis is striking when compared to the hydrolysis of sulfonyl chloride, as can be seen in Table 2. To compare rates of hydrolysis of TR-SC and TR-X-SE, the dyes were reacted with GAM IgG following incubation in a mostly aqueous environment (75% buffer, 25% DMF) for various periods of time. The degrees of labeling of the resulting conjugates were used to evaluate the relative extent of hydrolysis that occurred before the beginning of the conjugation reaction. After 5 min in aqueous solution, TR-SC failed to produce any detectable conjugate, whereas TR-X-SE continued to produce satisfactory yields of conjugates, even after an hour.

Qualitative evaluation of HEp-2 cells stained with streptavidin conjugates of TR-X-SE and/or TR-SC indicated that the streptavidin conjugate prepared with TR-

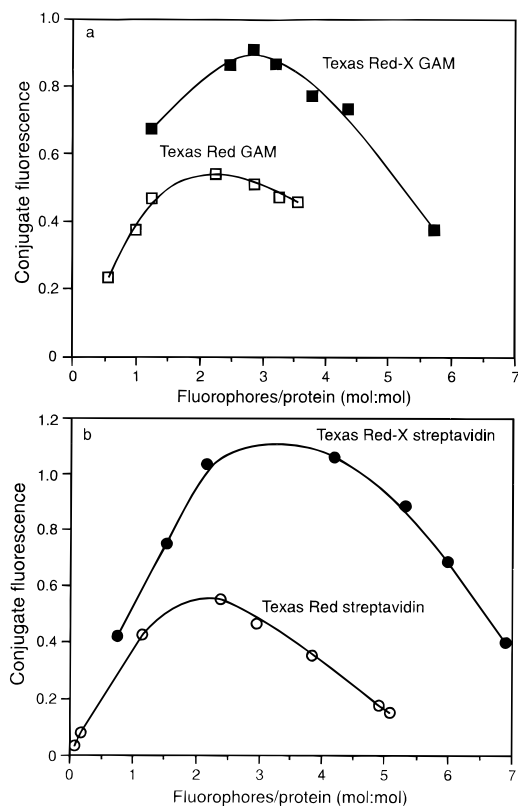


Figure 6. Increase in total fluorescence of GAM (panel a) and streptavidin conjugates (panel b) prepared with TR-SC or TR-X-SE. Conjugate fluorescence is obtained by multiplying the number of fluorophores/protein by the relative quantum yield, which is determined using sulforhodamine 101 as standard.

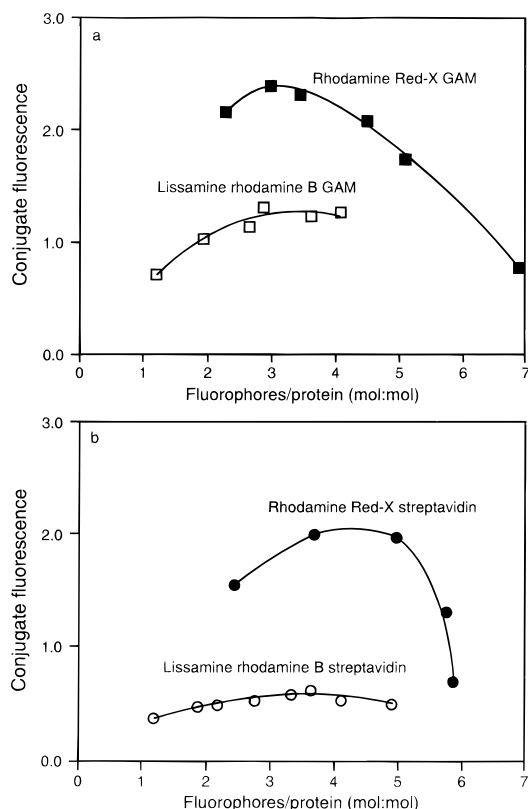


Figure 7. Results analogous to those illustrated in Figure 6 showing the difference in total fluorescence of conjugates prepared from LRB-SC and RhR-X-SE.

X-SE produces brighter staining of the nuclei with less nonspecific background than the streptavidin conjugate

Table 2. Stability of Texas Red Sulfonyl Chloride and Texas Red-X Succinimidyl Ester in Aqueous Solution^a

time (min)	degree of labeling (dyes:protein)	
	TR-SC	TR-X-SE
1	0.30	4.09
5	<0.1	3.64
10	<0.1	3.38
20	<0.1	2.96
30	<0.1	3.00
60	<0.1	2.78

^a To compare the rates of hydrolysis of TR-SC and TR-X-SE, the two compounds were dissolved at 10 mg/mL in 0.1 M sodium bicarbonate, pH 8.3, containing 25% DMF, and incubated for 1, 5, 10, 20, 30, and 60 min at room temperature. The dyes were then used at a molar ratio of 10 dyes per protein to label GAM. The degree of labeling of the resulting conjugates reported here shows that, within 5 min, the hydrolysis of TR-SC is complete and no conjugate is obtained, while TR-X-SE yields adequate labeling, even after 1 h in aqueous solution.

of TR-SC. The best signal-to-noise ratios were obtained with streptavidin-dye concentrations of 1 μ g/mL. Quantitative measurements averaged over three fields for each dye, conducted as part of the photobleaching study, indicated that cells stained with the streptavidin conjugate of TR-X-SE were approximately twice as bright as those stained with streptavidin conjugates of TR-SC (data not shown).

The photobleaching results from cells stained with streptavidin conjugates show that the photolysis rates of conjugates of TR-SC and TR-X-SE are nearly identical. Also, the photostabilities of these conjugates in solution, as measured in a spectrophotometer, were found to be identical (data not shown).

The thiol-reactive Texas Red iodoacetamide, bromoacetamide, and maleimide derivatives all successfully reacted with β -galactosidase, giving conjugates with degrees of labeling of 7–12 dyes per molecule and fluorescence quantum yield ranging from 0.2 to 0.5 relative to sulforhodamine 101 used as a standard. They produced some precipitation, though recovery of the protein remained acceptable. The pattern obtained by staining BAEC with TR-X phalloidin is very specific and bright (not shown). The pattern of staining is similar to the one obtained using rhodamine or fluorescein phalloidin. However, the longer wavelength emission of Texas Red phalloidin should be advantageous in multi-color staining of cell organelles or features.

A comparison of the efficiency of labeling an M13 primer with TR-SC or TR-X-SE showed that TR-X-SE conjugated with the oligonucleotide twice as efficiently as TR-SC at the same molar ratio, as determined by comparing the integrated absorbance of the product peak to that of the unlabeled oligonucleotide during HPLC (data not shown).

These synthetic modifications of the sulforhodamine 101 and sulforhodamine B fluorophores have improved the ease and reproducibility of conjugations and have provided conjugates of a higher quality and, in some cases, conjugates that could not have been made with the sulfonyl chloride derivatives, such as Texas Red-X phalloidin and thiol-labeled Texas Red β -galactosidase. Also, proteins that do not tolerate exposure to high pH, such as IgM, can now be successfully conjugated with TR-X-SE and RhR-X-SE, because the reaction can be performed at pH 7.2 by moderately increasing the dye-to-protein molar ratio. Thus, the availability of TR-X and RhR-X dyes in stable reactive forms considerably increases the range of conjugates that can be prepared that have red fluorescence emission.

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LITERATURE CITED

- Abuelyaman, A. S., Hudig, D., Woodard, S. L., and Powers, J. C. (1994) Fluorescent derivatives of diphenyl [1-(*N*-peptidylamino)alkyl]phosphonate esters: synthesis and use in the inhibition and cellular localization of serine proteases. *Bioconjugate Chem.* 5, 400–405.
- Banks, P. R., and Paquette, D. M. (1995) Comparison of three common amine reactive fluorescent probes used for conjugation to biomolecules by capillary zone electrophoresis. *Bioconjugate Chem.* 6, 447–458.
- Bannwarth, W., et al. (1991) Formation of carboxamides with *N,N,N',N'*-tetramethyl(succinimido)uronium tetrafluoroborate in aqueous/organic solvent systems. *Tetrahedron Lett.* 32, 1157–1160.
- Brinkley, J. M. (1992) A brief survey of methods for preparing protein conjugates with dyes, haptens, and cross-linking reagents. *Bioconjugate Chem.* 3, 2–13.
- Drexhage, K. H. (1990) Structure and properties of laser dyes. *Dye Lasers* (F. P. Schäfer, Ed.) 3rd ed., pp 155–200, Springer-Verlag, Berlin.
- Haugland, R. P. (1995) Coupling of monoclonal antibodies with fluorophores. *Methods In Molecular Biology, Vol. 45: Monoclonal Antibody Protocols* (W. C. Davis, Ed.) pp 205–221, Humana Press, Clifton, NJ.
- Haugland, R. P., You, W., Paragas, V. B., Wells, K. S., and DuBose, D. A. (1994) Simultaneous visualization of G- and F-actin in endothelial cells. *J. Histochem. Cytochem.* 42, 345–350.
- Mehta, N. B., Phillips, A. P., Fu Lui, F., and Brooks, R. E. (1960) Maleamic and citraconamic acids, methyl esters and imides. *J. Org. Chem.* 25, 1012–1015.
- Staines, W. A., Meister, G., Melander, T., Nagy, J. I., and Hokfelt, T. (1988) Three-color immunofluorescence histochemistry allowing triple labeling within a single section. *J. Histochem. Cytochem.* 36, 145–151.
- Titus, J. A., Haugland, R., Sharrow, S. O., and Segal, D. M. (1982) Texas Red, a hydrophilic, red-emitting fluorophore for use with fluorescein in dual parameter flow microfluorometric and fluorescence microscopic studies. *J. Immunol. Methods* 50, 193–204.
- Walker, M. (1994) The Mitsunobu reaction: a novel method for the synthesis of bifunctional maleimide linkers. *Tetrahedron Lett.* 35, 665–668.
- Wessendorf, M. W., Appel, N. M., Molitar, T. W., and Elde, R. P. (1990) A method for immunofluorescent demonstration of three coexisting neurotransmitters in rat brain and three coexisting neurotransmitters in rat brain and spinal cord, using the fluorophores fluorescein, Lissamine rhodamine, and 7-amino-4-methylcoumarin-3-acetic acid. *J. Histochem. Cytochem.* 38, 1859–1877.
- Wieland, Th., Hollósi, M., and Nassal, M. (1983) Über die Inhaltsstoffe des grünen Knollenblätterpilzes. LXI. δ -Aminophalloin, ein 7-Analogs des Phalloidins, und biochemisch nützliche, auch fluoreszierende Derivate. *Liebigs Ann. Chem.*, 1533–1540.
- Wong, S. S. (1991) Reactive groups of proteins and their modifying agents, in *Chemistry of Protein Conjugation and Crosslinking*, p 37, CRC, Boston, MA.

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3-(2-Pyridyldithio)propionic Acid Hydrazide as a Cross-Linker in the Formation of Liposome–Antibody Conjugates

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Liposome antibody conjugates are potentially useful as a means of targeting drugs to specific tissues. A new protocol for the conjugation of IgG to maleimide-containing liposomes was developed using 3-(2-pyridyldithio)propionic acid hydrazide (PDPH) as a cross-linker. Periodate-oxidized antibody was treated with PDPH to yield a hydrazone derivative. Deprotection with DTT produced a thiolated antibody which was then conjugated to liposomes containing *N*-[4-(*p*-maleidophenyl)butyryl]-1,2-*sn*-distearoylphosphatidylethanolamine. The liposome–antibody conjugates were found to have *in vitro* properties similar to those of conjugates formed by the traditional 3-(2-pyridyldithio)propionic acid *N*-hydroxysuccinimide ester (SPDP) protocol but were cleared less rapidly in circulation. The PDPH protocol presents a viable alternative to SPDP, particularly for antibodies sensitive to amine modification.

INTRODUCTION

The potential of liposomal drug delivery systems to target specific tissues by means of surface-bound vectors, such as antibodies, is of current interest to those working in the liposome field. There have been many reported protocols for the conjugation of antibodies and other proteins to liposomal surfaces (1); however, all fall essentially into four categories: (i) derivatization of the amino functions, (ii) derivatization of the carbohydrate functions, (iii) reduction of intrinsic disulfide linkages, and (iv) conjugation of a binding domain to the liposome surface, for example, protein A or avidin. Most current protocols involve the derivatization of the protein amino functions with an appropriate cross-linker such as SPDP.¹ There are potential drawbacks to using antibodies in this approach since the amino groups may be involved directly or indirectly in antigen recognition. The global distribution of lysine residues may also result in a random orientation on the liposome surface, potentially increasing the probability of clearance due to Fc recognition.

An alternative approach involves derivatization of the carbohydrate functions after oxidation with periodate. Protocols developed for affinity columns based on this approach have been reported as having superior antigen recognition when compared to those based on amino group or intrinsic disulfide derivatization (2).

Procedures using the carbohydrate approach for coupling antibodies to liposomes have been reported (3, 4).

These systems used a liposomally incorporated lipid hydrazide which was used to conjugate oxidized IgM (3) or IgG (4). Low reaction efficiencies were observed for the IgG system compared to an analogous thiol–maleimide-based system due to the slower rate of reaction in the hydrazide–aldehyde system.

We are currently investigating the parameters required for optimizing targeted liposomal systems relative to untargeted model systems. Targeted systems have to meet two basic criteria to show enhanced drug delivery, namely an acceptable affinity for the target antigen and a reasonable circulation lifetime relative to that of the untargeted systems. We had found that the SPDP-coupled IgG systems are cleared much more rapidly than controls and show reduced tumor accumulations *in vivo* relative to controls in spite of substantially better targeting *in vitro*. This observation may be explained in terms of increased clearance by the RES and phagocytic cell populations resulting from Fc fragment presentation. We have been investigating the selective derivatization of the carbohydrate functions of IgG in an effort to orient the antibody in a uniform manner as a means of addressing this problem. In the course of this work we have developed a new protocol that combines the coupling efficiencies of the thiol–maleimide systems with the selective modification allowed by the hydrazide method. In short, periodate-oxidized IgG was derivatized with a large excess of PDPH, an S-protected thiol hydrazide, to compensate for the lower reactivity of the hydrazide–aldehyde system. Deprotection of the thiol function with DTT generated an antibody thiolated in the carbohydrate region, thus allowing rapid selective conjugation to conventional MPB-PE liposomes (Figure 1). The conjugates generated according to the new PDPH protocol were assessed with respect to coupling efficiency, aggregation, liposome composition, *in vitro* targeting ability, and *in vivo* clearance by comparison with the corresponding conjugates generated according to the SPDP protocol.

EXPERIMENTAL METHODS

PDPH was obtained from BioAffinity Systems, Roscoe, IL. DTT, 5,5'-dithiobis(2-nitrobenzoic acid) (Ellman's reagent), HEPES, 1-amino-2-naphthol-4-sulfonic acid, human IgG, sodium periodate, Sephadex G-50, and Sepharose CL-4B were obtained from Sigma, St. Louis,

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¹ Abbreviations: SPDP, 3-(2-pyridyldithio)propionic acid *N*-hydroxysuccinimide ester; PDPH, 3-(2-pyridyldithio)propionic acid hydrazide; DTT, dithiothreitol; MPB-DSPE, *N*-[4-(*p*-maleidophenyl)butyryl]-1,2-*sn*-distearoylphosphatidylethanolamine; HEPES, *N*-(2-hydroxyethyl)piperazine-*N'*-(2-ethanesulfonic acid); BSA, bovine serum albumin; HBS, HEPES-buffered saline; SAS, sodium acetate buffered saline; MePEGs-2000-DSPE, *N*-[2'-(ω -monomethoxypolyethylene glycol₂₀₀₀)succinoyl]-1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine; MePEGs-2000-Acer, 1-*O*-[2'-(ω -monomethoxypolyethylene glycol₂₀₀₀)-succinoyl]-*N*-arachidoylsphingosine; SATA, S-acetylthioglycolic acid *N*-hydroxysuccinimide ester; DSPC, 1,2-distearoyl-*sn*-glycero-3-phosphocholine; RES, reticuloendothelial system.

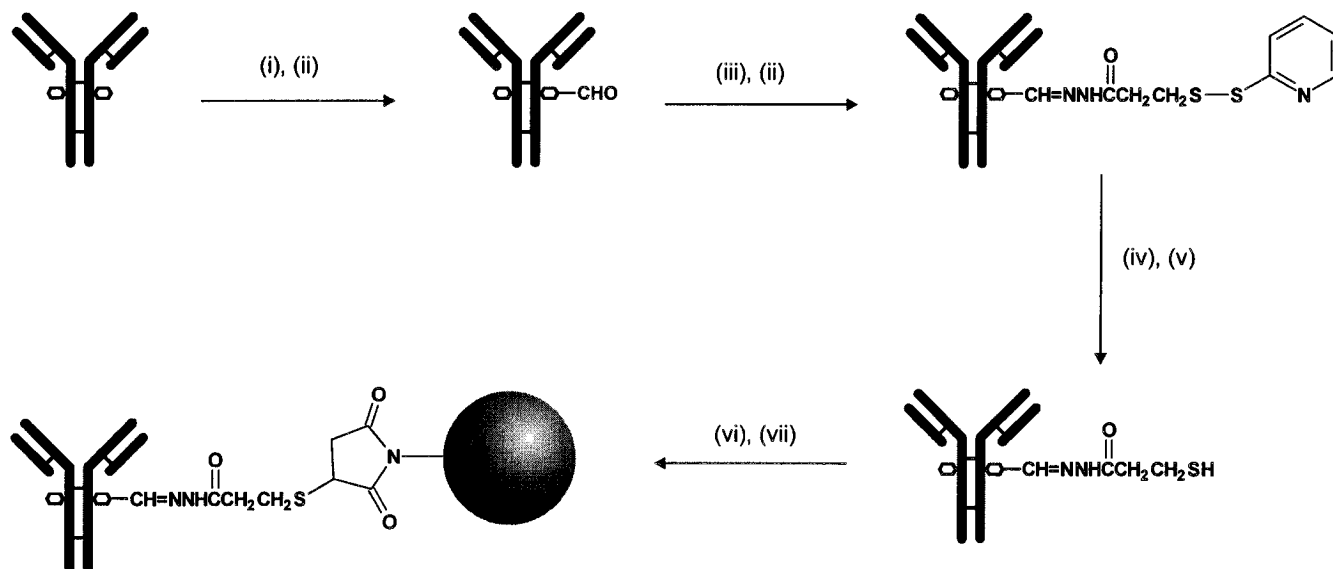


Figure 1. Synthesis of liposome–IgG conjugates: (i) NaIO_4 , 1 h, PBS pH 7; (ii) Sephadex G-50, SAS, pH 4.4; (iii) PDPH, 5 h; (iv) DTT, 20 min; (v) Sephadex G-50, HBS, pH 7.4; (vi) MPB-PE liposomes, 16 h; (vii) Sepharose CL-4B, HBS, pH 7.4

MO. Goat anti-human IgG was obtained from Jackson Laboratories, Bar Harbor, ME, mouse NR-LU-10 antibody was obtained from NeoRx Corp., Seattle, WA, and MPB-DSPE was obtained from Northern Lipids, Vancouver. SPDP and the micro BCA protein assay kit were obtained from Pierce, Rockford, IL. Ammonium molybdate, sodium chloride, NaHSO_3 , Na_2SO_3 , and anhydrous sodium acetate were obtained from BDH, Poole, U.K. HBS was prepared as 20 mM HEPES and 150 mM sodium chloride at pH 7.4. SAS was prepared as 100 mM sodium acetate and 50 mM sodium chloride at pH 4.4. Fiske reagent was prepared by dissolving 150 g of NaHSO_3 , 5 g of Na_2SO_3 , and 2.5 g of 1-amino-2-naphthol-4-sulfonic acid in 1 L of water. MePEG-2000-DSPE was prepared as previously described (5). MePEG-2000-Acer was obtained from Inex Pharmaceuticals. Human colon carcinoma LS180 cells were obtained from the American Tissue Culture Collection (Rockville, MD) and grown in minimum Eagle's medium (MEM, from Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS, from Intergen, Purchase, NY), 0.01 M HEPES, 2 mM glutamine, nonessential amino acids (from Sigma) and penicillin–streptomycin (1 unit/ μg , from Gibco). [^3H]Cholesterol hexadecyl ether was obtained from NEN (Boston, MA).

Preparation of Liposomes. Liposomes were prepared as described by Hope *et al.* (6). Briefly, lipid mixtures of the appropriate composition were dissolved in chloroform and concentrated to a homogeneous film under a nitrogen stream in a warm water bath. The film was then dried overnight in a lyophilizer. The dried lipid was hydrated in HBS at 65 °C, and the resultant multilamellar vesicles were frozen in liquid nitrogen and thawed at 65 °C five times before being extruded through stacked polycarbonate 100 nm filters (Nucleopore) using an extrusion press device (Lipex Biomembranes, Inc., Vancouver, BC) at 65 °C.

Thiolation of IgG Using SPDP. An IgG solution in PBS was treated for 20 min with 5 mol equiv of SPDP in HBS, prepared by diluting 80 μL of an ethanol stock solution (3.9 mg of SPDP/mL of ethanol) with 920 μL of HBS. The reaction mixture was passed down a Sephadex G-50 column equilibrated in SAS to isolate the derivatized antibody and replace the buffer. Fractions with an absorbance >1.0 at 280 nm were combined, and DTT was added by weight (3.8 mg of DTT/mL of solution). The

solution was stirred at room temperature for 20 min and then passed down a Sephadex G-50 column equilibrated in HBS to isolate the thiolated antibody and replace the buffer. The protein concentration was determined from the absorbance at 280 nm ($\text{mg/mL} = A_{280}/1.35$).

Thiolation of IgG Using PDPH. An IgG solution in PBS was oxidized with sodium periodate (1 mg/mL of final solution, dissolved in 0.2 mL of distilled water) at room temperature for 1 h. The antibody was isolated and the buffer exchanged by gel filtration (Sephadex G-50 equilibrated in SAS). Fractions with an absorbance at 280 nm >1.0 were combined and treated with 0.1 M PDPH in ethanol (40 μL of PDPH stock solution/mL of IgG solution) at room temperature with stirring for 5 h. The antibody was isolated by gel filtration (Sephadex G-50 equilibrated in SAS) and treated with DTT (3.86 mg/mL of IgG solution) for 20 min. The thiolated antibody was isolated and the buffer exchanged by gel filtration (Sephadex G-50 equilibrated in HBS). The protein concentration was determined from the absorbance at 280 nm ($\text{mg/mL} = A_{280}/1.35$).

Conjugation of Thiolated IgG to Liposomes. A solution of thiolated antibody was treated with liposomes at ratio of 75 or 150 μg of protein/ μmol of lipid with stirring for an appropriate time, usually 16 h. The reaction was stopped by passing the mixture down a Sepharose CL-4B column equilibrated with HBS. The protein and lipid concentrations were assayed to determine the degree of conjugation. The size of the conjugates was determined by a Nicomp particle sizer.

Lipid Assays. The lipid assay is based on phosphate content, except for the cell binding and *in vivo* experiments in which [^3H]cholesterol hexadecyl ether was used. Briefly, for the phosphate assay, samples with an estimated phosphate content in the range 50–150 nmol were digested in perchloric acid (0.7 mL) at 180 °C for 1 h. The samples were allowed to cool. Ammonium molybdate solution (7 mL, prepared by dissolving 4.4 g in 2 L of distilled water and 40 mL of sulfuric acid) and Fiske reagent (0.7 mL) were added and the solutions vortexed. The solutions were heated in a boiling water bath for 30 min, allowed to cool, and assayed relative to a standard curve (0, 50, 100, and 200 nmol of phosphate) at 815 nm. Phosphate assays were performed in triplicate.

Protein Assays. Protein is assayed using a modification of the protocol for the micro BCA protein assay kit

from Pierce. A set of BSA standards containing 2.5–20 μg of protein and samples containing 0.333 μmol of lipid were diluted with 5% Triton X-100 (100 μL) and sufficient distilled water to produce a total volume of 1.0 mL. The working reagent (1.0 mL) was then added and the solution vortexed to ensure mixing. The solutions were incubated at 60 $^{\circ}\text{C}$ for 1 h and allowed to cool, and the protein concentrations of the samples were determined relative to the standards from the absorbance at 562 nm. Control liposomes (without protein conjugated on the surface) consistently assay at less than 1 mg of protein/mmol of lipid. The protein assays were performed in triplicate whenever possible.

Sulfhydryl Assays. The protein concentration of the IgG solution to be assayed was determined from the absorbance at 280 nm. Ellman's reagent (60 μL of a 4 mg/mL solution in HBS) was added to 0.600 mL aliquots of a control and the samples in HBS and allowed to incubate at room temperature for 20 min. Sulfhydryl levels were determined from the absorbance at 412 nm ($\epsilon = 13\,600$) using the formula

$$\text{SH} = 1.1A_{412}/13600C_p$$

where A_{412} is the absorbance at 412 nm, C_p is the protein concentration, and SH is the number of thiol equivalents.

In Vitro Cell Binding of Liposome–NR-LU-10 Conjugates. Human colon carcinoma LS180 cells were aliquoted (10^8 cells/mL) into round-bottom polystyrene tubes and incubated for 1 h at 37 $^{\circ}\text{C}$ (with gentle shaking) with [^3H]cholesterol hexadecyl ether labeled NR-Lu-10–liposome conjugates or control liposomes at a 1 mM lipid concentration. The cells were washed three times with RPMI containing 10% FBS. After the third wash, the pellet was resuspended in RPMI (500 μL) and transferred to a scintillation vial. The amount of bound lipid was determined from the tritium count.

In Vivo Clearance of Liposome–NR-LU-10 Conjugates. Plasma clearance studies were performed by injecting immunoliposomes via the lateral tail vein to female CD1 mice (20–25 g). The injection, given in a volume of 200 μL , contained a total dose of 1 μmol of lipid. This corresponds to approximately 32 mg of lipid/kg of body weight. Four mice were used per time point. Liposomal lipid was monitored using the lipid marker [^3H]cholesteryl hexadecyl ether. Previous studies have shown this lipid to be nonexchangeable and nonmetabolizable (7). The circulating level of injected liposomal lipid was determined at 1, 4, and 24 h after iv injection. At the 1 and 4 h time points, mice were lightly anesthetized with methoxyfluorane and blood (~ 200 μL) was obtained via retro-orbital bleeding. The collected blood was placed into EDTA-coated microtainers (Becton-Dickson). The samples were centrifuged at 500g for 10 min. The amount of radioactivity associated with the plasma was determined by mixing with 5 mL of Pico-Fluor 15 scintillation cocktail and counted employing a Beckman LS6500 scintillation counter. At 24 h, animals were anesthetized (ip mixture of 160 mg/kg ketamine and 20 mg/kg xylazine) and blood was collected via cardiac puncture in heparinized syringes and centrifuged in EDTA-coated microtainers (as above). An aliquot of the plasma was used for scintillation counting.

RESULTS

Conjugation Protocol. Periodate oxidations of IgG are typically carried out at lower pH values (4–5); however, under these conditions we found that the reaction was difficult to control and often resulted in significant aggregation of the IgG. Consequently, we

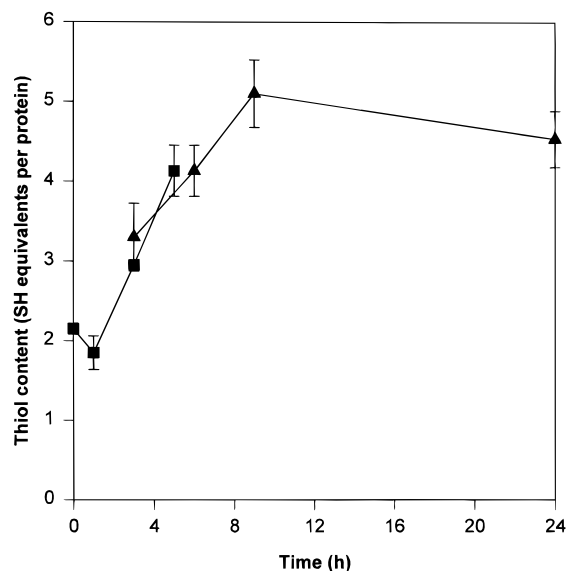


Figure 2. Effect of hydrazide incubation time on thiolation of human IgG. Oxidized human IgG was prepared by incubation with periodate for 1 h, followed by gel filtration (G-50, SAS). PDPH was added, and aliquots were taken at the indicated times. Unreacted PDPH was removed by gel filtration (G-50, HBS), and the samples were processed as described under Experimental Methods. Sulfhydryl levels were determined for two consecutive fractions as described under Experimental Methods. Each value is the mean and range for the two fractions. Data are presented for two separate experiments (▲ and ■).

chose to use a modified version of the protocol outlined by Hermanson *et al.* (8). The oxidations were performed over a period of 1 h, rather than 0.5 h, using IgG concentrations of 10–20 mg/mL. *In vitro* binding studies demonstrated that liposome conjugates produced using the oxidized IgG synthesized in this manner were equivalent in binding ability to conjugates formed using SPDP.

The PDPH reagent was stored as a suspension in ethanol and introduced into the reaction mixture in this form; however, an aqueous solution could be prepared immediately prior to use for proteins that are ethanol sensitive. The thiolation reaction was essentially complete after 5 h (Figure 2), resulting in approximately 4 thiol equiv. Longer reaction times yielded slightly higher levels of thiolation but resulted in significant protein aggregation. Some aggregation occurs at shorter incubation periods; however, the precipitate is easily removed by centrifugation. The degree of thiolation is typically determined by observing the release of 2-mercaptopyridine at 343 nm after treatment of the conjugate with DTT. We found that this method is unreliable since the DTT treatment changes the absorbance at 280 nm, resulting in small baseline changes at 343 nm which are of a similar order of magnitude to those expected for the released 2-mercaptopyridine anion. Consequently, we used Ellman's reagent to assay the sulfhydryl groups after DTT treatment. These experiments showed that the IgG contained 1–2 thiol equiv even without exposure to the PDPH reagent, suggesting that the DTT treatment damages some of the endogenous disulfide bonds. The DTT treatment was carried out at pH 4.5 since this reportedly minimizes damage to the disulfide bonds (9). To determine the extent of intrinsic damage by this procedure, we treated oxidized IgG with DTT for various time intervals and then assayed the resultant product for thiol equivalents (Figure 3). The results suggested that disulfide damage becomes significant at reaction times greater than 20 min. Approximately 1 thiol equiv of damage appeared to take place very rapidly and

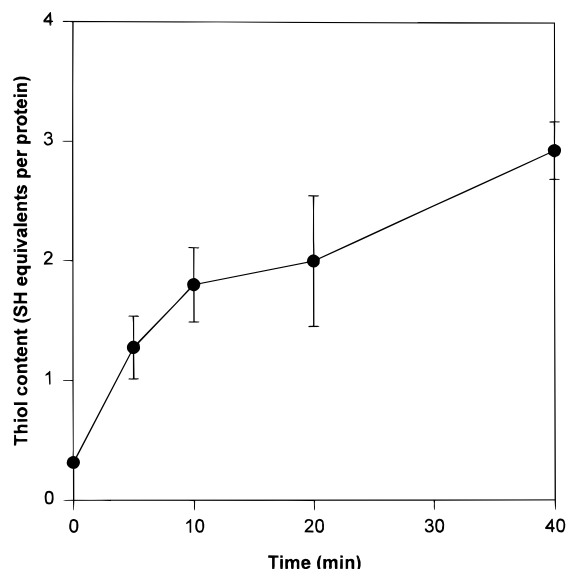


Figure 3. Effect of DTT exposure in SAS buffer at pH 4.4 on the intrinsic disulfide bonds in human IgG. Oxidized human IgG was prepared by incubation with periodate for 1 h, followed by gel filtration (G-50, SAS). Aliquots were taken and treated with DTT for the indicated times. The reaction was terminated and excess DTT removed by gel filtration (G-50, HBS). Sulfhydryl levels were determined for two consecutive fractions as described under Experimental Methods. Each value is the mean and range for the two fractions.

probably occurred when the sample was applied to the desalting column. These results imply that treatment with PDPH using our protocol introduces 2–3 thiol equiv into human IgG.

MPB-DSPE liposomes were generally treated with thiolated IgG overnight; however, a time course experiment using human IgG at 75 (data not shown) and 150 μg of protein/ μmol of lipid (Figure 4) indicated that the reaction was essentially complete after 1–2 h. Longer incubation times resulted in negligible increases in protein levels. A higher coupling efficiency was observed when using SPDP as opposed to PDPH in this system, both in the presence and in the absence of liposomal MePEGs-2000-DSPE. This effect was probably due to slightly higher thiolation levels in the SPDP–IgG conjugate and was not observed with systems using the mouse NR-LU-10 antibody. The coupling efficiency and proteoliposome aggregation was reduced when 2% MePEGs-2000-DSPE was included in the lipid composition. Similar effects have been previously reported for avidin–liposome systems (10). The PDPH protocol resulted in slightly lower levels of aggregation than the SPDP protocol in the absence of PEG lipids, suggesting that PDPH-modified IgG is somewhat less capable of cross-linking liposomes than SPDP-modified IgG. Further direct comparison of the PDPH and SPDP conjugates in the absence of PEG lipids was not attempted since the *in vivo* behavior is partly dependent on the size of the liposome conjugates. Control of the size distribution was possible in the presence of PEG lipids (10), thus allowing a direct comparison between the two protocols. A 2% PEG lipid concentration was chosen to allow relatively efficient conjugation and to minimize inhibition of antigen binding by the PEG.

The protein content of these systems was assayed by using a modified version of Pierce's micro BCA assay. Pierce claims that lipid may interfere with the assay; consequently, we conducted a number of control assays on unconjugated liposomes. These controls consistently assayed at $<1 \mu\text{g}$ of apparent protein/ μmol of lipid.

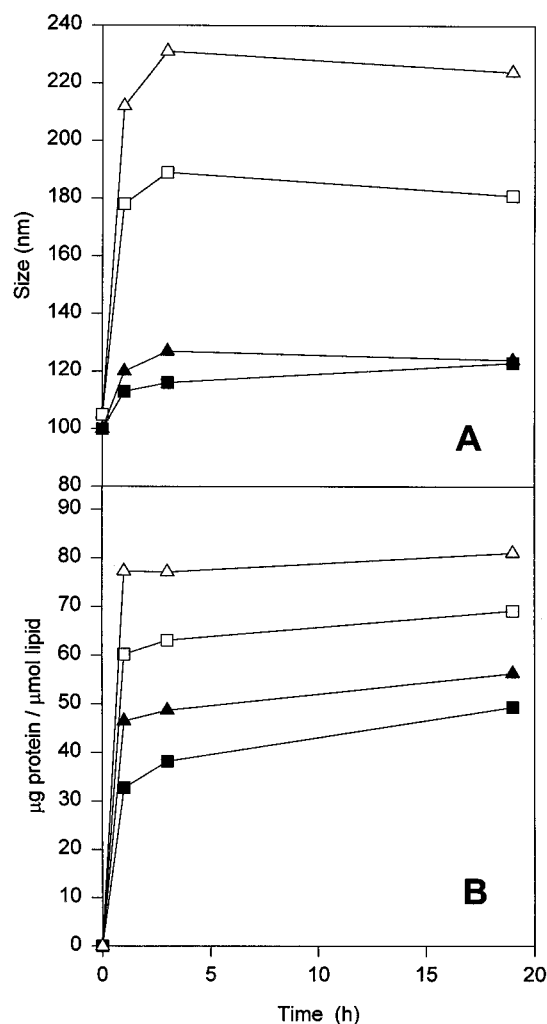


Figure 4. Effect of incubation time on protein conjugation to liposomes. Experiments were carried out as described under Experimental Methods. The liposome conjugation reactions were carried out with 150 μg of IgG/ μmol of lipid. Each value is the mean and range for the two consecutive fractions from the final gel column. Data are presented for DSPC/cholesterol/MPB-PE (54:45:1) liposomes with 2% MePEGs-2000-DSPE (solid triangles/squares) and without MePEGs-2000-DSPE (open triangles/squares), using the SPDP protocol (triangles) and the PDPH protocol (squares). Panels A and B show the effect of coupling time on the size and levels of conjugation, respectively.

In Vitro Binding Studies. The targeting capacity of conjugates prepared according to the PDPH protocol using the mouse NR-LU-10 antibody against human colon LS180 cells *in vitro* was determined by comparison with conjugates prepared according to the SPDP method and control liposomes (Figure 5). Freshly prepared conjugates using both the SPDP and PDPH protocols showed comparable enhanced accumulation relative to the controls, suggesting that, in this case, the PDPH protocol does not adversely affect the antigen binding properties of the conjugates. However, lower cell binding was observed for the PDPH conjugate after storage for 6 weeks at 4 °C. This raised the question of the stability of the conjugates since hydrazine linkages are known to hydrolyze over time (11). Short-term stability studies in buffer at 5 °C (data not shown) and 37 °C (Figure 6) showed that the conjugates are stable for at least a week. There is evidence of nonspecific absorption; however, it is relatively minor, accounting for about 6–7 $\mu\text{g}/\mu\text{mol}$ of initially bound protein.

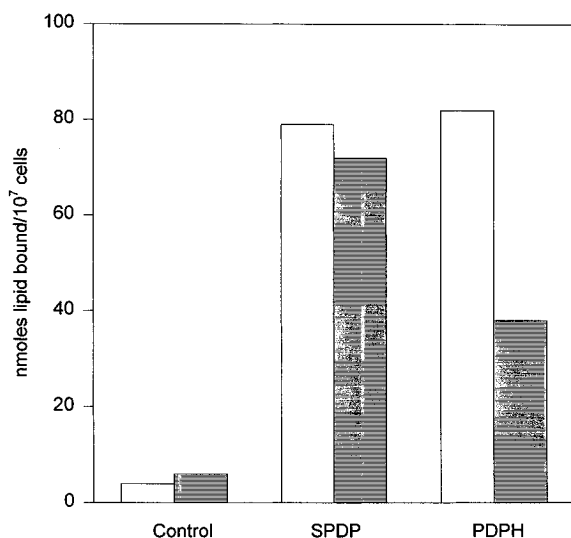


Figure 5. Binding of NR-LU-10 liposome conjugates (52% DSPC/45% cholesterol/2% MePEG-2000-Acer/1% MPB-PE) to LS180 cells. Experiments were conducted 1 day (open bars) and 6 weeks (shaded bars) after conjugate preparation, as described under Experimental Methods. The liposome conjugation reactions were carried out with 75 μ g of IgG/ μ mol of lipid.

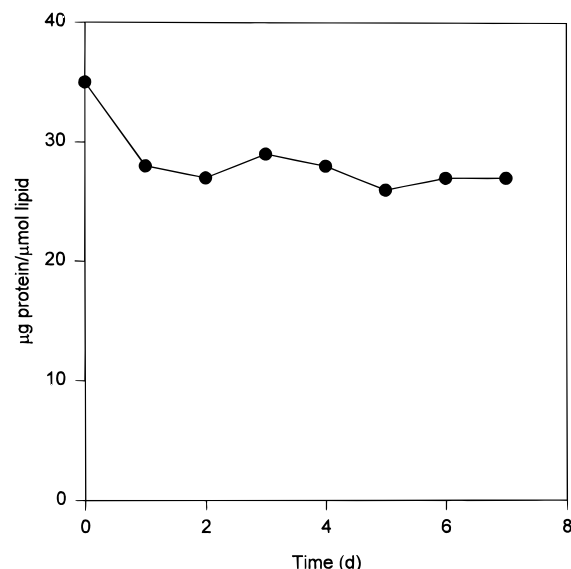


Figure 6. Stability of human IgG-liposome conjugates (52% DSPC/45% cholesterol/2% MePEG-2000-DSPE/1% MPB-PE) on exposure to HBS at 37 °C. The conjugate was prepared as described under Experimental Methods, with an initial protein to lipid ratio of 75 μ g/ μ mol. Aliquots were removed at the indicated times, except at $t = 0$ h, and passed down a Sepharose CL-4B column in HBS to remove nonconjugated IgG. Lipid and protein assays were conducted as described under Experimental Methods.

In Vivo Clearance. The clearance characteristics of IgG-liposome conjugates prepared according to the PDPH protocol were compared to those of control liposomes and conjugates prepared with SPDP using the NR-LU-10 antibody coupled to liposomes composed of DSPC/cholesterol/MePEG-2000-Acer/MPB-DSPE (52:45:2:1) or sphingomyelin/cholesterol/MePEG-2000-Acer/MPB-DSPE (52:45:2:1). Aliquots were taken from the reaction mixture at 1 and 16 h, worked up, and injected into mice to determine the effect of different protein loads. Blood samples were removed after 1, 4, and 24 h and analyzed for liposome levels (Figure 7). The circulation levels of the PDPH conjugates were consistently higher than those of the SPDP conjugates even though both systems had similar protein loads. The 1 and 16 h aliquot circulation

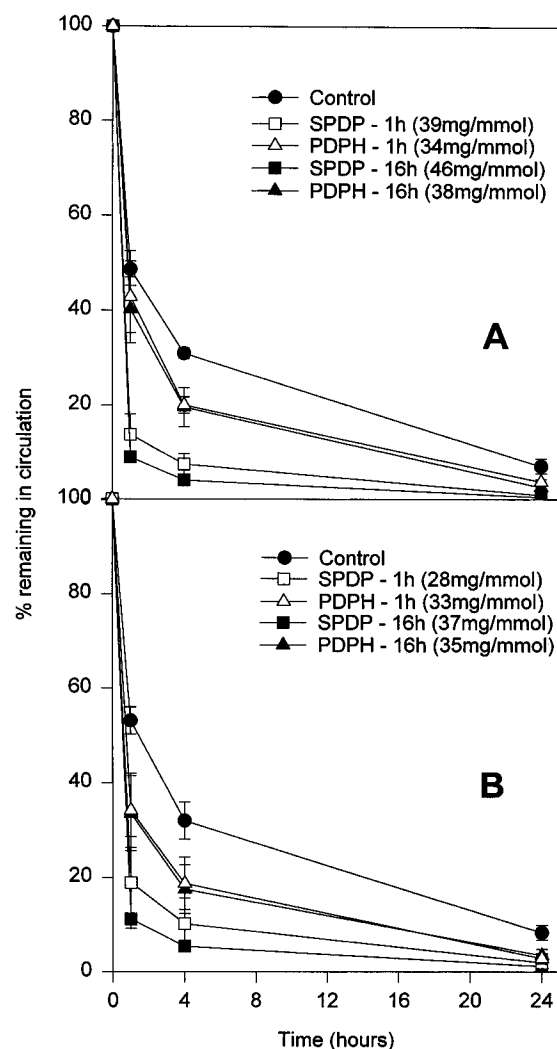


Figure 7. Clearance of NR-LU-10 liposome conjugates in CD1 mice. Panels A and B represent results from liposomes comprised of DSPC/cholesterol/MePEG-2000-Acer/MPB-PE (52:45:2:1) and sphingomyelin/cholesterol/MePEG-2000-Acer/MPB-PE (52:45:2:1), respectively. The solid circles represent control liposomes with no protein conjugated. Conjugates were prepared by the SPDP (squares) and PDPH (triangles) protocols. The liposome conjugation reactions were carried out with 75 μ g of IgG/ μ mol of lipid. The conjugation reactions were stopped at 1 h (open squares/triangles) and 16 h (solid squares/triangles) by removal of aliquots, followed by processing as described under Experimental Methods. Error bars represent standard deviation.

levels of the PDPH systems were comparable, but this was expected since both time points had equivalent protein loads. On the other hand, circulation levels for the analogous SPDP systems showed increased clearance for the 16 h system relative to the 1 h system for both liposome compositions. This effect is probably due to the small but significant increase in the protein loads for the 16 h SPDP systems. Interestingly, little difference was observed in the clearance behavior for the DSPC compositions relative to the analogous sphingomyelin compositions, even though it has been reported that sphingomyelin is superior to DSPC in this respect (12).

DISCUSSION

Our results show that mercaptohydrazides such as PDPH are viable alternatives to conventional cross-linkers, *i.e.* SPDP or SATA, for the conjugation of glycosylated proteins to liposomes. A number of related hydrazide cross-linkers have been reported, for example,

S-(2-thiopyridyl)-L-cysteine hydrazide (TCPH) (13) and *N*-acetylmercaptobutyric hydrazide (AMBH) (14). TCPH is structurally closely related to PDPH and could be expected to behave in a similar manner. Unprotected mercaptohydrazides such as AMBH are unsuitable since the free thiol function is susceptible to oxidation and may also reduce the disulfide bonds in IgG at the concentrations required for conjugation.

The lower reactivity of the hydrazide–aldehyde system of the PDPH protocol relative to the active ester–amine system of the SPDP protocol is easily compensated for by the large excess of hydrazide reagent used. Conjugation protocols in which the hydrazide reagent is incorporated into liposomes are at a disadvantage in this respect since the effective hydrazide concentration is limited by the lipid concentration. The PDPH protocol presented here does not have this disadvantage since the IgG–hydrazide reaction takes place prior to contact with the liposomes. The relatively long incubation time for the PDPH-oxidized IgG reaction could be further reduced by using higher PDPH concentrations than are used in this study.

We were particularly interested in using a cross-linker such as PDPH since it has been reported that IgG molecules attached to affinity gel supports via the carbohydrate moieties maintain the activity of both antigen binding sites, whereas procedures that utilize the amino functions on the protein surface resulted in significant loss of binding activity (2). There was little difference in the *in vitro* targeting capabilities of the IgG–liposome conjugates prepared according to the SPDP and PDPH protocols for the example used in this study. The result was not unexpected since it is known that the relationship between levels of conjugated antibody and *in vitro* targeting ability is not linear (15). This effect is due to the fact that in theory only one copy of an active antibody per liposome is required for binding. The targeting capacity of any liposome preparation then would level out once the population of liposomes without an active antibody conjugated to their surfaces becomes small, provided that low binding constants and kinetic factors do not play a significant role in the system being studied. In practice, we have found that 20–25 μg of antibody/ μmol of lipid is sufficient to effect optimal targeting *in vitro*. The SPDP and PDPH protocols appear to be equivalent in terms of *in vitro* targeting in the context of this study; however, this may not be true for all systems. It would be inappropriate to use the SPDP protocol for antibodies that are particularly sensitive to amine modification. Periodate is known to damage some amino acid residues, such as methionine, tyrosine, and tryptophan (3). Consequently, some antibodies may be sensitive to periodate treatment and would be unsuitable candidates for the PDPH protocol as well. The lower cell binding levels observed for the PDPH conjugate after 6 weeks of storage suggests that hydrolysis of the hydrazone bond will be a potential problem if the conjugate is not used soon after preparation. However, it is possible to stabilize the bond using sodium cyanoborohydride to reduce the hydrazone linkage if long-term storage is required (11).

The clearance characteristics of IgG–liposome conjugates are of great interest since rapid clearance reduces the probability that the conjugate will interact with the desired target tissue. The rapid clearance of these conjugates is thought to be due to RES and phagocytic cell uptake mediated through Fc receptors (16) or complement activation. The PDPH protocol results in conjugates with considerably improved clearance characteristics relative to those of the conjugates produced according

to the SPDP protocol. This suggests that a clearance recognition event may be affected for conjugates prepared according to the PDPH protocol. The effect may be due to the orientation of the IgG on the liposome surface or to disruptions to the effector functions of the Fc region. The oligosaccharide functions of IgG are located in an interstitial region between the two Fc chains (17, 18), where they are thought to play a role in the secondary structure of the Fc chains. It is known that oxidation or deglycosylation results in a reduction of the effector functions of IgG (FcR recognition and complement activation) (19–21); therefore, it is likely that the reduced clearance is due primarily to perturbation of the Fc secondary structure. The location of the oligosaccharide functions in the hinge region implies that the IgG molecules are conjugated sideways on the liposome surface. The SPDP protocol should conjugate the protein in a more random manner, particularly since the N terminus of the peptide chains is located in the binding domains. This implies that at least some copies are conjugated to the liposomes with the Fc fragment protruding from the surface. However, at this point it is not clear how significant this altered presentation on the liposome surface is with respect to clearance. Experiments to establish the exact mechanism for the reduced clearance are in progress and will be reported elsewhere.

Polyethylene glycol–lipid conjugates were incorporated into the liposome compositions since they have been shown to reduce clearance (5) and inhibit liposome aggregation during the IgG–liposome conjugation step (10). These lipids also reduce coupling efficiencies and have been reported to reduce binding to target cells (22). However, these negative factors are outweighed by the advantages of controlled size distributions and reduced clearance rates afforded by PEG lipids, and it is our opinion that these compounds are an essential component of any targeted liposomal system.

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LITERATURE CITED

- (1) Heath, T. D., and Martin, F. J. (1986) The development and application of protein-liposome conjugation techniques. *Chem. Phys. Lipids* 40, 347–358.
- (2) Domen, P. L., Nevens, J. R., Mallia, K., Hermanson, G. T., and Klenk, D. C. (1990) Site directed immobilization of proteins. *J. Chromatogr.* 510, 293–302.
- (3) Chua, M. M., Fan, S. T., and Karush, F. (1984) Attachment of immunoglobulin to liposomal membrane via protein carbohydrate. *Biochim. Biophys. Acta* 800, 291–300.
- (4) Hansen, C. B., Kao, G. Y., Moase, E. H., Zalipsky, S., and Allen, T. M. (1995) Attachment of antibodies to sterically stabilized liposomes: evaluation, comparison and optimization of coupling procedures. *Biochim. Biophys. Acta* 1239, 133–144.
- (5) Parr, M. J., Ansell, S. M., Choi, L. S., and Cullis, P. R. (1994) Factors influencing the retention and chemical stability of poly(ethyleneglycol)-lipid conjugates incorporated into large unilamellar vesicles. *Biochim. Biophys. Acta* 1195, 21–30.
- (6) Hope, M. J., Bally, M. B., Webb, G., and Cullis, P. R. (1985) Production of large unilamellar vesicles by a rapid extrusion procedure. Characterization of size distribution, trapped volume and ability to maintain a membrane potential. *Biochim. Biophys. Acta* 812, 55–65.

- (7) Derksen, J. T. P., Morselt, H. W. M., and Scherphof, G. L. (1987) Processing of different liposome markers after in vitro uptake of immunoglobulin-coated liposomes by rat liver macrophages. *Biochim. Biophys. Acta* 931, 33–40.
- (8) Hermanson, G. T., Mallia, A. K., and Smith, P. K. (1992) *Immobilized Affinity Ligand Techniques*, 230 pp, Academic Press, San Diego, CA.
- (9) Carlsson, J., Drevin, H., and Axén, R. (1978) Protein thiolation and reversible protein-protein conjugation. *Biochem. J.* 173, 723–737.
- (10) Harasym, T. O., Tardi, P., Longman, S. A., Ansell, S. M., Bally, M. B., Cullis, P. R., and Choi, L. S. L. (1995) Poly(ethylene glycol)-modified phospholipids prevent aggregation during covalent conjugation of proteins to liposomes. *Bioconjugate Chem.* 6, 187–194.
- (11) Kondejewski, L. H., Kralovec, J. A., Blair, A. H., and Ghose, T. (1994) Synthesis and characterization of carbohydrate-linked murine monoclonal antibody K20-human serum albumin conjugates. *Bioconjugate Chem.* 5, 602–611.
- (12) Webb, M. S., Harasym, T. O., Masin, D., Bally, M. B., and Mayer, L. D. (1995) Sphingomyelin-cholesterol liposomes significantly enhance the pharmacokinetics and therapeutic properties of vincristine in murine and human tumour models. *Br. J. Cancer* 72, 896–904.
- (13) Zara, J. J., Wood, R. D., Bredehorst, R., and Vogel, C. H. (1990) Site-specific heterobifunctional crosslinking reagents. World Intellectual Property Organization, International Publication WO 90/10621 (International Application PCT/US/01201).
- (14) Taylor, K. E., and Wu, Y. C. (1980) A thiolation reagent for cell surface carbohydrate. *Biochem. Int.* 4, 353–358.
- (15) Matthey, K. K., Heath, T. D., and Papahadjopoulos, D. (1984) Specific enhancement of drug delivery to AKR lymphoma by antibody-targeted small unilamellar vesicles. *Cancer Res.* 44, 1880–1886.
- (16) Aragonol, D., and Leserman, L. D. (1986) Immune clearance of liposomes inhibited by an anti-Fc receptor antibody in vivo. *Proc. Natl. Acad. Sci. U.S.A.* 83, 2699–2703.
- (17) Sutton, B. J., and Phillips, D. C. (1983) The three-dimensional structure of the carbohydrate within the Fc fragment of immunoglobulin G. *Biochem. Soc. Trans.* 11, 130–132.
- (18) Deisenhofer, J. (1981) Crystallographic refinement and atomic models of human Fc fragment and its complex with fragment B of protein A from *Staphylococcus aureus* at 2.9- and 2.8-Å resolution. *Biochemistry* 20, 2361–2370.
- (19) Nose, M., and Wigzell, H. (1983) Biological significance of carbohydrate chains on monoclonal antibodies. *Proc. Natl. Acad. Sci. U.S.A.* 80, 6632–6636.
- (20) Leatherbarrow, R., Rademacher, T., Dwek, R., Woof, J., Clark, A., Burton, D., Richardson, N., and Feinstein, A. (1985) Effector functions of a monoclonal aglycosylated mouse IgG2a: binding and activation of complement component C1 and interaction with human monocyte Fc receptor. *Mol. Immunol.* 22, 407–415.
- (21) Tao, M.-H., and Morrison, S. (1989) Studies of aglycosylated chimeric mouse-human IgG. Role of carbohydrates in the structure and effector functions mediated by the human IgG constant region. *J. Immunol.* 143, 2595–2601.
- (22) Klibanov, A. L., Maruyama, K., Beckerleg, A. M., Torchilin, V. P., and Huang, L. (1991) Activity of amphiphathic poly(ethylene glycol) 5000 to prolong the circulation time of liposomes depends on the liposome size and is unfavourable for immunoliposome binding to target. *Biochim. Biophys. Acta* 1062, 142–148.

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Novel Acid Labile COL1 Trityl-Linked Difluoronucleoside Immunoconjugates: Synthesis, Characterization, and Biological Activity¹

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LY207702 (**1**) is a difluorinated purine nucleoside that exhibits impressive antitumor activity in preclinical models. This agent, however, also possesses cardiotoxicity which limits the potential clinical utility of this novel drug candidate. We therefore developed linker chemistry whereby regioselective N6-tritylation of LY207702 (**1**) allowed this drug to be coupled to ϵ -lysine amino groups of mAb's reactive with human tumor-associated antigens. The resulting immunoconjugates **3** possessed conjugation ratios ranging from 5 to 7 mol of LY207702/mol of mAb, minimal aggregate content (5–10%), and good immunoreactivity. The electronic nature of substituents on the aromatic rings of the trityl group dictated the degree of acid lability of the trityl linker. Increased electronic stabilization of the transient trityl carbocation led to increase in the release rate of free drug, i.e., *m*-DMT **10a** = *p*-DMT **10b** > *p*-MMT **10d** > *p*-T **10f**. Consequently, the more acid labile DMT conjugates **3a** and **3b** proved to be the most potent cytotoxic agents, and the most stable *p*-T conjugate **3f** exhibited the least antitumor activity when evaluated *in vitro* and *in vivo*. *p*-MeT-linked conjugate **3e**, the most stable construct that retained excellent *in vivo* antitumor activity, was selected for more extensive evaluation. No detectable free drug or metabolite was observed in mouse plasma at a single intravenous dose of *p*-MeT conjugate **3e**, which was consistent with its predicted stability under physiological conditions. This construct did, however, exhibit significant antigen-mediated antitumor activity *in vivo*. No cardiotoxicity was detected in mice dosed with conjugate **3e** (6 mg/kg free drug content per day for 21 days) equivalent to ~8 times the total dose required for complete regression of well-established (~1 g) HC1 human colon tumor xenografts in nude mice. Cardiotoxicity was induced in 20% of free drug **1** treated group at the equivalent dose. Cardiomyopathy was, however, observed when the dose of conjugate **3e** was increased to 8 mg/kg per day for 21 days. These data suggest that antitumor activity of LY207702 (**1**) was maintained and its cardiotoxic potential reduced when this agent was administered to human tumor xenograft bearing nude mice as COL1–N6-*p*-MeT-207702 conjugate **3e**.

INTRODUCTION

A continuing challenge in the design of oncolytics for the treatment of human solid tumors is to enhance the cytotoxic effect and the selectivity of the agent toward tumor cells. A myriad of agents have been identified and shown to possess potent cytotoxicity against tumor cells; however, most of these agents are limited in their use due to the lack of selectivity toward tumor cells versus normal cells (1). Traditionally, structure–activity relationships (SAR)² have been employed in an attempt to overcome this problem, but this approach is not always successful. Conversion of an active drug into an inactive prodrug (2), which selectively dissociates to the active component at the tumor site, provides an alternative method of enhancing selectivity and is more generally applicable to the structurally diverse classes of antitumor agents. An extension of this concept whereby the drug is covalently attached to a tumor specific monoclonal antibody (mAb) provides an alternative method of delivering drug to the tumor site (3). The ability to attach

numerous drug molecules on each antibody also presents a unique opportunity for selective dose intensification of drug at the tumor. In general, drug immunoconjugates are comprised of three distinct components: (i) a cytotoxic drug, (ii) a tumor specific mAb for targeting, and (iii) the linker for attachment of the drug to the antibody. The role of each of these entities is critical in defining the overall properties of the drug immunoconjugate. Drug immunoconjugates have attracted much attention in recent years as agents for cancer therapy; however, much of the research has focused on optimization of the drug and the monoclonal antibody (4). The ability of the linker to release active drug in a timely and predictable fashion is also of paramount importance in the overall design of drug immunoconjugates but has received less attention (5).

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² Abbreviations: AcCl, acetyl chloride; AcOH, acetic acid; AST, aspartate transaminase; ALT, alanine transaminase; CEA, carcinoembryonic antigen; CR, conjugation ratio; DCC, dicyclohexylcarbodiimide; *m*-DMT, 3-carboxy-4',4''-dimethoxytrityl; *p*-DMT, 4-carboxy-4',4''-dimethoxytrityl; *p*-MMeT, 4-carboxy-4'-methoxy-4''-methyltrityl; *p*-MMT, 4-carboxy-4'-methoxytrityl; *p*-MeT, 4-carboxy-4'-methyltrityl; *p*-T, 4-carboxytrityl; DMF, dimethylformamide; EtOAc, ethyl acetate; IgG, immunoglobulin G; iPrNH₂, isopropylamine; iPA, isopropylamide; iPrOH, isopropyl alcohol; mAb, monoclonal antibody; MIR, molar input ratio; NHS, *N*-hydroxysuccinimide; PBS, phosphate-buffered saline; SAR, structure–activity relationship; TBAPC, tetra-*n*-butylammonium perchlorate; THF, tetrahydrofuran.

For drug immunoconjugates to selectively destroy tumor cells, it is essential that the release of cytotoxic drug from the conjugate should occur while the antibody is bound to its antigen; however, the construct should remain stable during systemic circulation. For noninternalizing antibodies the extracellular acidic tumor environment (6) and for internalizing antibodies translocation to the acidic lysosomal compartment (7) provide convenient trigger mechanisms for drug release from acid labile conjugates. This rationale has led to the development of several acid labile linkers, including *cis*-aconityl (8), hydrazone (9), hydrazide (10), and acetal (11) groups, for drug immunoconjugates. The trityl group is a well-known acid labile protecting group widely used in organic synthesis (12). Variations in acid lability can be readily achieved by appropriately substituting the aromatic rings of the trityl moiety (12). The versatile triphenylmethyl group has also found applications in solid phase synthesis of oligonucleotides (13) and peptides (14). It was apparent to us that the trityl moiety would be a particularly attractive linker candidate for use in the construction of drug immunoconjugates as the aromatic rings provide sites for appending substituents that allow (a) the control of drug release and (b) attachment of the mAb. Furthermore, the ability to readily attach a trityl group to a variety of pendant nucleophilic functionalities suggested that drug immunoconjugates could be prepared with a range of structurally diverse antitumor agents.

In this paper we report on the synthesis, characterization, and biological properties of novel acid labile COL1-N6-trityl-207702 conjugates **3** for their potential use in cancer therapy. COL1 is a murine IgG_{2a} mAb, isolated by Schlom and co-workers (15) that binds to an epitope of CEA that is highly expressed on tumor cell membranes and is only minimally present in normal tissue. COL1 did not appear to be internalized after binding to LS174T colon tumor cells as determined by acid dissociation of ¹²⁵I-labeled antibody (16). LY207702 (**1**) is a member of the 2',2'-difluoro-2'-deoxyribofuranosylpurine class of nucleoside antimetabolites which exhibits exquisite and unique antitumor activity in preclinical human tumor xenograft models, but also possesses cardiotoxicity (17). Our objective, therefore, was to determine if COL1-N6-trityl-207702 conjugates **3** could maintain the antitumor activity of LY207702 (**1**) while displaying less cardiotoxicity than the free drug.

EXPERIMENTAL PROCEDURES

All reactions described herein were performed under an inert atmosphere of dry nitrogen in flame-dried glassware unless otherwise noted. Tetrahydrofuran was freshly distilled from sodium benzophenone ketyl for use. All other solvents and reagents were used as supplied unless stated otherwise. In those cases where solvents and reagents were dried and purified, literature procedures were followed. Organic extracts were dried over Na₂SO₄ and concentrated *in vacuo* at room temperature. Melting points were determined on a 6427-H10 Thomas-Hoover melting point apparatus and are uncorrected. NMR spectra were obtained on a General Electric QE-300 instrument at 300 MHz for ¹H and 74.48 Hz for ¹³C in various solvents (specified) and are reported as parts per million (δ) values downfield from tetramethylsilane. Multiplicities of resonances are described as broad (b), singlet (s), doublet (d), triplet (t), quartet (q), or multiplet (m). IR spectra were run as KBr pellets or CHCl₃ solutions on a Nicolet 510P FT-IR. Optical rotations were measured on a Perkin-Elmer 241 spectrometer, and UV spectra were obtained on a Shimadzu 2101PC instrument. The mass spectral data were obtained on either a

VG-70SE or a Varian MAT-731 (for FD) and a VG-ZAB2SE or VG-ZAB3F (for FAB) spectrometer. "Flash chromatography" was performed on silica gel 60 230–400 mesh (40–63 μ m) according to the procedure of Still *et al.* (18). TLC was carried out on E. Merck silica gel 60 F₂₅₄ plates. Analytical HPLC separations were performed on a LDC system with a CM 4000 multiple solvent delivery system, spectroMonitor 5000 photodiode array detector set to record at 254 nm, a Hitachi D-2500 chromato-Integrator and a Gilson FC204 fraction collector. A μ Porasil 10 μ m column (3.9 $\times$ 150 mm) was used for normal phase (NP) and a Nova-Pak HR C₁₈ 6 μ m column (3.9 $\times$ 150 mm) for reverse phase (RP). A mobile phase of EtOAc:*n*-hexanes for NP and acetonitrile:ammonium acetate(aq) for RP was used at a flow rate of 1.0 mL/min. All solvents were filtered through a 0.22 μ m membrane and then thoroughly degassed under vacuum. Boric acid (12.37 g) was dissolved in Milli-Q-water (2 L) and the pH adjusted to 8.60 with 50% aqueous sodium hydroxide to give a 0.1 M borate buffer solution. Dulbecco's PBS powder (GIBCO) was dissolved in Milli-Q-water (1 L) to give a 0.1 M solution of pH 7.40 PBS. Monoclonal antibodies were purified on a protein-A column, and fractions containing protein were concentrated to ~15–25 mg/mL. mAb was dialyzed in a molecular porous membrane (Spectra/Por, MW cutoff = 12000–14000, capacity = 2 mL/cm) tubing against 0.1 M pH 8.60 borate buffer ($\times$ 100 volume) at 4 °C for 20–30 h and sterile filtered, and then the protein concentration was determined. Centrifugation was performed in a IEC Clinical centrifuge at 4000 rpm (2000G). Protein solutions were concentrated in an Amicon stirred-cell (series 8000) containing a disc membrane (MW cutoff 30000 and presoaked in water to remove azide) at 55 psi nitrogen pressure. Protein chromatography was performed using a FPLC Pharmacia LKB system equipped with a p-500 Pump, LCC-500 plus Controller, 100 Fraction collector, and a MII-UV monitor. Gel filtration purification was performed using a G-25 Sephadex medium (bead diameter 50–150 μ m dry) column eluting with PBS. Analytical runs were performed on a Superose 12 HR10/30 column eluting with 15% acetonitrile:PBS at a flow rate of 0.8 mL/min. Peak integrations were determined by area under the curve, where AUC = $\frac{1}{2}$ width $\times$ height. UV measurements were performed on a Beckmann DU-70 spectrophotometer. A 1 cm path length UV cell was used to measure absorbance of the product diluted in PBS. LY207702 (**1**) was prepared according to known procedures (19). All other chemicals were obtained from commercial sources. Physical chemical data were obtained at Lilly Research Laboratories.

4,4'-Methoxymethylbenzophenone (5c) (20). A solution of *p*-toluoyl chloride (1.55 g) in dry CH₂Cl₂ (3 mL) was added to a suspension of AlCl₃ (4 g) in dry CH₂Cl₂ (20 mL) at 0 °C and the mixture stirred at 0 °C for 1 h. A solution of anisole (1.08 g) in dry CH₂Cl₂ (3 mL) was added dropwise at a rate such that the temperature was maintained 0 °C. The orange mixture was stirred for a further 2 h at 0 °C and quenched with cold 1 N aqueous HCl (10 mL). Organics were separated, washed with saturated aqueous NaHCO₃ (50 mL) and H₂O (50 mL), dried, concentrated and purified by column chromatography (10% EtOAc:hexanes) to provide benzophenone **5c** as a white solid (1.85 g, 82%): mp 88.5–89.5 °C; IR (KBr) ν_{max} 1645, 1596, 1260, 760 cm⁻¹; ¹H (CDCl₃) δ 7.82 (d, *J* = 8 Hz, 2 ArH), 7.68 (d, *J* = 8 Hz, 2 ArH), 7.26 (d, *J* = 8 Hz, 2 ArH), 6.97 (d, *J* = 8 Hz, 2 ArH), 3.90 (s, OMe), 2.44 (s, Me) ppm. Anal. Calcd for C₁₅H₁₄O₂ requires M 226; C, 79.62; H, 6.24. Found: *m/z* 226 (100); C, 79.82; H, 6.35.

Tetra-*n*-butylammonium Perchlorate (TBAPC). A solution of KClO₄ (1.38 g) in H₂O (40 mL) was added to a solution of tetra-*n*-butylammonium sulfate (3.39 g) in H₂O (15 mL). A white flocculent precipitate immediately formed and was filtered, washed with H₂O, and dried over phosphorus pentoxide to yield the desired product as a white powder (1.61 g, 48%): IR (KBr) $\nu_{\max}$ 2967, 2879, 1475, 1093, 623 cm⁻¹; ¹H (CDCl₃) δ 3.26–3.20 (m, 8H, 4 CH₂N), 1.70–1.59 (m, 8H, 4 CH₂), 1.44 (dq, *J* = 6 and 8 Hz, 8H, 4 CH₂), 1.01 (t, *J* = 8 Hz, 6H, 2 Me) ppm. Anal. Calcd for C₁₆H₃₆NO₄Cl requires M 341.5; C, 56.20; H, 10.61; N, 4.10; Cl, 10.37. Found MS-FAB *m/z* 242.4 (M – ClO₄⁺, 100); C, 56.36; H, 10.43; N, 3.96; Cl, 10.25.

General Procedure A. A suspension of flame-dried magnesium turnings (48 mmol) in dry THF (25 mL) was sonicated for 20 min, and neat aryl bromide **4** (40 mmol) was added, followed by addition of a catalytic amount of iodine (10 mg), and the mixture was sonicated for 30 min to form the Grignard reagent. A suspension of the benzophenone **5** (44 mmol) in dry THF (10 mL) was added to the Grignard solution, and the mixture was stirred at room temperature for 2.5 h. The reaction was quenched with 5% aqueous KHSO₄ (50 mL) and extracted with EtOAc (3 × 20 mL). Combined organics were dried, concentrated, and purified by column chromatography (gradient elution 25–75% EtOAc:hexanes) to give carbinol **6**.

Racemic 2-[4-[(4-Methylphenyl)phenylhydroxymethyl]phenyl]-4,4-dimethyl-1,3-oxazoline (6e). 4-Methylbenzophenone (**5e**) (10.53 g) was reacted with the Grignard derived from 2-(4-bromophenyl)-4,4-dimethyl-1,3-oxazoline (**4**) (12.40 g), according to general procedure A, to give carbinol **6e** as a white solid (18.20 g, 99%): mp 68–72 °C; IR (KBr) $\nu_{\max}$ 2971, 1647, 1274 cm⁻¹; UV (EtOH) $\lambda_{\max}$ 251.5 (ϵ = 19 295), 204.5 (ϵ = 59 991) nm; ¹H (CDCl₃) δ 7.94 (br d, *J* = 8 Hz, 2 ArH), 7.38 (d, *J* = 8 Hz, 2 ArH), 7.36–7.24 (m, 5 ArH), 7.13 (s, 4 ArH), 4.15 (s, OCH₂), 2.80 (br s, OH), 2.36 (s, Me), 1.42 (s, 6H, 2 Me) ppm. Anal. Calcd for C₂₅H₂₅NO₂ requires M 372; C, 80.83; H, 6.78; N, 3.77. Found: *m/z* 372 (100); C, 80.55; H, 7.01; N, 3.73.

General Procedure B. Oxazoline **6** (22.4 mmol) was dissolved in 80% aqueous glacial AcOH (80 mL) and heated at reflux for 21 h. The solution was concentrated (50 °C) to an oily residue which was redissolved in 20% (w/v) NaOH in ethanol/water (1:1, 70 mL) and reheated at reflux for 3 h. The reaction solution was concentrated, diluted with water (50 mL), and acidified with 1 N aqueous HCl to pH 3. Product was extracted with EtOAc (4 × 10 mL), and the combined organics were dried and concentrated to give carboxylic acid **7**.

Racemic 4-Carboxy-4'-methyltriphenylhydroxymethane (7e). Oxazoline **6e** (18.0 g) was hydrolyzed according to general procedure B to give acid **7e** as a yellow solid (13.35 g, 87%): mp 91–94 °C; IR (CHCl₃) $\nu_{\max}$ 3380, 3020, 1692, 1282, 1009 cm⁻¹; UV (EtOH) $\lambda_{\max}$ 247 (ϵ = 12 558), 203.5 (ϵ = 45 569) nm; ¹H (CDCl₃) δ 8.03 (d, *J* = 8 Hz, 2 ArH), 7.44 (d, *J* = 8 Hz, 2 ArH), 7.35–7.23 (m, 5 ArH), 7.13 (s, 4 ArH), 2.36 (s, Me) ppm. Anal. Calcd for C₂₁H₁₈O₃ requires M 318.4; C, 79.23; H, 5.70. Found: *m/z* 318 (100); C, 78.87; H, 6.03.

General Procedure C. To a stirred suspension of arene carboxylic acid **7** (18.1 mmol) and NHS (18.1 mmol) in dry CH₂Cl₂ (100 mL) was added DCC (21.7 mmol) at room temperature and the mixture stirred for 4 h. The reaction mixture was filtered through Celite, washed with CH₂Cl₂, concentrated, and purified by column chromatography (gradient elution 25–50% EtOAc:hexanes) to give active ester **8**.

Racemic *N*-Succinimidyl 4-[(4-Methylphenyl)phenylhydroxymethyl]benzoate (8e). Carboxylic acid **7e** (13.20 g) was coupled to NHS (4.77 g) using DCC (10.27 g), according to general procedure C, to give active ester **8e** as a solid (15.65 g, 91%): mp 156–158 °C; IR (KBr) $\nu_{\max}$ 3467, 1771, 1731, 1212, 996 cm⁻¹; UV (EtOH) $\lambda_{\max}$ 249 (ϵ = 18 105), 206 (ϵ = 48 790) nm; ¹H (DMSO-*d*₆) δ 8.03 (d, *J* = 9 Hz, 2 ArH), 7.50 (d, *J* = 9 Hz, 2 ArH), 7.36–7.26 (m, 3 ArH), 7.23–7.19 (m, 2 ArH), 7.16–7.06 (br q, *J* = 8 Hz, 4 ArH), 2.89 (s, 4H, 2 CH₂), 2.30 (s, Me) ppm. Anal. Calcd for C₂₅H₂₁NO₅ requires M 415; C, 72.28; H, 5.10; N, 3.37. Found: *m/z* 415 (100); C, 71.95; H, 5.26; N, 4.06.

General Procedure D. Hydroxy ester **8** (4.98 mmol) was dissolved in freshly distilled AcCl (25 mL) and the solution heated at reflux for 5–24 h, cooled to room temperature, and concentrated to approximately one-third of its original volume. Dry Et₂O (20 mL) was added to form a precipitate, which was filtered, washed with further dry Et₂O, and dried under vacuum to give trityl chloride **9**. In those cases where product could not be precipitated, the reaction solution was concentrated to dryness and the product **9** azeotroped with dry CH₂Cl₂ (5 × 10 mL).

Racemic *N*-Succinimidyl 4-[(4-Methylphenyl)phenylchloromethyl]benzoate (9e). Hydroxy ester **8e** (2.50 g) was heated in AcCl according to general procedure D to provide trityl chloride **9e** as a yellow solid (2.35 g, 90%): IR (CHCl₃) $\nu_{\max}$ 1775, 1744, 1257, 1201, 1071, 996 cm⁻¹; ¹H (DMSO-*d*₆) δ 8.04 (d, *J* = 8 Hz, 2 ArH), 7.50 (d, *J* = 8 Hz, 2 ArH), 7.36–7.25 (m, 2 ArH), 7.25–7.17 (m, 3 ArH), 7.12 (q, *J* = 8 Hz, 4 ArH), 2.88 (s, 4 H, 2 CH₂), 2.28 (s, Me) ppm. Anal. Calcd for C₂₅H₂₀NO₄Cl requires M 433.5; C, 69.21; H, 4.65; N, 3.23. Found: *m/z* 415 (100); C, 69.50; H, 4.79; N, 3.47.

General Procedure E. To a solution of 2,6-diamino-9-(2'-deoxy-2',2'-difluoro- β -D-ribofuranosyl)purine (**1**) (0.331 mmol) in a solvent mixture of dry DMF:CH₂Cl₂ (1:1; 6 mL) was added TBAPC (0.397 mmol) and dry collidine (0.497 mmol) at room temperature. The solution was stirred for 5 min and trityl chloride **9** (0.331 mmol) added portionwise over ~3 min. The resulting solution was stirred at room temperature until all the starting material was consumed (monitored by TLC). The crude reaction was diluted with EtOAc (1 volume) and the solution directly applied onto a silica column and purified by chromatography (gradient elution EtOAc then 5–20% iPrOH/EtOAc) to give trityl nucleoside **2**.

***N*-Succinimidyl 3-[Bis(4-methoxyphenyl)][[2-amino-9-(2'-deoxy-2',2'-difluoro- β -ribofuranosyl)purin-6-yl]-amino]methyl]benzoate (2a).** Nucleoside **1** (100 mg) was reacted with *m*-DMT chloride **9a** (159 mg) in the presence of TBAPC (136 mg) and collidine (70 μ L) for 20 min, according to general procedure E, to give product **2a** as a white solid (190 mg; 77%): TLC *R_f* (10% MeOH/CH₂Cl₂) 0.42; IR (KBr) $\nu_{\max}$ 3379, 1740, 1606, 1253, 1072 cm⁻¹; UV (EtOH) $\lambda_{\max}$ 278 (ϵ = 9970.5), 225.5 (ϵ = 32 651), 203.5 (ϵ = 50 143) nm; ¹H (DMSO-*d*₆) δ (8.07 (s, 1 ArH), 7.90–7.77 (m, 2 ArH), 7.88 (s, H8), 7.50 (t, *J* = 9 Hz, 1 ArH), 7.27 (dd, *J* = 4 and 10 Hz, 4 ArH), 6.95 (s, NH), 6.80 (dd, *J* = 6 and 10 Hz, 4 ArH), 6.74 (br s, NH₂), 6.24 (d, *J* = 6 Hz, 3'OH), 5.60–5.44 (m, H1'), 5.19 (t, *J* = 4 Hz, 5'OH), 4.41–4.23 (m, H3'), 3.82–3.54 (m, 3H, H4'5'), 3.70 (s, 6H, 2 OMe), 2.84 (s, 4 H, 2 CH₂) ppm. Anal. Calcd for C₃₆H₃₃N₇O₉F₂ requires M 746; C, 57.99; H, 4.40; N, 13.15. Found: *m/z* 746 (100), 630 (10), 444 (15), 302 (10); C, 57.81; H, 4.73; N, 12.89.

***N*-Succinimidyl 4-[Bis(4-methoxyphenyl)][[2-amino-9-(2'-deoxy-2',2'-difluoro- β -ribofuranosyl)purin-6-yl]-amino]methyl]benzoate (2b).** Nucleoside **1** (100 mg)

was reacted with *p*-DMT chloride **9b** (159 mg) in the presence of TBAPC (136 mg) and collidine (70 μ L) for 30 min, according to general procedure E, to give product **2b** as a white powder (232 mg; 94%): TLC R_f (10% MeOH/CH₂Cl₂) 0.22; IR (KBr) $\nu_{\max}$ 3372, 2933, 1741, 1608, 1510, 1072 cm⁻¹; UV (EtOH) $\lambda_{\max}$ 261 (ϵ = 22 597), 226.5 (ϵ = 43 631) nm; ¹H (DMSO-*d*₆) δ 7.95 (d, *J* = 10 Hz, 2 ArH), 7.87 (s, H8), 7.62 (d, *J* = 8 Hz, 2 ArH), 7.27 (d, *J* = 8 Hz, 4 ArH), 6.91 (s, NH), 6.80 (dd, *J* = 3 and 10 Hz, 4 ArH), 6.72 (br s, NH₂), 6.25 (d, *J* = 6 Hz, 3'OH), 5.65–5.44 (m, H1'), 5.19 (t, *J* = 6 Hz, 5'OH), 4.44–4.26 (m, H3'), 3.83–3.63 (m, 3H, H4'5'5'), 3.70 (s, 6H, 2 OMe), 2.85 (s, 4H, 2 CH₂) ppm. Anal. Calcd for C₃₆H₃₃N₇O₉F₂ requires *M* 746; C, 57.99; H, 4.46; N, 13.15. Found: *m/z* 746 (100), 444 (12), 302 (3); C, 58.05; H, 4.82; N, 12.80.

***N*-Succinimidyl 4-[(4-Methoxyphenyl)(4'-methylphenyl)][2-amino-9-(2'-deoxy-2',2'-difluoro- β -ribofuranosyl)purin-6-yl]amino]methyl]benzoate (**2c**).** Nucleoside **1** (200 mg) was reacted with *p*-MMeT chloride **9c** (307 mg) in the presence of TBAPC (271 mg) and collidine (132 μ L) for 2 h, according to general procedure E, to give product **2c** as a yellow solid (404 mg; 84%): diastereomeric mixture; TLC R_f (10% MeOH/CH₂Cl₂) 0.49; IR (KBr) $\nu_{\max}$ 3376, 1772, 1740, 1606, 1256, 1073 cm⁻¹; UV (EtOH) $\lambda_{\max}$ 259 (ϵ = 26 254) nm; ¹H (DMSO-*d*₆) δ 7.95 (d, *J* = 8 Hz, 2 ArH), 7.88 (s, H8), 7.62 (d, *J* = 10 Hz, 2 ArH), 7.30–7.22 (m, 4 ArH), 7.05 (dd, *J* = 2 and 8 Hz, 2 ArH), 6.92 (s, NH), 6.79 (dd, *J* = 8 and 10 Hz, 2 ArH), 6.74 (br s, NH₂), 6.25 (d, *J* = 6 Hz, 3'OH), 5.64–5.43 (m, H1'), 5.20 (t, *J* = 6 Hz, 5'OH), 4.44–4.24 (m, H3'), 3.84–3.54 (m, 3H, H4'5'5'), 3.72 (s, OMe), 2.87 (s, 4H, 2 CH₂), 2.23 (s, Me) ppm. Anal. Calcd for C₃₆H₃₃N₇O₈F₂ requires *M* 730; C, 59.26; H, 4.56; N, 13.44. Found: *m/z* 730 (100), 428 (5), 302 (2); C, 59.20; H, 4.84; N, 13.48.

***N*-Succinimidyl 4-[(4-Methoxyphenyl)phenyl][2-amino-9-(2'-deoxy-2',2'-difluoro- β -ribofuranosyl)purin-6-yl]amino]methyl]benzoate (**2d**).** Nucleoside **1** (302 mg) was reacted with *p*-MMT chloride **9d** (450 mg) in the presence of TBAPC (271 mg) and collidine (160 μ L) for 2 h, according to general procedure E, to give product **2d** as a pale yellow powder (493 mg; 70%): diastereomeric mixture; TLC R_f (10% MeOH/CH₂Cl₂) 0.34; IR (KBr) $\nu_{\max}$ 3374, 1739, 1607, 1511, 1256, 1207, 1073 cm⁻¹; ¹H (DMSO-*d*₆) δ 7.95 (d, *J* = 8 Hz, 2 ArH), 7.87 (s, H8), 7.64 (d, *J* = 1 Hz, 2 ArH), 7.38 (d, *J* = 8 Hz, 2 ArH), 7.30–7.13 (m, 5 ArH), 7.02 (s, NH), 6.80 (dd, *J* = 2 and 7 Hz, 2 ArH), 6.75 (br s, NH₂), 6.25 (d, *J* = 6 Hz, 3'OH), 5.60–5.44 (m, H1'), 5.20 (t, *J* = 6 Hz, 5'OH), 4.42–4.26 (m, H3'), 3.82–3.52 (m, 3H, H4'5'5'), 3.70 (s, OMe), 2.87 (s, 4H, 2 CH₂) ppm; C₃₅H₃₁N₇O₈F₂ requires *M* 716, found *m/z* 716 (100).

***N*-Succinimidyl 4-[(4-Methylphenyl)phenyl][2-amino-9-(2'-deoxy-2',2'-difluoro- β -ribofuranosyl)purin-6-yl]amino]methyl]benzoate (**2e**).** Nucleoside **1** (200 mg) was reacted with *p*-MeT chloride **9e** (287 mg) in the presence of TBAPC (271 mg) and collidine (132 μ L) for 3 h, according to general procedure E, to give product **2e** as a white solid (604 mg; 65%): diastereomeric mixture; TLC R_f (10% MeOH/CH₂Cl₂) 0.50; IR (KBr) $\nu_{\max}$ 3379, 1773, 1740, 1606, 1467, 1205, 1073 cm⁻¹; ¹H (DMSO-*d*₆) δ 7.96 (br d, *J* = 6 Hz, 2 ArH), 7.86 (s, H8), 7.64 (d, *J* = 10 Hz, 2 ArH), 7.42–7.35 (m, 2 ArH), 7.30–7.12 (m, 5 ArH), 7.10–7.02 (m, 2 ArH), 7.14 (s, NH), 6.76 (br s, NH₂), 6.26 (d, *J* = 7 Hz, 3'OH), 5.58–5.45 (m, 3H, H4'5'5'), 2.87 (br s, 4H, 2 CH₂), 2.24 (s, Me) ppm. Anal. Calcd for C₃₅H₃₁N₇O₇F₂ requires *M* 699; C, 60.08; H, 4.47; N, 14.01; F, 5.43. Found: *m/z* 699 (100); C, 59.89; H, 4.30; N, 13.82; F, 4.99.

***N*-Succinimidyl 4-[Bisphenyl][2-amino-9-(2'-deoxy-2',2'-difluoro- β -ribofuranosyl)purin-6-yl]amino]methyl]benzoate (**2f**).** Nucleoside **1** (302 mg) was reacted with *p*-T chloride **9f** (402 mg) in the presence of TBAPC (401 mg) and collidine (160 μ L) for 1 h, according to general procedure E, to give product **2f** as a white solid (391 mg; 57%): TLC R_f (10% MeOH/CH₂Cl₂) 0.27; IR (CHCl₃) $\nu_{\max}$ 3379, 1772, 1739, 1607, 1467, 1207, 1073 cm⁻¹; UV (EtOH) $\lambda_{\max}$ 259 (ϵ = 24 723) nm; ¹H (DMSO-*d*₆) δ 7.97 (d, *J* = 9 Hz, 2 ArH), 7.88 (s, H8), 7.66 (d, *J* = 9 Hz, 2 ArH), 7.42–7.36 (m, 4 ArH), 7.28–7.10 (m, 6 ArH), 7.04 (s, NH), 6.76 (br s, NH₂), 6.15 (d, *J* = 6 Hz, 3'OH), 5.60–5.44 (m, H1'), 5.20 (t, *J* = 6 Hz, 5'OH), 4.40–4.24 (m, H3'), 3.80–3.54 (m, 3H, H4'5'5'), 2.88 (s, 4H, 2 CH₂) ppm; C₃₄H₂₉N₇O₇F₂ requires *M* 685, found *m/z* 685 (100).

General Procedure F. To a stirred solution of active ester **2** (0.147 mmol) in dry CH₂Cl₂ (3 mL) was added neat *i*PrNH₂ (0.734 mmol) at room temperature, and a precipitate formed almost immediately. The reaction mixture was stirred for a further 1 h, filtered, concentrated, and purified by column chromatography (gradient elution CH₂Cl₂–10% MeOH/CH₂Cl₂) to provide amide **10**.

***N*-Isopropyl-3-[bis(4-methoxyphenyl)][2-amino-9-(2'-deoxy-2',2'-difluoro- β -ribofuranosyl)purin-6-yl]amino]methyl]benzamide (**10a**).** Active ester **2a** (30 mg) was reacted with *i*PrNH₂, according to general procedure F, to give amide **10a** as a white solid (20.6 mg; 74%): TLC R_f (10% MeOH/CH₂Cl₂) 0.35; IR (CHCl₃) $\nu_{\max}$ 3365, 2931, 1608, 1509, 1465, 1251, 1178, 1034, 830, 592 cm⁻¹; UV (EtOH) $\lambda_{\max}$ 277.5 (ϵ = 14 006), 224.5 (ϵ = 47 152), 205 (ϵ = 70 300) nm; ¹H NMR (acetone-*d*₆) δ 8.03 (s, ArH), 7.83 (s, H8), 7.65 (d, *J* = 8 Hz, ArH), 7.52 (d, *J* = 8 Hz, ArH), 7.48 (d, *J* = 9 Hz, ArH), 7.26 (d, *J* = 8 Hz, 4 ArH), 6.80 (d, *J* = 8 Hz, 4 ArH), 6.35 (s, NH), 6.20 (br s, NH₂), 5.76–5.71 (m, H1'), 5.55 (br s, 3'OH), 4.82 (br s, 5'OH), 4.62–4.57 (m, H3'), 4.20–4.12 (m, CH), 3.93–3.74 (m, 3H, H4'5'5'), 3.74 (s, 6H, 2 OMe), 1.14 (*J* = 6.6 Hz, 6H, 2 Me) ppm; C₃₅H₃₇N₇O₆F₂ requires *M* 690, found *m/z* 690 (100).

***N*-Isopropyl-4-[bis(4-methoxyphenyl)][2-amino-9-(2'-deoxy-2',2'-difluoro- β -ribofuranosyl)purin-6-yl]amino]methyl]benzamide (**10b**).** Active ester **2b** (60 mg) was reacted with *i*PrNH₂, according to general procedure F, to give amide **10b** as a white solid (38 mg; 69%): TLC R_f (10% MeOH/CH₂Cl₂) 0.41; IR (KBr) $\nu_{\max}$ 3356, 1608, 1509, 1464, 1251, 1178, 1033 cm⁻¹; UV (EtOH) $\lambda_{\max}$ 227.5 (ϵ = 47 461), 204 (ϵ = 79 092) nm; ¹H (CDCl₃/MeOD) δ 7.83 (s, H8), 7.62 (d, *J* = 8 Hz, 2 ArH), 7.40 (d, *J* = 8 Hz, 2 ArH), 7.19 (d, *J* = 8 Hz, 4 ArH), 6.74 (d, *J* = 8 Hz, 4 ArH), 5.53–5.45 (m, H1'), 4.65–4.55 (m, 2H, CH, H3'), 4.35–4.15 (m, 3H, H4'5'5'), 3.74 (s, 6H, 2 OMe), 1.19 (d, *J* = 7 Hz, 6H, 2 Me) ppm; C₃₅H₃₇N₇O₆F₂ requires *M* 689, found *m/z* 689 (10), 389 (30), 302 (25).

***N*-Isopropyl-4-[(4-methoxyphenyl)(4'-methylphenyl)][2-amino-9-(2'-deoxy-2',2'-difluoro- β -ribofuranosyl)purin-6-yl]amino]methyl]benzamide (**10c**).** Active ester **2c** (50 mg) was reacted with *i*PrNH₂, according to general procedure F, to give amide **10c** as a white solid (43.6 mg; 95%): TLC R_f (10% MeOH/CH₂Cl₂) 0.41; IR (KBr) $\nu_{\max}$ 3360, 1607, 1510, 1465, 1252, 1076, 1036 cm⁻¹; UV (EtOH) $\lambda_{\max}$ 205 (ϵ = 72 094) nm; ¹H (MeOD-*d*₄) δ 7.95 (s, H8), 7.67 (d, *J* = 8 Hz, 2 ArH), 7.45 (d, *J* = 8 Hz, 2 ArH), 7.22 (t, *J* = 8 Hz, 4 ArH), 7.07 (d, *J* = 6 Hz, 2 ArH), 6.79 (dd, *J* = 2 and 8 Hz, 2 ArH), 5.60–5.47 (m, H1'), 4.40–4.27 (m, H3'), 4.23–4.10 (m, CH), 3.90–3.70 (m, 3H, H4'5'5'), 3.75 (s, OMe), 2.27 (s, Me), 1.21 (d, *J* = 7 Hz, 6H, 2 Me) ppm; C₃₅H₃₇N₇O₅F₂ requires *M* 673, found *m/z* 673 (100), 372 (20), 302 (40).

N-Isopropyl-4-[(4-methoxyphenyl)phenyl][2-amino-9-(2'-deoxy-2',2'-difluoro- β -ribofuranosyl)purin-6-yl]-amino]methyl]benzamide (10d). Active ester **2d** (50 mg) was reacted with $i\text{PrNH}_2$, according to general procedure F, to give amide **10d** as a white solid (36 mg; 78%); TLC R_f (20% MeOH/ CH_2Cl_2) 0.68; IR (KBr) ν_{max} 3373, 1609, 1511, 1466, 1253, 1077 cm^{-1} ; UV (EtOH) λ_{max} 224 ($\epsilon = 42\,444$), 204.5 ($\epsilon = 68\,723$) nm; ^1H (acetone- d_6) δ 7.80 (s, H8), 7.72 (d, $J = 6$ Hz, 2 ArH), 7.46 (d, $J = 6$ Hz, 2 ArH), 7.42–7.36 (m, 2 ArH), 7.32–7.14 (m, 5 ArH), 6.82 (d, $J = 8$ Hz, 2 ArH), 6.28 (s, NH), 6.10 (br s, NH_2), 5.80–5.65 (m, H1'), 5.42 (d, $J = 6$ Hz, OH), 4.72–4.50 (m, 2H, OH, H3'), 4.28–4.10 (m, CH), 3.86–3.70 (m, 3H, H4'5'5'), 3.76 (s, OMe), 1.21 (d, $J = 6$ Hz, 6H, 2 Me) ppm; $\text{C}_{34}\text{H}_{35}\text{N}_7\text{O}_5\text{F}_2$ requires M 659, found m/z 659 (100), 358 (15).

N-Isopropyl-4-[(4-methylphenyl)phenyl][2-amino-9-(2'-deoxy-2',2'-difluoro- β -ribofuranosyl)purin-6-yl]-amino]methyl]benzamide (10e). Active ester **2e** (50 mg) was reacted with $i\text{PrNH}_2$, according to general procedure F, to give amide **10e** as a white solid (36.1 mg; 78%); TLC R_f (10% MeOH/ CH_2Cl_2) 0.51; IR (CHCl_3) ν_{max} 3358, 1633, 1427, 1077 cm^{-1} ; UV (EtOH) λ_{max} 204 ($\epsilon = 62\,080$) nm; ^1H (MeOD- d_4) δ 7.88 (s, H8), 7.67 (d, $J = 8$ Hz, 2 ArH), 7.46 (d, $J = 10$ Hz, 2 ArH), 7.34 (d, $J = 8$ Hz, 2 ArH), 7.26–7.12 (m, 5 ArH), 7.05 (d, $J = 7$ Hz, 2 ArH), 5.60–5.46 (m, H1'), 4.41–4.24 (m, H3'), 4.23–4.10 (m, CH), 3.92–3.64 (m, 3H, H4'5'5'), 2.28 (s, Me), 1.22 (d, $J = 6$ Hz, 2 Me) ppm; $\text{C}_{34}\text{H}_{35}\text{N}_7\text{O}_4\text{F}_2$ requires M 644, found m/z 644 (65).

N-Isopropyl-4-[bisphenyl][2-amino-9-(2'-deoxy-2',2'-difluoro- β -ribofuranosyl)purin-6-yl]amino]methyl]benzamide (10f). Active ester **2f** (50 mg) was reacted with $i\text{PrNH}_2$, according to general procedure F, to give amide **10f** as a white solid (32 mg; 70%); TLC R_f (10% MeOH/ CH_2Cl_2) 0.58; mp 190 $^\circ\text{C}$ dec; IR (KBr) ν_{max} 3362, 1633, 1467, 1077 cm^{-1} ; UV (EtOH) λ_{max} 205 ($\epsilon = 65\,359$) nm; ^1H (acetone- d_6) δ 7.80 (s, H8), 7.73 (d, $J = 8$ Hz, 2 ArH), 7.48 (d, $J = 8$ Hz, 2 ArH), 7.42–7.37 (m, 4 ArH), 7.32–7.15 (m, 6 ArH), 6.36 (s, NH), 6.12 (br s, NH_2), 5.78–5.64 (m, H1'), 5.43 (d, $J = 8$ Hz, OH), 4.74–4.52 (m, 2H, OH, H3'), 4.26–4.16 (m, CH), 3.96–3.72 (m, 3H, H4'5'5'), 1.20 (d, $J = 6$ Hz, 6H, 2 Me) ppm; $\text{C}_{33}\text{H}_{33}\text{N}_7\text{O}_4\text{F}_2$ requires M 630, found m/z 630 (70), 328 (20).

Antibody Purification. Ascites fluid, derived from the hybridoma cells, was used as the antibody source for the studies described in this paper. The mAbs were purified from ascites fluid using Prosep-A protein A chromatography (Bioprocessing, Inc., Princeton, NJ). Briefly, ascites fluid (containing about 5 mg/mL IgG) was diluted (50:50 v/v) with 1 M glycine/0.3 M NaCl, pH 8.6 buffer and filtered. This antibody-containing solution was applied to the Prosep-A column which had been previously equilibrated in 1 M glycine/0.15 M NaCl, pH 8.6 buffer. After all unbound material was washed out, the protein A bound IgG was eluted with 0.1 M citrate, pH 3.5 buffer and immediately neutralized with 1 M Tris-HCl, pH 8.6 buffer. Protein was diluted with PBS (dilution factor (df) = 20) and UV absorbance measured at 279 nm. Protein concentrations were determined using the Beer-Lambert Law (where $A_{279} = 1.43$ at 1 mg/mL for monoclonal antibody). Purified IgG was subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (Phastgel, Pharmacia, Piscataway, NJ) using reducing (12.5% gel in the presence of β -mercaptoethanol) and nonreducing (7.5% gel in the absence of β -mercaptoethanol) conditions.

General Conjugation Procedure. To a stirred 0.1 M borate buffer (pH = 8.64) solution of protein (1.442 mL of 10.4 mg/mL = 15 mg; 1.00×10^{-7} mol) was slowly

(over 3–5 min) added to a solution of N6-trityl-207702 active ester **2** (0.55 mg; 8.00×10^{-7} mol) in redistilled dimethylformamide (117 μL , 7.5% of total volume) at room temperature. The reaction mixture turned cloudy and was allowed to stir for a further 1 h. The clear solution was centrifuged at 2000g for 10 min and the supernatant then purified on a G-25 Sephadex (medium) HR 16/50 column (pre-equilibrated with PBS). The product was eluted with PBS, and the fractions containing product were combined and concentrated. The final product was sterile filtered and then analyzed by Superose 12 column for aggregate content and free drug content. The conjugation ratio (CR), protein and drug concentrations, and protein yield were determined from the UV spectrum. The protein concentration [mAb] in mg/mL was calculated using the formula

$$[\text{Ab}] = A_{279} \text{df} / 1.43 \quad (\text{where } A_{279} = 1.43 \text{ at } 1 \text{ mg/mL for monoclonal antibody})$$

and the drug concentration [drug] in mg/mL from the Beer-Lambert law:

$$[\text{drug}] = A / \epsilon l$$

amide (10)	$\epsilon/10^4$
(a) m-DMT	0.993
(b) p-DMT	2.06
(c) p-MMeT	1.47
(d) p-MMT	1.71
(e) p-MeT	1.87
(f) p-T	2.22

where ϵ is the molar absorptivity (molar extinction coefficient), l is the path length of the sample (cm), [drug] is the concentration of the drug (mol dm^{-3}), and A is the absorbance of the solution (optical density, OD) at $\lambda_{\text{max}} = 254$ nm for **3b–f** and 263 nm for **3a**.

Conjugation ratio (CR) is defined as the number of moles of drug per mole of antibody. For LY207702 (**1**) MW = 302 and mAb MW = 150 000:

$$\text{CR} = \{[\text{drug}]/302\} / \{[\text{Ab}]/150\,000\}$$

Evaluation of mAb and Conjugates Binding Activity. Competitive binding analyses were performed as follows: serial dilutions (250 $\mu\text{g/mL}$, 125 $\mu\text{g/mL}$, ...) of mAb or conjugate **3** were analyzed for their ability to displace the binding of 50 $\mu\text{g/mL}$ COL1-FITC, prepared by the method described by Harlow and Lane (21), to LS174T human colon carcinoma cells (obtained from the American Type Culture Collection, Rockville, MD). Cell surface fluorescence was quantitated by flow cytometry. Indirect immunofluorescence was accomplished by incubating half log dilutions of mAbs or conjugates (starting at 50 $\mu\text{g/mL}$) with LS174T tumor cells. After unbound antibody was washed away, FITC-conjugated rabbit anti-mouse IgG serum (Jackson Immunoresearch Laboratories, Inc., West Grove, PA) was added and cell surface binding was also quantitated by flow cytometry. The level of immunofluorescence observed in both assays is given as linear equivalent relative fluorescence intensity (LERFI) units.

In Vitro Cytotoxicity. *In vitro* cytotoxicity was determined using a [^3H]leucine incorporation assay as previously described (22).

Nude Mouse Xenografts. The CEA-expressing human colon carcinoma cell line, HC1 (23), was used in the *in vivo* evaluation of free and conjugated LY207702. Approximately 8 mm³ fragments were trocar implanted

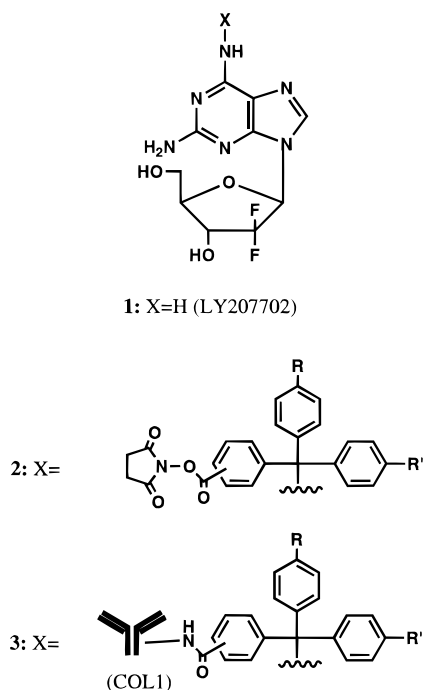


Figure 1. Purine difluoronucleoside, LY207702 (**1**), its trityl derivatives **2**, and immunoconjugates **3**.

Table 1. Trityl Derivatives

		R	R'
(a)	<i>m</i> -DMT	OMe	OMe
(b)	<i>p</i> -DMT	OMe	OMe
(c)	<i>p</i> -MMeT	OMe	Me
(d)	<i>p</i> -MMT	OMe	H
(e)	<i>p</i> -MeT	Me	H
(f)	<i>p</i> -T	H	H

into recipient mice (female, 20–25 g, Charles River Breeding Laboratories, Boston, MA). All animal experiments were done in accordance with the rules and regulations within the AAALAC approved animal research facility at Lilly Research Laboratories.

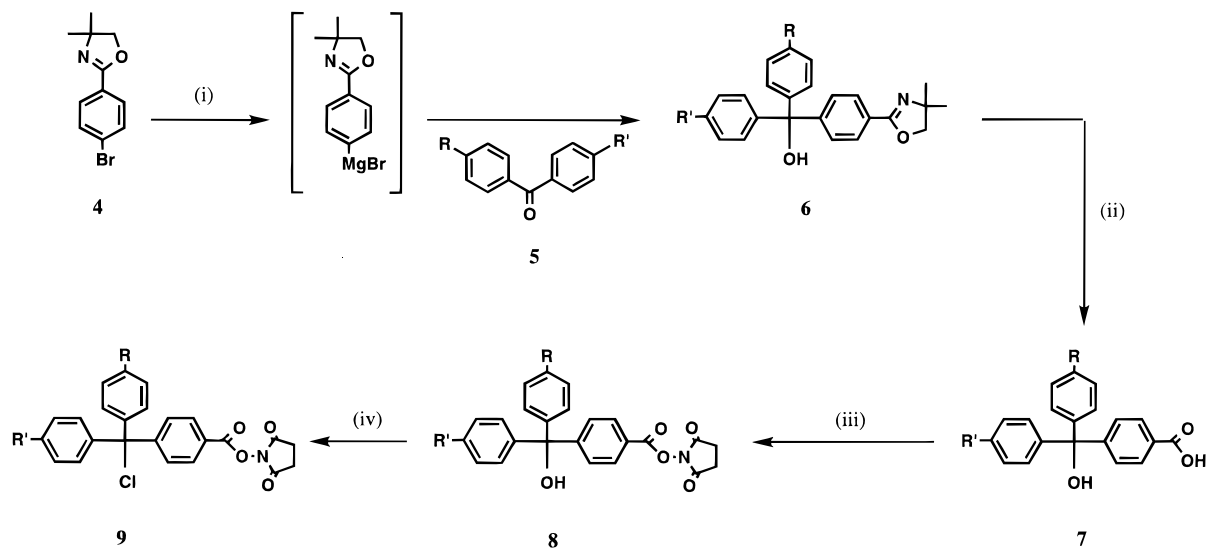
RESULTS AND DISCUSSION

Chemistry. The synthesis of drug conjugates **3** was achieved by initial tritylation of LY207702 (**1**) followed

by coupling of the resulting drug linker **2** to a monoclonal antibody (Figure 1) (24). Trityl linkers used in this study included dimethoxytrityl (DMT), monomethoxymethyltrityl (MMeT), monomethoxytrityl (MMT), methyltrityl (MeT), and trityl (T) each of which possess a carboxy substituent in the para (*p*) or meta (*m*) position on one of the aromatic rings (Table 1). Assuming that the acid mediated dissociation of trityl derivatives proceeds *via* initial protonation of the heteroatom attached to the trityl group followed by cleavage to give a transient trityl carbocation, the rate of dissociation should be proportional to the stability of the trityl carbocation (25). On the basis of this simplified electronic argument, linkers **a**, **b**, **d**, and **f** were expected to provide a range of acid stabilities and therefore were proposed for initial investigation.

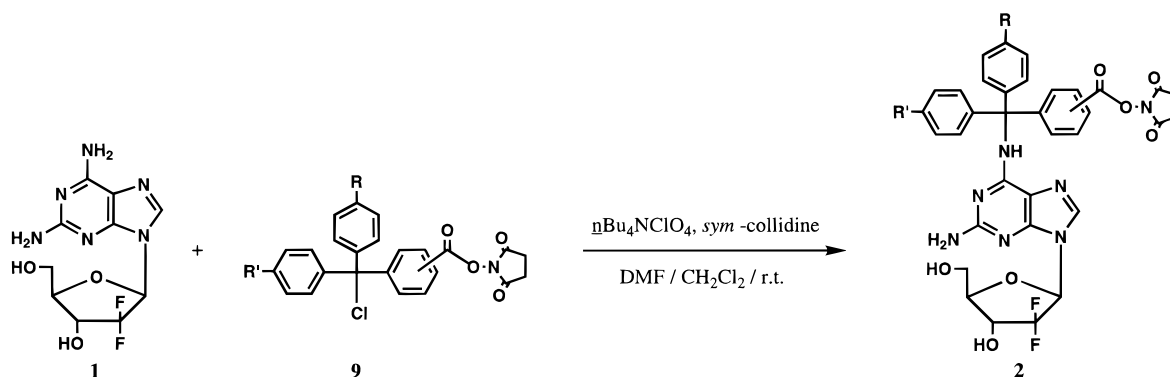
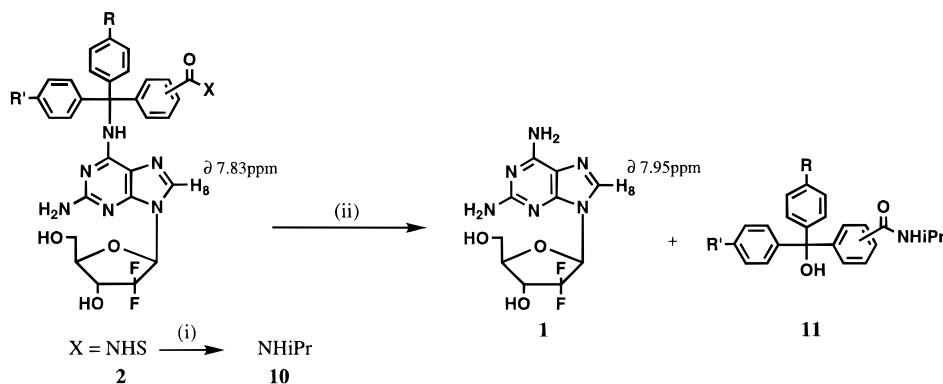
Trityl chloride derivative **9**, previously reported by Gildea (26), was chosen as the bis, differentially reactive electrophile for the sequential introduction of LY207702 (**1**) and mAb in the preparation of conjugate **3**. Thus, addition of the Grignard reagent, derived from aryl bromide **4**, to benzophenone **5** gave carbinol **6** (Scheme 1). Hydrolysis of the 2-oxazoline ring in **6** led to the corresponding carboxylic acid **7**, which was then converted to the corresponding *N*-hydroxysuccinimide active ester **8** under standard DCC coupling conditions. Treatment of alcohol **8** with refluxing acetyl chloride provided the desired trityl chloride **9**. *N*-Tritylation, for protection during synthesis, is commonly achieved by reacting an amine with a trityl chloride at room temperature usually in pyridine as solvent (27). The less reactive aromatic amines present in purine heterocycles can also be alkylated under similar conditions (28). In the presence of primary hydroxyl groups, however, excess trityl chloride and elevated temperatures lead to the formation of bis-*N*,*O*-tritylated product (29). In one instance, the use of 1.1 equiv of trityl chloride led to preferential *O*-tritylation in the presence of a purine N6-amino group (30). It has been shown that even the extremely unreactive guanosine N2-amino group can also be tritylated under standard conditions (31). It was, therefore, remarkable that reaction of unprotected difluoronucleoside, LY207702 (**1**), with 1.1 equiv of trityl chloride **9**, under conditions developed by Reddy and co-workers (32), cleanly produced the desired N6-tritylated nucleoside **2** as the sole product in 54–93% isolated yield (Scheme 2). It is worthy to note

Scheme 1^a

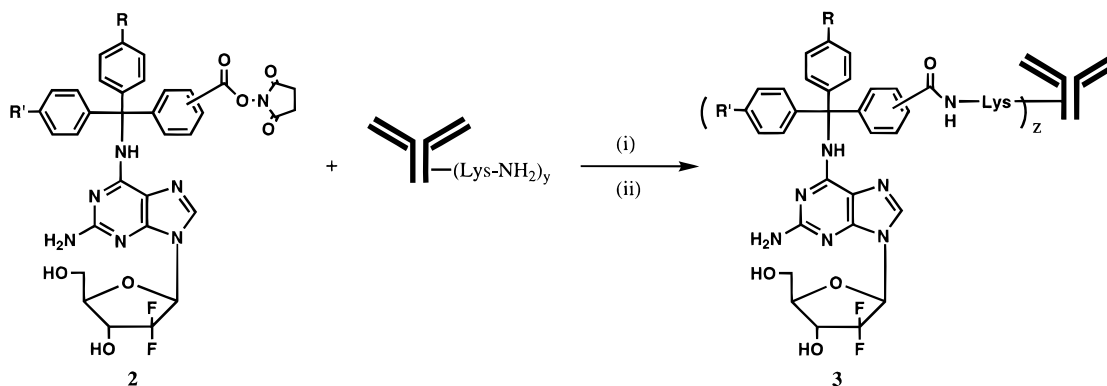


^a (i) (a) Mg/THF, room temperature, sonication; (b) benzophenone **5**, THF, room temperature; (ii) (a) 80% aqueous AcOH, 55–60 °C; (b) 20% NaOH in aqueous EtOH; (c) 6 M HCl; (iii) DCC, NHS, CH₂Cl₂; (iv) AcCl, 60 °C.

Scheme 2

Scheme 3^a

^a (i) *i*PrNH₂, CH₂Cl₂, room temperature; (ii) ¹H NMR, ~6 mM in 0.1 M KH₂PO₄ in D₂O/MeOH-*d*₄.

Scheme 4^a

COL1-(N6-Trityl-207702)_z

where *z* = Conjugation Ratio (CR)

^a (i) **2**, molar equivalents = 8, COL1 (20–30 mg/mL) in 0.1 M borate buffer, pH ~8.6, 7.5% DMF, room temperature, 1 h; (ii) centrifuge, purify on Sephadex G-25 column (eluting with pH 7.4 PBS), and concentration/sterile filter.

that exclusive regioselective tritylation of N6 amino group of **1** and selective alkylation versus acylation was achieved under these conditions (24). Subsequent acylation of isopropylamine with active ester **2** was found to be rapid and quantitative, at ambient temperatures, to provide the corresponding trityl amide **10** (Scheme 3). This encouraging result indicated that the acylation of ε-amino lysine groups on the mAb with NHS ester **2** should also proceed with ease under mild conditions that would be necessary in the final step of the synthesis of conjugate **3**.

Release Rates of LY207702 1 from Trityl-207702 Amides 10. To determine the effect of acid-mediated release of drug on biological activity of trityl-linked conjugates, it was necessary to first identify a range of trityl linkers with differing acid labilities. Trityl amides

10a–f were, therefore, subjected to hydrolysis under various pH conditions and the rate of consumption of amide **10** measured (33). In these experiments, a solution of amide **10** in deuterated methanol was diluted with a solution of 0.1 M KH₂PO₄ in D₂O buffered at pH 5.40, 6.40, and 7.40 to achieve a final concentration of ca. 6 mM (Scheme 3). The solutions were heated at 37 °C in an NMR tube and hydrolysis conveniently monitored by ¹H NMR spectroscopy. The starting trityl nucleoside derivative **10** and released free drug **1** were readily identified by the presence of a distinct 8 H singlet at 7.83 and 7.95 ppm, respectively. Integration of the respective peaks enabled the rate of consumption of amide **10** and appearance of **1** to be determined. It was found that the rate of formation of free drug **1** was equal to the rate of consumption of drug linker **10**. The formation of the

Table 2. Half-Lives (h) of N6-Trityl Amides under Various pH Conditions

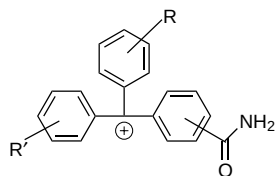
pH	<i>m</i> -DMT (10a)	<i>p</i> -DMT (10b)	<i>p</i> -MMeT (10c)	<i>p</i> -MMT (10d)	<i>p</i> -MeT (10e)	<i>p</i> -T (10f)
5.40	2.35	3.71	2.40	16.7	26.5	stable ^e
6.40	23.3	14.8	43	253	368 ^c	stable ^e
7.40	133.3 ^a	146.3	229	800 ^b	stable ^d	stable ^e

^a Extrapolated figures. ^b 14% dissociation after 100 h. ^c See footnote 5. ^d 5% dissociation after 631 h. ^e No dissociation up to day 33.

expected hydrolysis products, LY207702 (**1**) and carbinol **11**, was also detected by TLC analysis and confirmed by isolation and characterization. The data from the release studies is represented in graphical form (amount of **10** (%) vs time (h)) for drug linkers **10a–f** at (a) pH 5.40, (b) pH 6.40, and (c) pH 7.40 (Figure 2a–c). In all cases, a linear relationship was observed, suggesting that release of drug **1** from drug linker **10** proceeds in a pseudo-first-order manner under these conditions. In order to gain a better understanding of the relative acid lability of these trityl linkers, the release rates were determined from these plots and expressed as half-lives, $t_{1/2}$ (h), the results of which are summarized in Table 2. Indeed, as predicted earlier, a wide range (e.g. $t_{1/2}$ = 2.35 h for **10a** and >800 h for **10f** at pH 5.40) in release rates was observed for these trityl linkers. Moreover, for a particular trityl linker, the release rates were found to be pH dependent, where 5.40 > 6.40 > 7.40, suggesting that the rate of initial protonation of **10** significantly influences the rate of hydrolysis. Qualitative analysis of the data indicate that an increased stabilization of the transient trityl carbocation leads to increased rates of hydrolysis (i.e. **10a** = **10b** > **10c** > **10d** > **10f** > **10e**). Since substituents R and R' on the aromatic rings of the trityl group play a major role in stabilizing the trityl cation, it is, therefore, reasonable to conclude that the substituents also govern the release rates. The half-lives do not, however, completely correlate with the calculated charge⁴ on the presumed intermediate trityl cation,

³ Some cytotoxicity determinations differ from those in ref 23 due to the availability of further data. In those cases where a conjugate was prepared multiple times, the average cytotoxicity of all the conjugates is quoted.

⁴ Calculation of the charge on the methyl carbon atom of substituted trityl carbocation was determined by quantum chemistry performed on Silicon Graphics Indigo 2 Workstation with MOPAC program using the AM1 Hamiltonian through the interface provided by the program CERIU2. All of the structures for MOPAC calculations were geometry preoptimized using the molecular mechanics program with CERIU2. The optimized geometry was used by MOPAC on the Workstation for single point calculation of the partial atomic charges as the carbocation after the removal of the methine hydrogen (keyword: charge=+1). The charge values are listed below:



trityl cation	charge calcd
<i>m</i> -DMT	0.3948
<i>p</i> -DMT	0.4135
<i>p</i> -MMeT	0.4213
<i>p</i> -MMT	0.4351
<i>p</i> -MeT	0.4469
<i>p</i> -T	0.5163

⁵ This figure was redetermined using more data points to give an accurate half-life for amide **10e**. This figure significantly differs from that which was determined in ref 24 by extrapolation from a few early time points. It is apparent that extrapolation, in this manner, leads to overestimation of the half-lives.

Table 3. Physical Chemical and Cytotoxicity Data for COL1–N6-Trityl-207702 Conjugates

	CR	protein yield (%)	IC ₅₀ (μg/mL)
(1) LY207702	—	—	0.302
(3) COL1–N6-trityl-207702			
(a) <i>m</i> -DMT	6.44	52	0.352
(b) <i>p</i> -DMT	7.44	65	0.288
(c) <i>p</i> -MMeT	7.49	44	2.71
(d) <i>p</i> -MMT	5.73	61	0.966
(e) <i>p</i> -MeT	5.20	47	6.04
(f) <i>p</i> -T	4.44	50	>10
COL1	—	—	>330

suggesting that the hydrolysis of trityl groups is also influenced by other factors such as ionic strength (34) and counterion of the media, concentration, temperature as well as added nucleophiles (35). The presence of these and other factors at the site of release in a relevant biological system (e.g. extracellular environment of tumor cells in a cytotoxicity assay) is unknown, and therefore, precise conditions are difficult to reproduce. Nevertheless, the qualitative correlation between the electronic nature of R and R' and the relative release rates of trityl linked drug provides a useful and predictive tool for linker design.

Preparation and Characterization of Conjugates

3. The final step in the synthesis of drug immunoconjugate **3** required the formation of a stable amide bond between ϵ -amino group of lysine residues on mAb COL1 and the carboxy group of drug linker **2**. Accordingly, a solution of **2** in dimethylformamide was added to an aqueous pH 8.6 buffered solution of mAb at room temperature and stirred for 1–2 h. The initial cloudy reaction mixture became a clear, homogeneous solution, indicating that the reaction was complete. TLC analysis of the reaction mixture for the consumption of active ester **2**, however, provided a more accurate method of judging reaction progress. Final conjugates **3** were isolated by size exclusion chromatography and then analyzed by UV spectroscopy to determine the conjugation ratio (CR), protein and drug concentrations, and overall yield. These mild conjugation conditions led to high levels of drug (55–94%) incorporation and yielded conjugates **3** with conjugation ratio of 4–7 (Table 3). Total protein recovery was satisfactory (44–65%) at final concentrations of 20–30 mg/mL; however, at higher concentrations of conjugate, precipitation was observed leading to significant reduction in yield. It is very likely that the hydrophobic nature of the trityl group renders these conjugates less soluble in aqueous media. Protein aggregation usually leads to nonspecific binding and precipitation, both of which are undesirable properties for conjugates. The aggregation content in conjugates **3** was readily assessed using an analytical Superose-12 column. As an example, a chromatogram of COL1–N6-*p*-MeT-207702 (**3e**), which represents a typical profile for these conjugates, is shown in Figure 3. In general, conjugates **3a–f** consisted of 92–96% of the desired monomeric form of the protein with the remainder comprised of low molecular weight (3–6%) and high molecular weight (1–2%) aggregates. This method of analysis was also able to show that no free drug **1** was present in the final preparations. Furthermore, no significant aggregates or degradation products

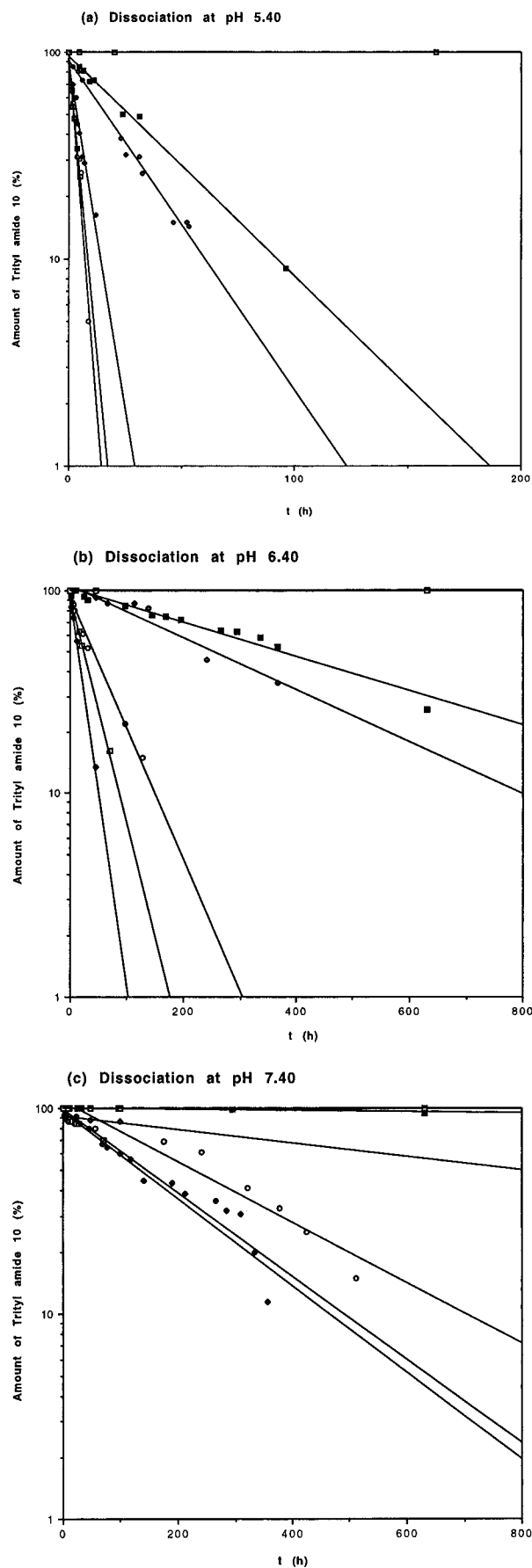


Figure 2. Release of LY207702 **1** from Trityl-207702 amides **10a** (□), **10b** (◆), **10c** (○), **10d** (◇), **10e** (■) and **10f** (□) with time of incubation at (a) pH 5.40, (b) pH 6.40, and (c) pH 7.40 (37 °C).

were detected when conjugates **3a–f** were subjected to analysis by SDS–PAGE under reducing and nonreducing conditions (data not shown). Conjugate **3**, which was isolated in 0.1 M phosphate-buffered solution (PBS) and stored under sterile conditions at 4 °C, remained unchanged for several months with respect to levels of free drug **1** and aggregate. The quality of the conjugates, prepared on scales ranging from 10 mg to 10 g was consistent and sufficient for biological studies and did not require further purification. Noncompetitive and competitive assays using CEA positive LS174T cells showed that construct **3** retained good antigen binding characteristics in comparison to unconjugated mAb COL1, examples of which are shown in parts a and b of Figure 4, respectively. Correspondingly, conjugate **3** prepared with an irrelevant IgG mAb did not bind to LS174T or HC1 tumor human colon carcinoma cells (data not shown), indicating that components in the construct other than the mAb do not contribute to antigen binding.

In order to minimize immunogenic response during human therapy with murine-based drug conjugates, it is desirable to deliver maximal quantities of drug using minimal amounts of antibody. One method of producing conjugates with higher drug loading (CR) is to increase the molar equivalents of drug. Coupling of **2** (16-fold molar excess) to mAb D612 (36), indeed, gave a conjugate with a higher drug load (CR = 11.3), however, a 10% yield and ~40% high MW aggregate content deemed the conjugate unacceptable for use. Furthermore, fractionation of this conjugate, using a hydrophobic interaction column, gave species with even higher CR (~20) but unfortunately also of diminished quality. These results reflect the limitations of lysine coupling methodology and probably also the physical properties of mAb COL1. The procedure described using a drug-linker molar equivalents of 8 was deemed optimal and gave COL1–N6-Trityl-207702 conjugate **3** with consistent and acceptable properties suitable for biological evaluation.

Biological Properties. Cytotoxicity³ of conjugates **3a–f**, LY207702 (**1**), and unconjugated mAb COL1 was determined by measuring [³H]leucine uptake in CEA positive LS174T cells after incubation for 48 h and expressed as IC₅₀ values (Table 3). The most active conjugates, **3a** and **3b**, which were equipotent to free drug **1**, corresponded to drug linkers **10a** and **10b** which released drug **1** at the highest rate under acidic conditions (see Table 2). The *p*-MeT conjugate **3e** was found to be less active than *p*-MMT **3d**, but significantly more active than *p*-T conjugate **3f**. With the exception of conjugate **3c**, the order of cytotoxicity of the conjugates, **3a** = **3b** > **3d** > **3e** > **3f**, correlated well with the rate of release of drug **1** from amides **10**. The higher than expected IC₅₀ for conjugate **3c** could be due to factors other than electronics which influence release of drug from the conjugate; however, these were not identified in these studies. A quantitative analysis of the cytotoxicity data (Table 3), however, indicates that incubation of conjugates **3a** and **3c** at 37 °C for 48 h led to 100% dissociation to free drug **1**. Likewise, conjugates **3c**, **3d**, **3e**, and **3f** were hydrolyzed 71%, 33%, 5%, and 0%, respectively. In contrast, incubation of prodrugs **10a–f** for 48 h at 37 °C in methanolic aqueous phosphate buffer, for example, at pH 6.40 gave 78%, 88%, 53%, 8%, 8%, and 0% free drug, respectively (see Figure 2b). This comparison assumes, firstly, that cytotoxicity of conjugate **3** is solely derived from the release and action of LY207702 (**1**) and, secondly, that the pH experienced by conjugate **3** is 6.40 in the cellular assay. Since both unconjugated COL1 and tritylated COL1, a byproduct from the hydrolysis of conjugate **3**, did not exhibit

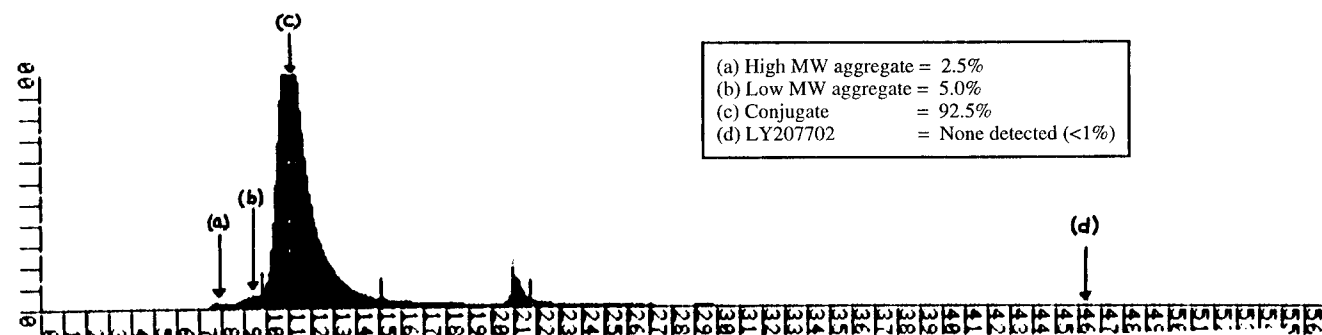


Figure 3. Profile of COL1-N6-*p*-MeT-207702 conjugate **3e** on an analytical Superose 12 column. Conjugate (100 μ L, df = 10 in PBS), with CR = 4.6, [COL1] = 24.9 mg/mL, [LY207702] = 0.231 mg/mL, was eluted with 15% CH₃CN/PBS at flow rate = 0.8 mL/min and detected at λ = 279 nm. High MW = $>1 \times 10^6$, low MW = $\sim 3 \times 10^5$, conjugate MW = 150 000.

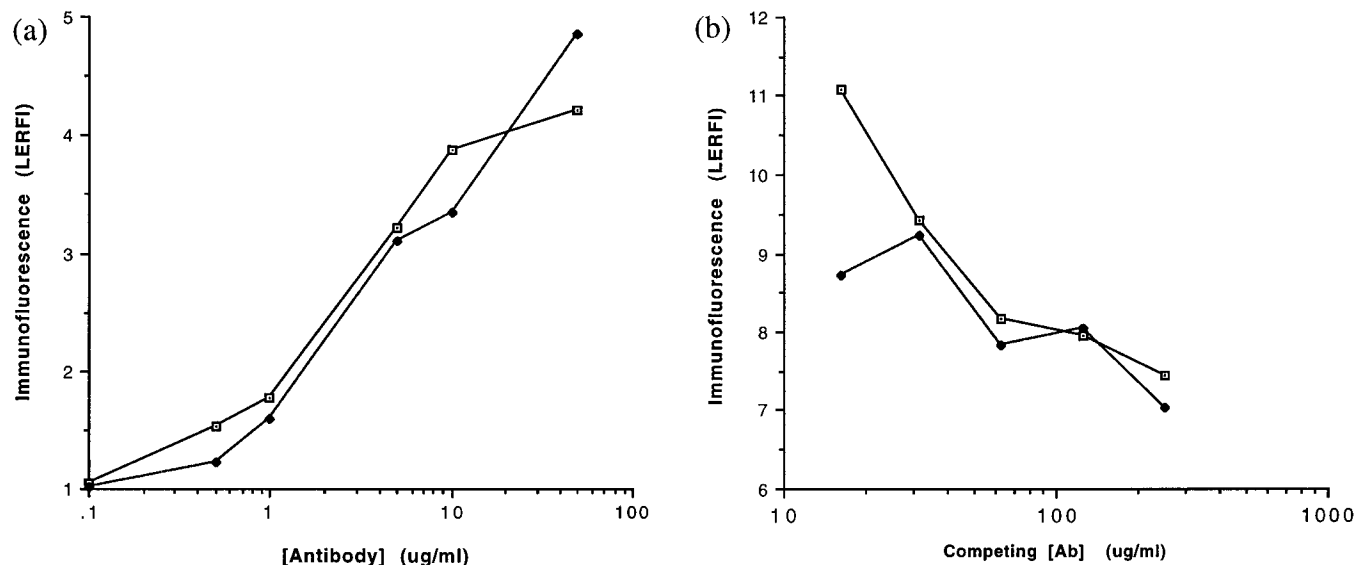


Figure 4. Immunoreactivity of COL1-N6-trityl-207702 conjugates **3** (◆) versus mAb COL1 (□) to LS174T cells determined by (a) indirect immunofluorescence of COL1-N6-*p*-MeT-207702 **3e** and (b) competitive binding analysis of COL1-N6-*p*-DMT-207702 **3b**.

cytotoxicity, it is believed that cytotoxicity of conjugate **3** is, in fact, due to release of LY207702 (**1**). This is consistent with the knowledge that COL1 is a noninternalizing antibody (16), and therefore, the cytotoxicity demonstrated by conjugate **3** most likely results from the release of drug in the extracellular environment of the tumor cells and that LY207702 (**1**) is rapidly internalized and subsequently phosphorylated to its active metabolites (37). Although there is convincing literature evidence that the tumor pH is on average lower than normal tissue pH, the exact difference is dependent on tumor type and method of measurement (6). Gerweck (6) recently reported the extracellular pH of tumors in several patients and found that the pH ranged from 6.4 to 7.0. It is, therefore, difficult to design accurate release studies without knowing the pH of the tumor environment. It should be noted that the release studies were not performed on the actual conjugates **3**, primarily due to difficulties encountered in assaying for free drug **1**. The release of drug from the conjugate may possibly be influenced by additional factors, such as steric and ionic effects of amino acid residues at the site of the drug-linker attachment on the mAb. The above comparison between release rates of **1** from prodrug amide **10** and the cytotoxicity of the corresponding conjugates **3** does, however, reveals a qualitative relationship between the electronic nature of the substituents on the trityl linker **a-f** and cytotoxicity of the trityl-linked conjugates **3a-f**. This correlation provides a useful means of ranking the *in vitro* cytotoxicity for a series of trityl-linked

conjugates, a property which should allow a more convergent approach to the design of an optimal trityl-linked conjugate.

The sensitivity of LY207702 (**1**) to the HC1 human colon carcinoma and expression of high levels of CEA on the tumor cells made this model suitable for determining the antitumor activity and targeting properties of conjugate **3**. The nature of the antigen, for example shedding which would lead to circulating levels of CEA, in the HC1 tumor model was not examined in these studies. Selective targeting, however, has previously been achieved using a different mAb drug conjugate that also recognizes CEA (38). The release studies indicate that conjugate **3** requires an acidic environment to release free drug **1**; however, the pH of HC1 tumors was not determined due to the complexity encountered in measuring tumor tissue versus adjacent normal tissue as well as extracellular (pHe) versus intracellular (pHi). Assumptions were, therefore, made that the extracellular tumor pH in the HC1 model would be more acidic than normal tissue (6) and that this pH would be sufficient to cleave acid labile conjugate **3** while bound to the target antigen. This hypothesis was tested using antitumor efficacy as an end point. To assess antitumor activity, nude mice were, first, trocar implanted subcutaneously with HC1, and the tumor was allowed to establish (~ 0.5 g). Mice were separated into groups of eight and treated iv with drug **1** or conjugate **3** twice weekly for 2 weeks, and tumors were measured weekly postimplantation. Initial studies with *p*-DMT-linked conjugate **3b** showed a good dose

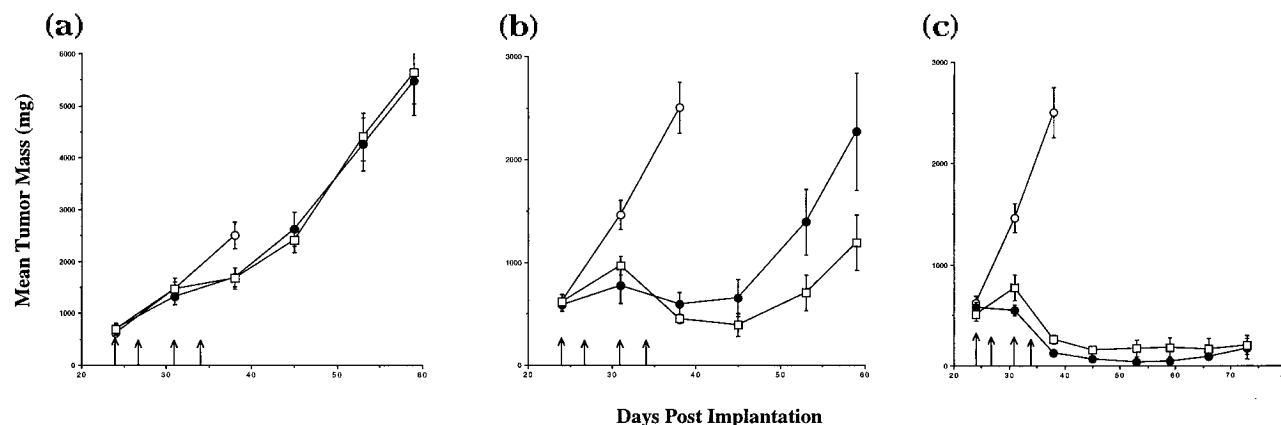


Figure 5. Effect of LY207702 (**1**) and COL1-N6-*p*-DMT-207702 conjugate **3b** on HC1 human colon carcinoma. Tumor was trocar implanted sc in nude mice and allowed to establish to ~0.5 g. Mice were separated into treatment groups of eight and dosed iv with LY207702 (**1**) (□), conjugate **3b** (●), and saline control (○) twice weekly for 2 weeks (indicated by arrows) at (a) 1 mg/kg (drug equivalents), (b) 2 mg/kg, and (c) 4 mg/kg. Tumors were measured weekly postimplantation.

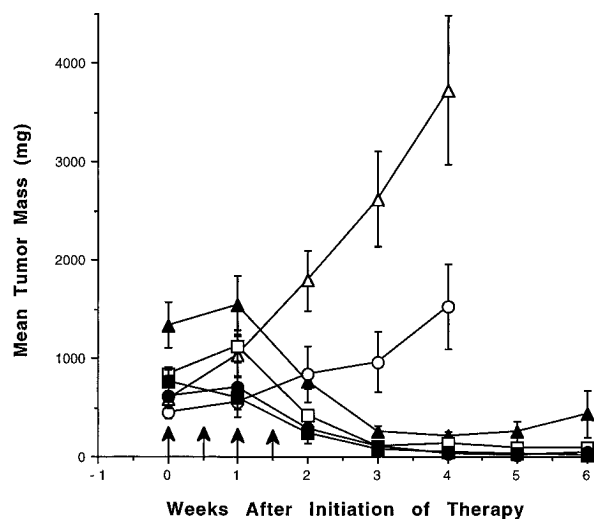


Figure 6. Comparison of antitumor activity of COL1-N6-trityl-207702 conjugates **3** and LY207702 (**1**) against HC1 human colon carcinoma. Tumors were allowed to establish and nude mice (groups of eight) were treated iv with saline control (Δ), LY207702 **1** (□) or conjugate **3b** (●), **3d** (■), **3e** (▲), **3f** (○) at 4 mg/kg (drug equivalents) twice weekly for 2 weeks (indicated by arrows). Tumors were measured weekly postimplantation.

response whereby conjugates **3b** and free drug **1** exhibited no activity at 1 mg/kg (drug equivalent), inhibition at 2 mg/kg, and complete and sustained regression at 4 mg/kg (Figure 5). It is interesting to note that the similar *in vivo* activity of conjugate **3b** and free drug **1** is consistent with the *in vitro* cytotoxicity data (Table 3). Antitumor activity of conjugates **3b**, **3d**, **3e**, and **3f** was then assessed in a higher tumor burden (0.5–1.2 g) HC1 model at the 4 mg/kg dose schedule. In these experiments, the least *in vitro* cytotoxic *p*-T conjugate **3f** displayed the least *in vivo* antitumor activity, exhibiting only moderate inhibition of tumor growth, while the more cytotoxic conjugates **3b**, **3d**, and **3e** all regressed the large tumor volumes (Figure 6). The complete tumor regression displayed by conjugates **3b**, **3d**, and **3e**, and free drug **1** reflects the remarkably high level of antitumor activity of this novel purine nucleoside in this resistant tumor xenograft (17). Particularly impressive was the sustained tumor regression observed several weeks after the last dose of conjugates **3b**, **3d**, and **3e** was administered. *p*-MeT conjugate **3e** treated group did, however, show signs of tumor regrowth at 5–6 weeks after initiation of therapy. In contrast, *p*-DMT **3b** and *p*-MMT **3d** conjugates, which were equally efficacious, main-

tained complete tumor regression until the end of the experiment (6 weeks). The larger initial tumor size in the conjugate **3e** experiment makes it difficult to draw a true comparison between the antitumor activities of conjugates **3b**, **3d**, and **3e**. The *in vivo* activities of free drug **1** and conjugates **3b** and **3d** were essentially indistinguishable; however, the faster tumor regrowth of conjugate **3e** indicates that this may be the least active of these. The lack of a good correlation between the *in vitro* activity and *in vivo* activity of conjugate **3** is probably attributed to several factors, including (i) the use of a more drug sensitive HC1 tumor cell line used *in vivo* versus the less responsive LS174T cells used *in vitro*, (ii) the unoptimized *in vivo* dosing schedule, and (iii) the unknown tumor pH of LS174T (*in vitro*) and HC1 (*in vivo*) which, if different, would give rise to different release rates of the same conjugate **3**.

A hallmark of drug conjugates is the ability to selectively deliver drug to the tumor and thereby reduce systemic toxicity. For this to be effective, a drug conjugate possessing a linker which releases drug while bound to the tumor antigen is desirable. A series of experiments were, therefore, conducted comparing antitumor activity of antigen-positive (COL1) and antigen-negative (IgG) binding conjugates **3** to identify a trityl-linked conjugate **3** which demonstrated activity primarily through antigen binding. Accordingly, conjugates **3b**, **3d**, **3e**, and **3f** were administered at a dose of 4 mg/kg twice weekly for 2 weeks to HC1 tumor-bearing mice, and antitumor activity was measured. Surprisingly, in all cases, *p*-DMT **3b**, *p*-MMT **3d**, and *p*-MeT **3e** linked conjugates, there were no significant differences in antitumor activity between the irrelevant (IgG) and relevant (COL1) conjugates in this large (~1 g) tumor burden model (data not shown). Immunofluorescent analysis of enzymatically dissociated tumor cells obtained from mice treated with COL1-*p*-MeT-207702 or irrelevant IgG-*p*-MeT-207702, however, demonstrated that HC1 tumor cell binding occurred only in the COL1 conjugate **3e** treated animals (data not shown) (22). It is conceivable, however, that the more labile irrelevant IgG conjugate **3b** may be rapidly releasing drug systemically prior to antigen binding and essentially acting in the same manner as administering free drug **1**. However, the origin of activity displayed by the supposedly more stable (see Table 2) irrelevant IgG conjugates **3d** and **3e** is unclear. Since the concept of targeting drugs using mAbs relies on specific binding of the conjugate to the target antigen with subsequent release of drug, the "mistiming" of these two events would lead to loss of the desired effect. It is also possible that

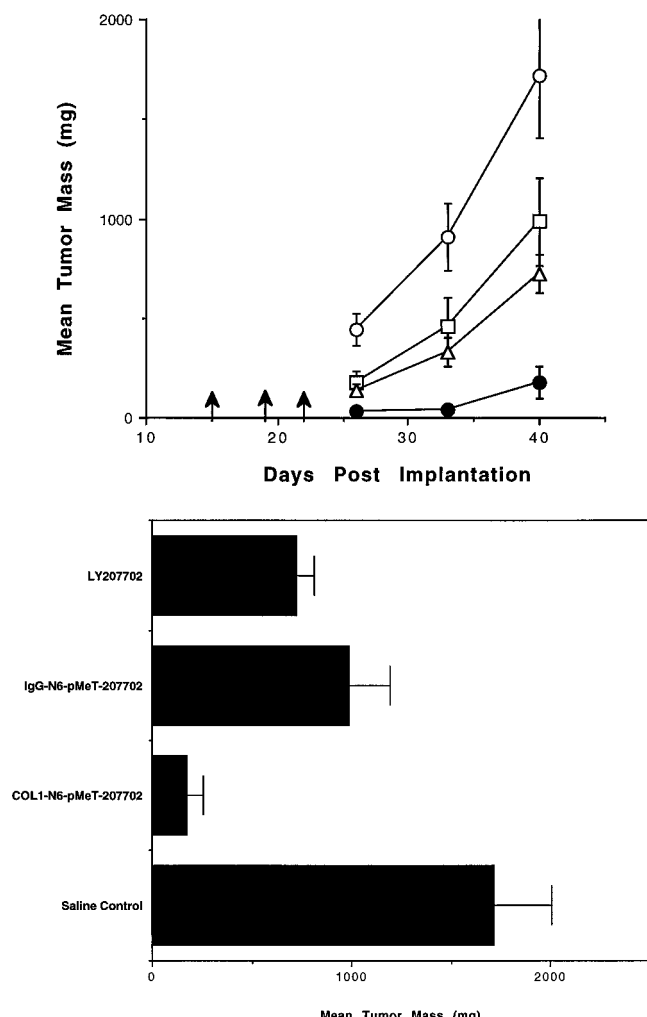


Figure 7. Comparison of antitumor activity of mAb-N6-*p*-MeT-207702 conjugates **3e** in HC1 tumors: COL1 (relevant) versus IgG (irrelevant) implanted sc with HC1 tumors on day 1. Mice with palpable tumors were selected on day 14 and separated into treatment groups of seven. (a) Tumor mass measured weekly for groups dosed with saline control (○), LY207702 (**1**) (△), relevant COL1 conjugate **3e** (●), and irrelevant IgG conjugate **3e** (□) at 4 mg/kg drug equivalents iv on days 15, 19, and 22. (b) Tumor mass measured on day 40 for each of the treatment groups.

the lipophilic trityl constructs **3** may bind nonspecifically to tumor cells exposed to an acidic environment which causes drug to be released at the tumor site. These and other unknown factors, which have not been investigated, may influence drug release *in vivo* and account for the observed results with irrelevant conjugate. Another possibility is that low levels of irrelevant conjugate were localized to the large tumor masses *via* Fc receptor-mediated binding. Once localized, the exquisite activity of released LY207702 (**1**) against HC1 may have been sufficient to cause tumor regression. In order to determine if tumor size was a contributing factor to the apparent cause of antigen-mediated activity of the mAb-trityl-207702 conjugates **3**, we evaluated these constructs in a lower tumor burden model. Consequently, a similar study was performed in which the HC1 tumor was implanted, and 14 days later mice bearing palpable tumors were separated and treated with conjugates **3b**, **3d**, **3e**, and **3f** under identical dosing conditions and schedule as described above. In this model, a clear difference in antitumor activity between irrelevant (IgG) and relevant (COL1) *p*-MeT-linked conjugates **3e** was observed (Figure 7a). A repeat of this experiment, with a different batch of conjugate **3e**, gave the same results.

The significantly higher level of activity observed with COL1 conjugate **3e** compared to the irrelevant conjugate, most pronounced on day 40 of the experiment (Figure 7b), suggested that antigen-mediated binding significantly contributes to its antitumor activity. It is also interesting to note that in this experiment COL1 conjugate **3e** was more active than LY207702 (**1**). Conjugates **3b**, **3d**, and **3f** did not, however, demonstrate this level of specificity, suggesting that *p*-MeT is the optimal linkage in this series of conjugates evaluated under the low tumor model conditions. These data provided the basis for selecting *p*-MeT-linked conjugate **3e** for further evaluation.

In an effort to establish the *in vivo* metabolic stability of *p*-MeT-linked conjugate **3e**, it was administered to nontumor-bearing nude mice and plasma levels of LY207702 (**1**) measured. It is, however, known that LY207702 (**1**) is an excellent substrate for adenosine deaminase which rapidly converts it to the corresponding difluoroguanosine analogue (LY223592) (37). Thus, when a single iv dose of 8 mg/kg of LY207702 (**1**) was administered to female nude mice, a peak concentration of ~1 µg/mL of metabolite LY223592 was observed 30 min after dosing, followed by a decrease in levels ($t_{1/2}$ = ~30 min) as a result of excretion of drug. In contrast, no detectable levels (detection limit = 200 ng/mL) of LY207702 (**1**) or its metabolite LY223592 were observed 30, 60, or 120 min after a single, 8 mg/kg (drug equivalents) iv dose of conjugate **3e**, suggesting that direct release of drug is not rapid ($t_{1/2}$ > 1 h). It is possible that antitumor activity observed with irrelevant *p*-MeT conjugate (Figure 7a) could be due to release of drug at levels not detected (i.e. <200 µg/mL) in this assay. Nevertheless, the combination of relative stability in mouse plasma and the observed antigen mediated antitumor activity of COL1 *p*-MeT-linked conjugate **3e** implied that this construct may possess an improved therapeutic index compared to LY207702 (**1**) and warranted further investigation.

Toxicological Evaluation of COL1-N6-*p*-MeT-207702 Conjugate 3e. To gain further insight into the therapeutic index, studies were conducted to directly compare toxicity profiles of *p*-MeT conjugate **3e** and LY207702 (**1**). In particular, emphasis was placed on the detection of irreversible cardiac toxicity, originally observed during the development of LY207702 (**1**) and which lead to its demise as a clinical candidate (17). Consequently, HC1 tumor-bearing (~0.5 g), male, nude mice were dosed ip at 6 mg/kg daily for 21 days with free drug **1** and conjugate **3e**. Histopathological analysis revealed 2/9 mice in the free drug **1** treated group possessed moderate focal necrosis of the heart, while no cardiotoxicity was detected in the conjugate **3e** treated group. These initial findings were particularly encouraging, since the total dose of conjugate **3e** was equivalent to ~8 times the efficacious dose (see Figure 6). It should be noted, however, that the route of administration was altered from iv to ip to allow the delivery of the large quantities of antibody. In spite of this change, excellent antitumor activity was still observed in both treatment groups (data not shown). Other toxicities found in the free drug **1** group included inflammation and hemorrhaging of colon tissue in 1/10 mice. All treated groups showed hypospermatogenesis, a toxicity of the testis expected from the use of antiproliferative agents. Hematological toxicities included increases in total leukocyte counts and decrease in thrombocyte counts in both treated groups. Changes noted in the clinical chemistry included minor elevation of ALT liver enzyme in the conjugate **3e** group, while both ALT and AST were significantly elevated in the free drug **1** group suggestive

of an inflammatory response and hepatocellular leakage. The upper limit of the therapeutic index was reached when the dose of conjugate **3e** was increased to 8 mg/kg for 21 days (~10 times the total dose required for complete regression of established HC1 tumors) and cardiotoxicity was observed in all the nontumor-bearing mice. Free drug **1**, at the same dose, also induced severe cardiotoxicity in all treated mice. An increased degree of toxicity of these novel agents in nontumor-bearing mice versus tumor-bearing mice was noted, but these differences are not yet understood.

In summary, difluoronucleoside LY207702 (**1**) was conjugated to mAb COL1 through a series of acid labile trityl linkers. *In vitro* cytotoxicity of the resulting conjugates **3** correlated well with rate of release of drug from the corresponding drug linkers **10**, but only a tentative relationship was observed with the *in vivo* antitumor activity in a human HCl colon xenograft. *p*-MeT-linked conjugate **3e** was identified as possessing significant antitumor activity mediated through specific antibody-antigen binding interactions. Substantial antitumor effects, however, were also observed that appeared to be independent of antigen binding. The differential effects of antigen versus non-antigen-mediated antitumor activity may be influenced by the nature of the target (i.e. internalizing versus noninternalizing) recognized by the tumor selective mAb. This parameter was not examined in this present study. Conjugate **3e** also demonstrated reduced cardiotoxicity (although not completely abrogated) compared to that of free drug **1**. The attachment of LY207702 to mAb COL1 using a trityl-based linker *p*-MeT has, therefore, provided a means of markedly improving the therapeutic index of LY207702 (**1**). Our study thus provides a useful foundation for the design and evaluation of trityl-based linkers as a means of identifying potent and selective monoclonal antibody drug conjugates.

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Supporting Information Available: Physical chemical data on compounds **6–9 (a–d,f)** (5 pages). Ordering information is given on any current masthead page.

LITERATURE CITED

- (1) Foye, W. O., Ed. (1995) *Cancer Chemotherapeutic Agents*, American Chemical Society, Washington, DC.
- (2) (a) Bundgaard, H., Ed. (1985) *Design of Prodrugs*, Elsevier Science, Amsterdam. (b) Roche, E. B., Ed. (1977) *Design of Biopharmaceutical Properties through Prodrugs and Analogues*, American Pharmaceutical Association, Washington, DC. (c) Stella, V. J., Mikkelsen, T. J., and Pipkin, J. D. (1980) *Drug Delivery Systems* (Juliano, R. L., Ed.) p 112, Oxford University Press, Oxford. (d) Senter, P. D. (1990) Activation of prodrugs by antibody-enzyme conjugates: a new approach to cancer therapy. *FASEB J.* 4, 188–193.
- (3) (a) Baldwin, R. W. (1985) Monoclonal Antibody Targeting of Anticancer Agents: Muhlbock Memorial Lecture. *Eur. J. Cancer Clin. Oncol.* 21 (11), 1281–1285. (b) Reisfeld, R. A., and Cherish, D. A. (1985) Human tumor-associated antigens: targets for monoclonal antibody-mediated cancer therapy. *Cancer Surv.* 4 (1), 271–290. (c) Ghose, T. I., Blair, A. H., Vaughan, K., Kulkarni, P. (1983) Antibody directed drug targeting in cancer therapy. *Targeted Drugs*; (E. P. Goldberg, Ed.) pp 1–22, John Wiley and Sons, New York. (d) Ram, B. P., and Tyle, P. (1987) Immunoconjugates: Applications in Targeted Drug Delivery for Cancer Therapy. *Pharm. Res.* 4 (3), 181–188.
- (4) Koppel, G. A. (1990) Recent Advances with Monoclonal Antibody Drug Targeting for the treatment of Human Cancer. *Bioconjugate Chem.* 1, 13–23.
- (5) Barton, R. L., Briggs, S. L., and Koppel, G. A. (March 1991) Monoclonal Antibody Drug Targeting. *Drug News Perspect.* 4 (2), 73–88.
- (6) (a) Gerweck, L. E., and Seetharaman, K. (1996) Cellular pH Gradient in Tumors versus Normal Tissue: Potential Exploitation for the Treatment for Cancer. *Cancer Res.* 56, 1194–1198. (b) Lavie, E., Hirschberg, D. L., Scriber, G., Thor, G., Hill, L., Hellstrom, I., and Hellstrom, K-E. (1991) Monoclonal antibody L6-Daunomycin conjugates constructed to release free drug at lower pH tumor tissue. *Cancer Immunol. Immunother.* 33, 223–230.
- (7) Poznansky, M., and Juliano, R. L. (1984) Biological approaches to the controlled delivery of drugs: a critical review. *Pharmacol. Rev.* 36, 277–336.
- (8) (a) Shen, S. C., and Reiser, J. P. (1981) *cis*-Aconityl spacer between daunomycin and macromolecular carriers: A model of pH-sensitive linkage releasing drug from a lysosomotropic conjugate. *Biochem. Biophys. Res. Commun.* 102, 1048–1054. (b) Yang, H. M., and Reisfeld, R. A. (1988) Doxorubicin conjugates with a monoclonal antibody directed to a human melanoma-associated proteoglycan suppresses the growth of established tumor xenografts in nude mice. *Proc. Natl. Acad. Sci. U.S.A.* 85, 1189–1193.
- (9) (a) Kaneko, T., Willner, D., Monkovic, I., Knipe, J. O., Braslawsky, G. R., Greenfield, R. S., and Vyas, D. M. (1991) New hydrazone derivatives of Adriamycin and their Immunoconjugates- a correlation between acid stability and cytotoxicity. *Bioconjugate Chem.* 2 (3), 133–141. (b) Greenfield, R. S., Kaneko, T., Duanes, A., Edson, M. A., Fitzgerald, K. A., Olech, L. J., Grattan, J. A., and Braslawsky, G. R. (1990) Evaluation in vitro of adriamycin immunoconjugates synthesized using an acid sensitive hydrazone linker. *Cancer Res.* 50, 6600–6607. (c) Braslawsky, G. R., Edson, M. A., Pearce, W., Kaneko, T., and Greenfield, R. S. (1990) Antitumor activity of adriamycin (hydrazone linked) immunoconjugates compared to free adriamycin and specificity of tumor cell killing. *Cancer Res.* 50, 6608–6614. (d) Mueller, B. M., Wrasidlo, W. A., and Reisfeld, R. A. (1990) Antibody conjugates with Morpholino-doxorubicin and acid-cleavable linkers. *Bioconjugate Chem.* 1, 325–330.
- (10) Lagazza, B. C., Nicols, C. L., Briggs, S. L., Cullinan, G. J., Johnson, D. A., Starling, J. J., Baker, L. A., Bumol, T. F., and Corvalan, J. R. F. (1989) New antitumor monoclonal antibody-Vinca conjugates LY203725 and related compounds: Design, preparation, and representative in vivo activity. *J. Med. Chem.* 32, 548–555.
- (11) Srinivasachar, K., and Neville, D. M., Jr. (1989) New protein cross-linking reagents that are cleaved by mild acid. *Biochemistry* 28, 2501–2509.
- (12) Greene, T. W., Ed. (1981) *Protective Groups in Organic Synthesis*, pp 34–37 (hydroxy group protection), pp 273–274 (amino group protection), J. Wiley and Sons, New York.
- (13) Sekine, M., Iwase, R., Masuda, N., and Hata, T. (1988) Synthesis of oligoribonucleotides by use of 4,4',4''-Tris(acyloxy)trityl groups for protection of 6-amino group of adenosine. *Bull. Chem. Soc. Jpn.* 61, 1669–1677.
- (14) Zikos, C. C., and Ferderigos, N. G. (1994) (R,S) 2-Fluoro (chloro) 4'-carboxy-triphenyl methanol. Novel acid labile type handles for solid phase peptide synthesis. *Tetrahedron Lett.* 35 (11), 1767–1768.
- (15) (a) Muraro, R., Wunderlich, D., Thor, A., Lundy, J., Noguchi, P., Cunningham, R., and Schlom, J. (1985) Definition by monoclonal antibodies of a repertoire of carcinoembryonic antigen differentially expressed in human colon carcinomas versus normal adult tissues. *Cancer Res.* 45, 5769–5780. (b) Kuroki, M., Greiner, J. W., Simpson, J. F., Primus, F. J., Guadagni, F., and Schlom, J. (1989) Serologic mapping and biochemical characterization of the carcinoembryonic antigen epitopes using fourteen distinct monoclonal

- antibodies. *Int. J. Cancer* 44, 208–218. (c) Ikeda, S., Kuroki, M., Masahide, K., Haruno, M., Oikawa, S., Nakazato, H., Kosaki, G., and Matsuoka, Y. (1992) Epitope mapping of the carcinoembryonic antigen with various related recombinant proteins expressed in Chinese Hamster Ovary cells and 25 distinct monoclonal antibodies. *Mol. Immunol.* 29 (2), 229–240.
- (16) Starling, J., and Law, K., Lilly Research Laboratories, Indianapolis, IN, unpublished work.
- (17) Patel, V. F., Hertel, L. W., Grindey, G. B., Grossman, C. S., et al. (1995) Medicinal Chemistry of Difluoropurines. *Seminars Oncol.* 22 (4), 54–60.
- (18) Still, W. C., Kahn, M., and Mitra, A. J. (1978) Rapid chromatographic technique for preparative separations with moderate resolution. *J. Org. Chem.* 43, 2923–2925.
- (19) LY207702 was prepared according to the procedures of Hertel, L. W., Chou, T. S., and Jones, C. D., Lilly Research Laboratories, Indianapolis, IN, unpublished work.
- (20) Gapinski, M. D., Mallett, B. E., Froelich, L. L., and Jackson, W. T. (1990) Benzophenone Dicarboxylic acid antagonists of Leukotriene B₄. Structure-activity relationships of the lipophilic side chain. *J. Med. Chem.* 33, 2807–2813.
- (21) Harlow, E., and Lane, D. (1988) *Antibodies: A laboratory manual*, pp 353–355, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
- (22) Starling, J. J., Hinson, H. A., Marder, P., Maciak, R. S., and Laguzza, B. C. (1988) Rapid internalization of antigen-immunoconjugate complexes is not required for antitumor activity of monoclonal antibody-drug conjugates. *Antibody Immunoconjug. Radiopharm.* 1, 311–324.
- (23) Houghten, J. A., and Taylor, D. M. (1978) Maintenance of biological and biochemical characteristics of human colorectal tumors during serial passage in immune-deprived mice. *Br. J. Cancer*, 37, 199–212.
- (24) Patel, V. F., Hardin, J. N., Starling, J. J., and Mastro, J. M. (1995) Novel trityl linked drug immunoconjugates for cancer therapy. *Bioorg. Med. Chem. Lett.* 5 (5), 507–512.
- (25) Rocek, J., and Riehl, A. (1966) Concerning structural limitations in the relationship between trityl cation stability and reactivity. *J. Am. Chem. Soc.* 88 (20), 4747–4750.
- (26) Glidea, B. D., Coull, J. M., and Koster, H. (1990) A versatile acid-labile linker for modification of synthetic biomolecules. *Tetrahedron Lett.* 31 (49), 7095–7098.
- (27) Morin, C., and Vidal, M. (1992) Synthesis of a Spermidine-derived conjugating agent. *Tetrahedron* 48 (42), 9277–9282.
- (28) (a) Webb, R. R., II, and Martin, J. C. (1987) A convenient synthesis of S-HPMA. *Tetrahedron Lett.* 28 (42), 4963–4964. (b) Kim, C. U., Luh, B. Y., and Martin, J. C. (1992) A novel synthesis of 1-Oxa-HPMPA: A potent antiviral agent against Herpesviruses. *Tetrahedron Lett.* 33 (1), 25–28. (c) Murai, Y., Shiroto, H., Ishizaki, T., Limori, T., Kodama, Y., Ohtsuka, Y., and Oishi, T. (1992) A synthesis and X-ray analysis of 2'-C, 3'-C- and 5'-C-Methyl-sangivmycins. *Heterocycles* 33 (1), 391.
- (29) Pankiewicz, K. W., Krzeminski, J., Ciszewski, L. A., Ren, W.-Y., and Watanabe, K. A. (1992) A synthesis of 9-(2-deoxy-2-fluoro- β -D-arabinofuranosyl)adenine and Hypoxanthine. An effect of C3'-Endo to C2'-Endo Conformational shift on the reaction course of 2'-hydroxyl group with DSAT. *J. Org. Chem.* 57, 553–559.
- (30) Bazin, H., and Chattopadhyaya, J. (1985) A convenient preparation of 3'-Deoxyadenosine (Cordycepin) and 9-[3'(R)-Deutro- β -D-2'(R)-pentofuranosyl]-adenine. *Synthesis* 1108–1111.
- (31) Prisbe, E. J., Martin, J. C., McGee, P. C., Barker, M. F., Smee, D. F., Duke, A. E., Matthews, T. R., and Verheyden, J. P. H. (1986) Synthesis and antiherpes virus activity of Phosphate and Phosphonate derivatives of 9-[(1,3-Dihydroxy-2-propoxy)methyl]guanine. *J. Med. Chem.* 29, 671–675.
- (32) Reddy, M. P., Rampal, J. B., and Beaucage, S. L. (1987) An efficient procedure for the solid phase tritylation of nucleosides and nucleotides. *Tetrahedron Lett.* 28 (1), 23–26.
- (33) Patel, V. F., Hardin, J. N., Grindey, G. B., and Schultz, R. M. Tritylated oncolytics as prodrugs. *Bioorg. Med. Chem. Lett.* 5 (5), 513–518.
- (34) Bleasdale, C., Golding, B. T., Lee, W. H., Maskill, H., Riseborough, J., and Smits, E. (1994) The mechanism of deamination of Methoxy substituted tritylammonium ions in methanolic aqueous acid. *J. Chem. Soc., Chem. Commun.*, 93–94.
- (35) McClelland, R. A., Banait, N., and Steenken, S. (1986) Electrophilic reactivity of the triphenylmethyl carbocation in aqueous media. *J. Am. Chem. Soc.* 108, 7023–7027.
- (36) Muraro, R., Nuti, M., Natali, P. G., Biogotti, A., Simpson, J. F., Primus, F. J., Colcher, D., Greiner, J. W., and Schlom, J. A. (1989) A monoclonal antibody (D612) with selective reactivity for malignant and normal gastro-intestinal epithelium. *Int. J. Cancer* 43, 598–607.
- (37) Hertel, L. W., Grossman, C. S., Kroin, J. S., Mineishi, S., Chubb, S., Nowak, B., and Plunkett, W. (1989) Synthesis, cytotoxicity and metabolism of the 2',2'-Difluoroanalogs of Deoxyadenosine (dFdA) and Deoxyguanosine (dFdG). *Nucleosides Nucleotides* 8 (5, 6), 951–955.
- (38) Starling, J. J., Maciak, R. S., Hinson, N. A., Nichols, C. L., Briggs, S. L., Laguzza, B. C., Smith, W. and Corvalan, J. R. F. (1992) In vivo antitumor activity of a panel of four monoclonal antibody-Vinca alkaloid immunoconjugates which bind to three distinct epitopes of Carcinoembryonic antigen. *Bioconjugate Chem.* 3, 315–322.

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SUPPLEMENTARY MATERIAL

2-[3-(Bis-(4-methoxyphenyl)-hydroxymethyl)phenyl]-4,4-dimethyl-1,3-oxazoline 6a. 4,4'-Dimethoxybenzophenone **5a** (10.65g) was reacted with the Grignard reagent derived from 2-(3-bromophenyl)-4,4-dimethyl-1,3-oxazoline **4** (10.20g), according to General procedure A, to give carbinol **6a** as a white powder (11.0g,66%): m.p. 198-200°C; IR (KBr) $\nu_{\max}$ 3316,2960,1511,1251,1177,1074,1037,825 cm^{-1} ; ^1H (CDCl_3) δ 8.08 (brs, 1ArH), 8.04-7.92 (m, 1ArH), 7.41-7.29 (m, 2ArH), 7.18 (d, $J=10\text{Hz}$, 4ArH), 6.83 (d, $J=10\text{Hz}$, 4ArH), 4.12 (s, CH_2), 3.78 (s, 6H, 2OMe), 2.80 (s, OH), 1.40 (s, 6H, 2Me) ppm; Anal. calcd. for $\text{C}_{26}\text{H}_{27}\text{NO}_4$ requires M_{417} ; C, 74.80; H, 6.52; N, 3.36%; Found m/z 417 (100); C, 74.59; H, 6.76; N, 3.45 %.

2-[4-(Bis(4-methoxyphenyl)-hydroxymethyl)phenyl]-4,4-dimethyl-1,3-oxazoline 6b. 4,4'-Dimethoxybenzophenone **5b** (2.66g) was reacted with the Grignard derived from 2-(4-bromo-phenyl)-4,4-dimethyl-1,3-oxazoline **4** (2.55g), according to General Procedure A, to give carbinol **6b** as a yellow solid (2.58g,62%): m.p. 138-139°C (*Lit.* 140-142°C); IR (KBr) $\nu_{\max}$ 2968,1510,1251,1177 cm^{-1} ; ^1H (CDCl_3) δ 7.88 (d, $J=8\text{Hz}$, 2ArH), 7.36 (d, $J=8\text{Hz}$, 2ArH), 7.16 (d, $J=9\text{Hz}$, 4ArH), 6.84 (d, $J=9\text{Hz}$, 4ArH), 4.11 (s, CH_2), 3.81 (s, 6H, 2MeO), 1.38 (s, 6H, 2Me) ppm; Anal. calcd. for $\text{C}_{26}\text{H}_{27}\text{NO}_4$ requires M_{417} ; C, 74.80; H, 6.52; N, 3.35%; Found m/z 417 (100); C, 74.53; H, 6.60; N, 3.19%.

Racemic 2-[4-((4-methoxyphenyl)-(4-methylphenyl)-hydroxymethyl)phenyl]-4,4-dimethyl-1,3-oxazoline 6c. 4,4'-Methoxymethylbenzophenone **5c** (4.89g) was reacted with the Grignard derived from 2-(4-bromophenyl)-4,4-dimethyl-1,3-oxazoline **4** (5.00g), according to General Procedure A, to give carbinol **6c** as white powder (5.73,72%): m.p. 64-68°C; IR (KBr) $\nu_{\max}$ 2970,1647,1510,1252,1245 cm^{-1} ; ^1H (CDCl_3) δ 7.98 (brd, $J=6\text{Hz}$, 2ArH), 7.39 (d, $J=8\text{Hz}$, 2ArH), 7.15-7.05 (m, 6ArH), 6.82 (d, $J=8\text{Hz}$, 2ArH), 4.16 (brs, OCH_2), 3.80 (s, OMe), 2.36 (s, Me), 1.43 (s, 6H, 2Me) ppm; Anal. calcd. for $\text{C}_{26}\text{H}_{27}\text{NO}_3$ requires $M_{401.5}$; C, 77.78; H, 6.78; N, 3.49%; Found m/z 401 (100); C, 77.44; H, 7.09; N, 3.06%.

Racemic 2-[4-((4-methoxyphenyl)-phenyl-hydroxymethyl)phenyl]-4,4-dimethyl-1,3-oxazoline 6d. 4-Methoxybenzophenone **5d** (9.34g) was reacted with the Grignard derived from 2-(4-bromophenyl)-4,4-dimethyl-1,3-oxazoline **4** (10.20g), according to General Procedure A, to give carbinol **6d** as a white solid (13.20g,85%): m.p. 62-65°C; IR (KBr) $\nu_{\max}$ 2969,1647,1511,1304,1252,1019 cm^{-1} ; ^1H (CDCl_3) δ 7.88 (d, $J=8\text{Hz}$, 2ArH), 7.36 (d, $J=8\text{Hz}$, 2ArH), 7.32-7.29 (m, 5ArH), 7.16 (d, $J=8\text{Hz}$, 2ArH), 6.85 (d, $J=8\text{Hz}$, 2ArH), 4.10 (s, CH_2), 3.80 (s, OMe), 2.80 (s, OH), 1.37 (s, 6H, 2Me) ppm; Anal. calcd. for $\text{C}_{25}\text{H}_{25}\text{NO}_3$ requires M_{387} ; C, 77.49; H, 6.50; N, 3.61%; Found m/z 387 (100); C, 77.73; H, 6.69; N, 3.61%.

2-[4-(Bis-phenyl-hydroxymethyl)phenyl]-4,4-dimethyl-1,3-oxazoline 6f. Benzophenone **5f** (8.02g) was reacted with the Grignard derived from 2-(4-bromophenyl)-4,4-dimethyl-1,3-oxazoline (10.20g) **4** according to General Procedure A, to give carbinol **6f** as a white powder (10.20g, 71%): m.p. 147-150°C; IR (KBr) $\nu_{\max}$ 2972, 1647, 1210 cm^{-1} ; ^1H (d_6 -acetone) δ 7.83 (d, $J=8\text{Hz}$, 2ArH), 7.38 (d, $J=8\text{Hz}$, 2ArH), 7.36-7.15 (m, 10ArH), 4.12 (s, OCH_2), 2.80 (s, OH), 1.30 (s, 6H, 2Me) ppm; Anal. calcd. for $\text{C}_{24}\text{H}_{23}\text{NO}_2$ requires M_{357} ; C, 80.64; H, 6.49; N, 3.92%; Found m/z 357 (100); C, 80.55; H, 6.58; N, 4.12%.

3-Carboxy-4',4''-dimethoxytriphenylhydroxymethane 7a. Oxazoline **6a** (11.00g) was hydrolyzed according to General Procedure B to give acid **7a** as a solid (9.60g, 100%): IR (KBr) $\nu_{\max}$ 3009, 2940, 1698, 1606, 1510, 1253 cm^{-1} ; ^1H (d_6 -acetone) δ 7.68 (d, $J=8\text{Hz}$, 2ArH), 7.10 (d, $J=8\text{Hz}$, 4ArH), 6.82 (d, $J=8\text{Hz}$, 4ArH), 6.75 (d, $J=8\text{Hz}$, 2ArH), 3.77 (s, 6H, 2OMe) ppm; $\text{C}_{22}\text{H}_{20}\text{O}_5$ requires $M_{364.4}$; Found m/z 364 (100), 347 (35), 333 (65).

4-Carboxy-4',4''-dimethoxytriphenylhydroxymethane 7b. Oxazoline **6b** (11.20g) was hydrolyzed according to General Procedure B to give acid (**7b**) as a yellow solid (9.60g, 100%): m.p. 102-104°C (Lit. 105-107°C); IR (KBr) $\nu_{\max}$ 3001, 1691, 1510, 1251, 1176 cm^{-1} ; U.V. (EtOH) $\lambda_{\max}$ 275 ($\epsilon=55750$), 232 ($\epsilon=30959$), 204.5 ($\epsilon=65718$) nm; ^1H (d_6 -DMSO) δ 12.85 (brs, CO_2H), 7.87 (d, $J=9\text{Hz}$, 2ArH), 7.33 (d, $J=9\text{Hz}$, 2ArH), 7.08 (d, $J=8\text{Hz}$, 4ArH), 6.87 (d, $J=8\text{Hz}$, 4ArH), 6.40 (s, OH), 3.73 (s, 6H, 2OMe) ppm; Anal. calcd. for $\text{C}_{22}\text{H}_{20}\text{O}_5$ requires M_{364} ; C, 72.52; H, 5.53%; Found m/z 364 (100); C, 72.09; H, 5.67%.

Racemic 4-Carboxy-4'-methoxy-4''-methyltriphenylhydroxymethane 7c. Oxazoline **6c** (5.50g) was hydrolyzed according to General Procedure B to give acid **7c** as a solid (4.74g, 100%): IR (KBr) $\nu_{\max}$ 3214, 1767, 1713, 1510, 1252 cm^{-1} ; ^1H (d_6 -DMSO) δ 12.20 (brs, CO_2H), 11.94 (brs, OH), 7.95 (d, $J=8\text{Hz}$, 2ArH), 7.42 (d, $J=8\text{Hz}$, 2ArH), 7.15-7.04 (m, 6ArH), 6.85 (d, $J=9\text{Hz}$, 2ArH), 3.73 (s, OMe), 2.20 (s, Me) ppm; Calcd. for $\text{C}_{22}\text{H}_{20}\text{O}_4$ requires M_{348} ; Found m/z 348 (85).

Racemic 4-Carboxy-4'-methoxytriphenylhydroxymethane 7d. Oxazoline **6d** (800mg) was hydrolyzed according to General Procedure B to give acid **7d** as a solid (640mg, 93%): m.p. 57-62°C; IR (CHCl_3) $\nu_{\max}$ 3028, 3010, 1695, 1511, 1252 cm^{-1} ; ^1H (CDCl_3) δ 8.04 (d, $J=8\text{Hz}$, 2ArH), 7.46 (d, $J=8\text{Hz}$, 2ArH), 7.34-7.25 (m, 5ArH), 7.14 (d, $J=8\text{Hz}$, 2ArH), 6.84 (d, $J=8\text{Hz}$, 2ArH), 3.80 (s, OMe) ppm; Anal. calcd. for $\text{C}_{21}\text{H}_{18}\text{O}_4$ requires M_{334} ; C, 75.43; H, 5.43%; Found m/z 334 (100); C, 75.68; H, 5.69%.

4-Carboxytriphenylhydroxymethane 7f. Oxazoline **6f** (8.00g) was hydrolyzed according to General Procedure B to give acid **7f** as a white solid (6.56g, 96%): m.p. 200-202°C; IR (CHCl_3) $\nu_{\max}$ 3025, 3013, 1695, 1285 cm^{-1} ; ^1H (d_6 -acetone) δ 11.20 (brs, CO_2H), 7.99

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(d,J=9Hz,2ArH), 7.50 (d,J=9Hz,2ArH), 7.37-7.25 (m,10ArH), 2.80 (brs, OH) ppm; Anal. calcd for $C_{20}H_{16}O_3$ requires M 304; C,78.93; H,5.30%; Found m/z 304 (100);C,78.94;H,5.37%.

N-Succinimidyl-3-[bis-(4-methoxyphenyl)hydroxymethyl]benzoate 8a. Carboxylic acid **7a** (6.00g) was coupled to NHS (1.19g) using DCC (4.09g), according to General Procedure C, to give active ester **8a** as a yellow solid (4.23g,55.6%): m.p. 154-156°C; IR ($CHCl_3$) ν_{max} 3600,3010,1743,1510,1253,1070 cm^{-1} ; ^1H (d_6 -acetone) δ 8.20 (brs,1ArH), 8.05 (d,J=6Hz,1ArH), 7.68 (d,J=6Hz,1ArH), 7.57 (t,J=7Hz,1ArH), 7.21 (d,J=8Hz,4ArH), 6.88 (d,J=8Hz,4ArH), 3.80 (s,6H,2Me), 2.95 (s,4H,2CH₂), 2.80 (s,OH) ppm; Anal. calcd. for $C_{26}H_{23}NO_7$ requires M 461;C,67.67;H,5.02; N,3.03%;Found m/z 461 (100);C,67.86;H,5.22;N,3.30%.

N-Succinimidyl-4-[bis-(4-methoxyphenyl)hydroxymethyl]benzoate 8b. Carboxylic acid **7b** (728mg) was coupled to NHS (230mg) using DCC (454mg), according to General Procedure C, to give active ester (**8b**) as a white solid (880mg,95%): m.p. 184-186°C; IR ($CHCl_3$) ν_{max} 3600, 3050,1744, 1570 1254, 1179 cm^{-1} ; ^1H ($CDCl_3$) δ 8.06 (d,J=8Hz,2ArH), 7.50 (d,J=8Hz,2ArH), 7.15 (d,J=8Hz,4ArH), 6.84 (d,J=8Hz,4ArH),3.80 (s,6H,2OMe), 2.87 (brs,4H, 2CH₂) ppm; Anal. calcd. for $C_{26}H_{13}NO_7$ requires M 461.5;C,67.67;H,5.02; N;3.03%;Found m/z 461 (100),444 (10);C, 67.94;H,5.15;N,3.24%.

Racemic N-Succinimidyl-4-[4'-methoxyphenyl-4''methylphenyl-hydroxymethyl] benzoate 8c. Carboxylic acid **7c** (4.40g) was coupled to NHS (1.46g) using DCC (3.13g), according to General Procedure C, to give active ester **8c** as a yellow solid (2.85g,51%): m.p. 90-94°C; IR ($CHCl_3$) ν_{max} 3600,3012,1743,1510,1254,1074 cm^{-1} ; ^1H (d_6 -acetone) δ 8.07 (d,J=8Hz,2ArH), 7.60 (d,J=8Hz,2ArH), 7.24-7.12 (m,6ArH), 6.87 (d,J=8Hz,2ArH),3.80 (s,6H,2 OMe), 2.96 (s,4H,2CH₂), 2.31 (s,Me) ppm; Anal. calcd. for $C_{21}H_{23}NO_6$ requires M 445.5; C,70.10;H,5.20; N,3.14%;Found m/z 446 (100), 429 (5);C,69.84;H,5.53; N,3.34%.

Racemic N-Succinimidyl-4-[4'-methoxyphenyl-phenyl-hydroxymethyl] benzoate 8d. Carboxylic acid **7d** (6.00g) was coupled to NHS(2.07g) using DCC (4.46g), according to General Procedure C, to give active ester **8d** as a solid (5.98g,77%): m.p. 85-90°C; IR ($CHCl_3$) ν_{max} 3600,3010,1744,1254 cm^{-1} ; ^1H ($CDCl_3$) δ 8.08 (d,J=8Hz, 2ArH), 7.52 (d,J=9Hz, 2 ArH), 7.37-7.23 (m, 5ArH), 7.14 (d,J=10Hz,2ArH), 6.86 (d,J=10Hz,2ArH), 3.82 (s,OMe), 2.90 (s,4H,2CH₂) ppm; Anal. calcd. for $C_{25}H_{21}NO_6$ requires M 431; C,69.60;H,4.91; N3.25%; Found m/z 431 (100);C,69.36;H,5.04;N,3.29%.

N-Succinimidyl-4-[bis-phenyl-hydroxymethyl] benzoate 8f. Carboxylic acid **7f** (5.50g) was coupled to NHS (2.08g) using DCC (4.48g), according to General Procedure C, to give active ester **8f** as a solid (6.88g,95%): m.p. 151-153°C; IR ($CHCl_3$) ν_{max} 3030,

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1774,1742, 1448, 1374,1256,1202,1046,996 cm^{-1} ; ^1H (d_6 -acetone) δ 8.08 (d,J=8Hz, 2ArH), 7.50 (d,J=8Hz,2ArH),7.39-7.30 (m,6ArH), 7.30-7.22 (m,4ArH),2.90 (brs,4H,2CH₂) ppm; Anal. calcd. for C₂₄H₁₉NO₅ requires M401;C,71.10;H,4.77;N,3.49%;Found m/z 401 (100); C, 72.10;H,5.00;N;3.69%.

N-Succinimidyl-3-[bis-(4-methoxyphenyl)chloromethyl]benzoate 9a. Hydroxyester **8a** (2.00g) was heated in AcCl according General Procedure D to provide trityl chloride **9a** as a reddish-orange solid (1.52g,73%): m.p. 165-167°C; IR (CHCl₃) ν_{max} 1774,1744,1510, 1255,1179,1070 cm^{-1} ; ^1H (CDCl₃) δ 8.10 (d,J=10Hz,1ArH), 8.05 (s,ArH), 7.62 (d,J=10Hz, ArH),7.50-7.42 (m,ArH), 7.12 (d,J=10Hz,4ArH), 6.82 (d,J=10Hz,4ArH), 3.80 (s,6H,2OMe), 2.90 (brs,4H,2CH₂) ppm; Anal. calcd. for C₂₆H₂₂NO₆Cl requires M480;C,65.07;H,4.62; N,2.92; Cl,7.09%;Found m/z (FAB,Lil/Sulpholane)444.2(M-Cl,50);C,64.86;H,2.90;N,2.90; Cl, 7.09%

N-Succinimidyl-4-[bis-(4-methoxyphenyl)chloromethyl]benzoate 9b. Hydroxyester **8b** (420mg) was heated in acetyl chloride according General Procedure D to provide trityl chloride **9b** as a white crystalline solid (320mg,73%): m.p. 202-203°C; IR (CHCl₃) ν_{max} 174,1744,1510,1255,1071 cm^{-1} ; ^1H (CDCl₃) δ 8.80 (d,J=9Hz,2ArH), 7.46 (d,J=9Hz,2ArH), 7.14 (d,J=9Hz,4ArH), 6.85 (d,J=9Hz,4ArH), 3.84 (s,6H,2OMe), 2.93 (brs,2CH₂) ppm; Anal. calcd. for C₂₆H₂₂NO₆Cl requires M480;C,65.07;H,4.62;N,2.66;Cl,7.39%; Found m/z (FAB, Lil/ Sulpholane) 444.2 (M-Cl, 40); C,65.23; H,4.63; N,2.66; Cl,7.58%.

Racemic N-Succinimidyl-4-[4'-methoxyphenyl-4''methylphenyl-chloromethyl] benzoate 9c. Hydroxyester **8c** (1.00g) was heated in ACCL according General Procedure D to provide trityl chloride **9c** as a white solid (843mg,81%):m.p. 194-196°C; IR (CHCl₃) ν_{max} 1774,1744, 1609,1510,1256,1182,1070,996 cm^{-1} ; ^1H (d_6 -DMSO) δ 8.04 (d,J=8Hz,2ArH), 7.47 (d,J=8Hz,2ArH), 7.15-7.06 (m,6ArH), 6.88 (d,J=8Hz,2ArH), 3.71 (s,OMe), 2.90 (s,4H,2CH₂), 2.28 (s,Me) ppm; Anal. calcd. for C₂₆H₂₂NO₅Cl requires M464; C,67.31;H,4.78;N,3.02;Cl, 7.64%; Found m/z (FAB,Lil/Sulpholane) 428.2 (M-Cl,40); C,67.57;H,4.75;N,3.05;Cl,7.75%.

Racemic N-Succinimidyl-4-[4'-methoxyphenyl-phenyl-chloromethyl] benzoate 9d. Hydroxyester **8d** (2.00g) was heated in AcCl according General Procedure D to provide trityl chloride **9d** as a white solid (1.15g,55%): m.p. 161-163°C;IR (CHCl₃) ν_{max} 1774, 1744,1511, 1253,1071,996 cm^{-1} ; ^1H (CDCl₃) δ 8.04 (d,J=8Hz,2ArH), 7.52 (d,J=8Hz,2ArH), 7.37-7.18 (m,5ArH), 7.10 (d,J=9Hz,2ArH), 6.90 (d,J=9Hz,2ArH), 3.74 (s,OMe), 2.90 (s,4H,2CH₂) ppm; Anal. calcd. for C₂₅H₂₀NO₅Cl requires M450;C,66.74;H,4.48; N,3.11;Cl,7.88%; Found m/z 450 (25), 414 (55);C,67.01;H,4.50; N3.12;Cl,7.72%.

N-Succinimidyl-4-[bis-phenyl-chloromethyl] benzoate 9f. Hydroxyester **8f** (2.00g) was heated in AcCl according General Procedure D to provide trityl chloride **9f** as a white solid

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(1.20g,57%):m.p. 173-175 °C; IR (CHCl₃) $\nu_{\max}$ 1175,1744,1071,997 cm⁻¹; ¹H (CDCl₃) δ 8.08 (d,J=8Hz,2ArH), 7.45 (d,J=8Hz,2ArH), 7.29-7.18 (m,4ArH), 2.92 (s,4H,2CH₂) ppm; Anal. calcd. for C₂₄H₁₈NO₄Cl requires M419;C,68.66;H,4.32;N,3.34;Cl,8.44%; Found m/z 419 (12), 384 (M-Cl,85);C,68.75; H;4.36;N,3.26, Cl,8.55%.

Comparison of Four Bifunctional Chelates for Radiolabeling Monoclonal Antibodies with Copper Radioisotopes: Biodistribution and Metabolism

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The bifunctional chelating agents (BFCs), 6-[*p*-(bromoacetamido)benzyl]-1,4,8,11-tetraazacyclotetradecane-1,4,8,11-tetraacetic acid (BAT), 6-[*p*-(isothiocyanato)benzyl]-1,4,8,11-tetraazacyclotetradecane-1,4,8,11-tetraacetic acid (SCN-TETA), 4-[(1,4,8,11-tetraazacyclotetradec-1-yl)methyl]benzoic acid (CPTA), and 1-[(1,4,7,10,13-pentaazacyclopentadec-1-yl)methyl]benzoic acid (PCBA), were synthesized and conjugated to the anti-colorectal monoclonal antibody (mAb), 1A3, and antibody fragments, 1A3-F(ab')₂, for radiolabeling with ^{64,67}Cu and comparison in animal models. *In vivo* metabolism studies were carried out in liver and kidneys in order to correlate the nature of the metabolites formed to the uptake and retention of the radiolabel in each organ. Animal biodistribution studies were performed in Golden Syrian hamsters bearing the GW39 human colon cancer tumors and in normal Sprague–Dawley rats. All conjugates showed good tumor uptake in hamsters. Biodistribution in rats showed that ⁶⁴Cu-BAT-2IT-1A3 had the lowest liver and kidney uptake of the intact 1A3 conjugates (*p* < 0.03), whereas in hamsters, there were no significant differences in liver and kidney uptake between the four intact BFC-1A3 conjugates. Tumor-bearing hamsters injected with ⁶⁴Cu-CPTA-1A3-F(ab')₂ and ⁶⁴Cu-PCBA-1A3-F(ab')₂ had from 3 to 7 times greater uptake in the kidneys than hamsters given ⁶⁴Cu-labeled BAT and SCN-TETA 1A3-F(ab')₂ conjugates, while rats injected with ⁶⁴Cu-CPTA-1A3-F(ab')₂ and ⁶⁴Cu-PCBA-1A3-F(ab')₂ had nearly twice the uptake. The *in vivo* metabolism of the mAbs 1A3 and 1A3-F(ab')₂ radiolabeled with ⁶⁷Cu through the SCN-TETA, CPTA, and PCBA BFCs was investigated by excising the livers and kidneys of normal rats from 1–5 days post-injection of the radiolabeled conjugates. Liver and kidney homogenates were analyzed by size exclusion chromatography and thin layer chromatography (TLC). The size exclusion chromatography data showed that all of the ⁶⁷Cu-labeled 1A3-F(ab')₂ conjugates were >85% degraded in the kidneys to small molecular weight metabolites by 1 day post-injection. In contrast, in the liver at 1 day post-injection, greater than 70% of the ⁶⁷Cu-labeled 1A3 conjugates were unmetabolized. By day 5, a 35 kDa peak appeared in the liver of rats injected with the ⁶⁷Cu-labeled 1A3 conjugates, possibly due to transchelation of the ⁶⁷Cu to proteins. Superoxide dismutase chromatographically elutes at the same retention time as this ⁶⁷Cu-labeled metabolite. The TLC data indicate that the low molecular weight metabolite (<5 kDa) of both ⁶⁷Cu-CPTA-1A3 and ⁶⁷Cu-CPTA-1A3-F(ab')₂ conjugates co-chromatographed with a ⁶⁷Cu-CPTA- ϵ -lysine standard. Our data suggest that chelate charge and lipophilicity play a large role in kidney retention of ^{64,67}Cu-labeled BFC-1A3-F(ab')₂ conjugates, while transchelation of the copper label appears to be the major factor for liver accumulation of ^{64,67}Cu-labeled BFC-1A3 conjugates.

INTRODUCTION

The specificity of mAbs¹ for their antigens has led to their use in targeting cancer cells. Radiolabeled mAbs have been used for the early detection and therapy of cancer (1, 2). Monoclonal antibodies labeled with copper isotopes have applications in both diagnosis and therapy. ⁶⁷Cu emits abundant β^- (100%) with the subsequent emission of γ photons for both therapeutic and imaging applications (3–5), while ⁶⁴Cu decays by β^+ and β^- which

also allows its use in both PET imaging (6–8) and radiotherapy (9–11).

It has been demonstrated that the BFC used to complex radiometals to mAbs has a significant effect on the biodistribution in animal models (12–15). The purpose of this study was to gain a better understanding of how the nature of the BFC affects biodistribution of Cu-labeled mAbs. Four BFCs (BAT, SCN-TETA, CPTA, and PCBA (Figure 1)) that differ in charge, lipophilicity, and covalent linkage to the mAb were synthesized and

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¹ Abbreviations: mAb, monoclonal antibody; PET, positron emission tomography; BFC, bifunctional chelating agent; BAT, 6-[*p*-(bromoacetamido)benzyl]-1,4,8,11-tetraazacyclotetradecane-1,4,8,11-tetraacetic acid; SCN-TETA, 6-[*p*-(isothiocyanato)benzyl]-1,4,8,11-tetraazacyclotetradecane-1,4,8,11-tetraacetic acid; CPTA, 4-[(1,4,8,11-tetraazacyclotetradec-1-yl)methyl]benzoic acid; PCBA 1-[(1,4,7,10,13-pentaazacyclopentadecyl-1-yl)methyl]benzoic acid; 2IT, 2-iminothiolane; FPLC, fast protein liquid chromatography; EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide; sNHS, *N*-hydroxysulfosuccinimide; IR, immunoreactivity; SOD, superoxide dismutase; Fmoc, fluorenylmethoxycarbonyl; boc, butoxycarbonyl; HOBt, hydroxybenzotriazole.

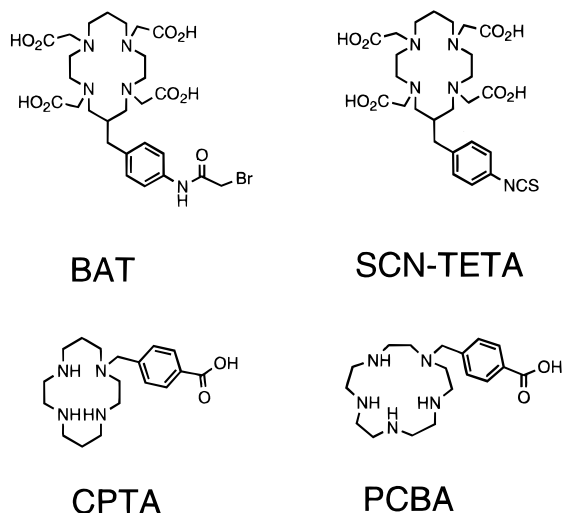


Figure 1. Structures of the bifunctional chelating agents used for labeling $^{64,67}\text{Cu}$ to mAb 1A3 and 1A3-F(ab')₂.

conjugated to intact mAb and mAb-F(ab')₂ and labeled with ^{64}Cu or ^{67}Cu . The biodistribution in two animal models and *in vivo* metabolism by normal rats of $^{64,67}\text{Cu}$ -labeled BFC-mAb conjugates are compared.

The four BFCs were conjugated to the anti-colorectal mAb 1A3 and 1A3-F(ab')₂ fragments. This antibody has been previously conjugated with BAT, labeled with ^{64}Cu , and evaluated in tumor-bearing hamsters and normal rats (16, 17). Tumor uptake for ^{64}Cu -BAT-2IT-1A3 and ^{64}Cu -BAT-2IT-1A3-F(ab')₂ was shown to be superior to both the ^{111}In - and ^{125}I -labeled 1A3 and 1A3-F(ab')₂ (16), and both ^{64}Cu agents have been investigated clinically as PET imaging agents for colorectal cancer (6–8). One drawback of ^{64}Cu -BAT-2IT-1A3-F(ab')₂ was the high accumulation in the kidneys of both animal models (17) and humans (7). Since the nature of the BFC appears to play an important role in the biodistribution of metal-labeled BFC-mAb conjugates, in the present study, we compare the *in vivo* behavior of ^{64}Cu -labeled mAbs conjugated with the BFCs SCN-TETA, CPTA, PCBA, and BAT.

The BFCs are primarily conjugated to the mAbs through the ϵ -amine of lysines (18), although conjugation to other amino acids bearing a nucleophilic side chain or the N-terminus cannot be excluded. The bifunctional chelate SCN-TETA was chosen to compare to BAT because the only difference in structure between the two BFCs is the covalent linkage to the mAbs. SCN-TETA forms a thiourea bond when linked to primary amines, while BAT is conjugated to mAbs using the linking agent 2IT which forms a thioether bond between BAT and the mAb (19). The Cu(II) complexes of BAT and SCN-TETA bear a net formal charge of 2–.

The CPTA and PCBA BFCs both form Cu(II) complexes with a net charge of 2+, and they are conjugated to mAbs via an amide linkage. The CPTA BFC has been used by Smith-Jones and co-workers in labeling ^{67}Cu to AB35, a mAb directed against carcinoembryonic antigen (CEA) (20). This radioimmunoconjugate was evaluated in tumor-bearing mice and showed high tumor uptake (20). CPTA is readily synthesized in a one-step reaction and forms a stable complex with copper *in vivo* (20). PCBA is a BFC which is similar in structure to CPTA and can also be synthesized in a single step. The macrocyclic portion of PCBA is 15aneN5 which forms a stable complex with Cu(II) ($\log K = 26.73$) (21). The stability constant of Cu(II)–15aneN5 is similar to that of Cu(II)–cyclam ($\log K = 28.09$) (21) and greater than the stability

constant of Cu(II)–TETA (the macrocyclic chelate portion of the BFCs BAT and SCN-TETA) ($\log K = 21.87$) (22). The similarity in complex stability of Cu(II)–15aneN5 to Cu(II)–cyclam and the fact that the 15aneN5 macrocycle is less lipophilic than cyclam made PCBA an interesting chelate for comparative purposes.

Metabolism studies on ^{111}In -labeled antibodies and polypeptides have been conducted to gain a better understanding of the mechanisms involved in the uptake and retention of activity in nontarget organs (i.e., liver and kidneys) (23–30). We previously identified ^{111}In -DTPA- ϵ -lysine as the major small molecular weight metabolic product of ^{111}In -labeled 1A3 and 1A3-F(ab')₂ conjugates in the livers and kidneys of normal rats (30). In the present study, we compared the liver and kidney metabolism of ^{67}Cu -labeled SCN-TETA, CPTA, and PCBA conjugates of 1A3 and 1A3-F(ab')₂ in normal rats to correlate the nature of the metabolites with the accumulation of radioactivity in nontarget organs (30).

MATERIALS AND METHODS

1-Ethyl-3-[3-(dimethylamino)propyl]carbodiimide-HCl (EDC) and *N*-hydroxysulfosuccinimide (sNHS) were purchased from Pierce Chemical Co. (Rockford, IL). Centricon 30 concentrators were purchased from Amicon Inc. (Beverly, MA). Bio-Spin 6 chromatography columns were from Bio-Rad Laboratories (Hercules, CA). Sephadex G-25/50, diisopropylcarbodiimide (DIC), and superoxide dismutase (SOD) were purchased from Sigma Chemical Co. (St. Louis, MO). ϵ -Fmoc- α -Boc-lysine and *p*-(benzyloxy)benzyl alcohol resin were purchased from NovaBiochem (LaJolla, CA). Ultrapure ammonium dihydrogen phosphate ($\text{NH}_4\text{H}_2\text{PO}_4$) was purchased from Johnson Matthey (Royston, England). Ultrapure (>99%) ammonium acetate (NH_4OAc) and ammonium citrate (NH_4Cit) were purchased from Fluka (Ronkonkoma, NY). Disodium hydrogen phosphate (Na_2HPO_4) and 4-(dimethylamino)pyridine (DMAP) were purchased from Fisher Chemical Co. (Fair Lawn, NJ). All other chemicals used were purchased from Aldrich Chemical Co. (Milwaukee, WI). All solutions were made using distilled deionized water (Milli-Q; $\sim 18\text{ M}\Omega$ resistivity). Bio-Spin and Sephadex G-25/50 columns were equilibrated with 0.1 M NH_4OAc (pH 5.5) prior to use. The mAbs 1A3 and 1A3-F(ab')₂ were purified from serum-free medium by Invitron (St. Louis, MO) using proprietary methods. Adult, female Sprague-Dawley rats were purchased from Sasco (Omaha, NE), and Golden Syrian hamsters were purchased from Charles River (Wilmington, MA). A Tekmar tissue homogenizer (Cincinnati, OH), a Branson Sonifier 185 cell disrupter, and a Sorvall RC2-B centrifuge were used in the metabolism experiments. A Pharmacia Superose 12 gel filtration column was used in conjunction with a Pharmacia/LKB FPLC system (Piscataway, NJ) for metabolite analyses. A Beckman Gamma 8000 automated well type γ counter was used to count the FPLC fractions. Radio-TLC was carried out using a Bioscan System 200 Imaging Scanner. ^{67}Cu (115.6–215.9 Ci/mmol) was purchased from Brookhaven National Laboratories. ^{64}Cu (4572–15240 Ci/mmol) was produced and purified at the University of Missouri–Columbia Research Reactor as previously described (16, 31).

Ligand Syntheses. A method similar to Moi et al. and Moran et al. was used in the synthesis of nitrobenzyl-TETA, the direct precursor to BAT and SCN-TETA (32, 33). Briefly, the diethyl malonate anion was generated with NaH in THF and alkylated with *p*-nitrobenzyl bromide to give 2-(*p*-nitrobenzyl)diethyl malonate. The cyclic amide was formed under high dilution conditions

in EtOH from the diester and *N,N'*-bis(2-aminoethyl)-1,3-diaminopropane. After refluxing for 8 weeks, the cyclic amide was isolated by slow evaporation of the solvent and subsequently reduced with $\text{BH}_3\cdot\text{THF}$. The cyclic amine was alkylated with 4 equiv of *tert*-butyl bromoacetate in acetonitrile (ACN) at room temperature. The reaction mixture was concentrated, and the *tert*-butyl groups were removed with trifluoroacetic acid. Nitrobenzyl-TETA was purified by anion exchange chromatography. Nitrobenzyl-TETA was then converted to BAT through a method described by McCall et al. (19) and converted to SCN-TETA through a method described by McMurry et al. (34).

CPTA was synthesized according to the method of Studer and Kaden (35), while PCBA was synthesized using a similar method described by Motekaitis et al. (21).

CPTA- ϵ -lysine was synthesized using a solid-phase procedure modeled after the previously described solid-phase synthesis of DTPA- ϵ -lysine (29). DIC (164 μL , 1.05 mmol), HOBT (142 mg, 1.05 mmol), DMAP (12.8 mg, 0.105 mmol), and ϵ -Fmoc- α -Boc-lysine (492 mg, 1.05 mmol) were added to 5.2 mL of DMF and stirred for 5 min at room temperature. To this was added 750 mg of the *p*-(benzyloxy)benzyl alcohol resin (hydroxyl substitution level of 0.7 mmol/g), and the mixture was stirred at room temperature overnight (~16 h). Then, the mixture was filtered and rinsed three times with 5 mL of DMF and three times with 10 mL of MeOH. The level of lysine substitution on the resin was determined using a UV absorbance test. DMF (100 μL) and piperidine (100 μL) were added to 1–2 mg of the substituted resin, and the mixture was incubated at room temperature for 30 min. After dilution with 4.8 mL of MeOH, the absorbance of fulvene was measured at $\lambda = 301 \text{ nm}$ ($\epsilon = 7800 \text{ M}^{-1} \text{ cm}^{-1}$). Triplicate measurements yielded an amino acid substitution level of 0.24 mmol/g. The ϵ -Fmoc group was removed by stirring in 150 mL of 1:1 DMF:piperidine for 1 h at room temperature. The mixture was filtered, and the resin was rinsed with DMF followed with MeOH. CPTA (200 mg, 0.60 mmol), HOBT (80.9 mg, 0.60 mmol), and DIC (94.3 μL , 0.60 mmol) were dissolved in 5 mL of DMF and added to the Fmoc-deprotected resin (625 mg, 0.15 mmol). After reaction overnight at room temperature, the mixture was filtered and the resin rinsed as above. The CPTA- ϵ -lysine was cleaved from the resin over a 75 min period using 2 mL of 95:5 trifluoroacetic acid (TFA): water, simultaneously removing the α -Boc group. The mixture was filtered, concentrated, and diluted to 1.0 mL with $\text{H}_2\text{O}/0.1\%$ TFA.

A portion of the CPTA- ϵ -lysine was then purified by reversed-phase HPLC (Vydac 201HS1010, $10 \times 250 \text{ mm}$, C-18) with a 10 min isocratic elution at 10% A, followed by a linear gradient of 10% to 80% A over the next 10 min at 2.0 mL/min, where A = ACN/0.1% TFA and B = $\text{H}_2\text{O}/0.1\%$ TFA. Fractions were collected and analyzed by RP-HPLC at $\lambda = 272 \text{ nm}$ using 2% A as the starting eluant and followed by a linear gradient of 10% to 80% A over 10 min. The desired product eluted at 21.3 min and was greater than 98% pure by RP-HPLC. The identity of the product was confirmed by electrospray MS $m/e \text{ (M + H)}^+ = 463.65 \text{ g/mol}$, calcd = 463.63 g/mol.

Copper Challenge Experiments. ^{67}Cu -labeled TETA, CPTA, and PCBA were prepared by incubating the ligands in 0.1 M NH_4OAc (pH 5.5) with ^{67}Cu acetate for 1 h at room temperature. A 10-fold molar excess of CuCl_2 to ligand was added and the mixture allowed to incubate at room temperature. At various time points, 2 μL of the ^{67}Cu -TETA and ^{67}Cu -CPTA solutions were applied to silica gel TLC plates and eluted using 1:1 MeOH:10% NH_4OAc in water. The silica plates were then dried and

analyzed with a radio-TLC scanner to determine the amount of copper complex that remained. At various time points, 100 μL of the ^{67}Cu -PCBA solution was passed through a 0.45 μm Chelex chelating filter. Uncomplexed ^{67}Cu bound to the filter, while ^{67}Cu -PCBA passed through the filter.

Conjugation of BAT, CPTA, PCBA, and SCN-TETA to 1A3 and 1A3-F(ab')₂. BAT was conjugated to 1A3 or 1A3-F(ab')₂ in 0.1 M $\text{NH}_4\text{H}_2\text{PO}_4$ (pH 8). An aqueous solution of BAT was added to the buffered antibody in a 20:1 molar ratio (BAT:mAb) followed by the addition of 2IT in 50 mM triethanolamine hydrochloride in a 10:1 molar ratio (2IT:mAb). The solution was incubated at 37 °C for 30 min and purified using a Sephadex G-25/50 spin column (36) equilibrated in 0.1 M NH_4Cit (pH 5.5). BAT-2IT-1A3-F(ab')₂ was purified by FPLC as previously described (17). The conjugated mAbs were stored at -80 °C until needed (16).

CPTA was conjugated using an adaptation of the method described by Smith-Jones (20). CPTA (5.0 mg, 0.010 mmol), EDC (9.2 mg, 0.048 mmol), and sNHS (5.0 mg, 0.0230 mmol) were dissolved in 359 μL of 0.1 M $\text{NH}_4\text{H}_2\text{PO}_4$ (pH 7.0) and incubated at room temperature. After 1 h, another 359 μL of buffer was added and the solution was vortexed. A volume equal to a 50-fold molar excess of the ligand over antibody was added to the antibody (0.050 mM 1A3 and 0.065 mM 1A3-F(ab')₂) at room temperature. The samples were incubated at room temperature for 2 h before they were purified by either a Bio-Spin or Sephadex G-25/50 spin column purification (36). Reaction volumes that were greater than 100 μL and masses of greater than 1 mg of mAb were purified using a Sephadex column, while smaller volumes and amounts of mAb were purified using a Bio-Spin column. PCBA was conjugated to 1A3 or 1A3-F(ab')₂ similarly to CPTA mAbs as described above.

1A3 and 1A3-F(ab')₂ were concentrated using a Centricon 30 and then diluted with Na_2HPO_4 (pH 10.5) prior to conjugation with SCN-TETA. The concentrated solutions were collected and diluted to give approximately 0.027 and 0.043 mM solutions for 1A3 and 1A3-F(ab')₂, respectively. SCN-TETA was dissolved in H_2O to give a 1 mM solution. A volume equal to a 1.2 M excess of this solution was added to the antibody solutions, vortexed, and incubated at room temperature for 1 h. The conjugate was then added to a Centricon 30, diluted to 2.0 mL with 0.1 M NH_4OAc (pH 5.5), and centrifuged. This procedure was repeated and the conjugate collected from the membrane. Purities of all compounds were confirmed by FPLC. The BFC-mAb conjugates were stored at -80 °C until needed.

Radiolabeling of BAT, SCN-TETA, CPTA, and PCBA-1A3 and 1A3-F(ab')₂ with ^{64}Cu and ^{67}Cu . $^{64}\text{CuCl}_2$ or $^{67}\text{CuCl}_2$ in 0.1 M HCl was converted to $^{64,67}\text{Cu}$ acetate by a 10-fold dilution with 0.1 M NH_4OAc (pH 5.5). The $^{64,67}\text{Cu}$ acetate solution was then added to the desired chelate-antibody conjugate and incubated at room temperature for 30 min. The resulting radiolabeled conjugates were purified using Bio-Spin columns equilibrated with 0.1 M NH_4OAc (pH 5.5). The immunoreactivities of the resulting conjugates were determined as previously described (16).

Radiolabeling of SOD with ^{64}Cu and Analysis by FPLC. SOD was labeled with ^{64}Cu using the same method as described above for labeling mAbs. The resulting ^{64}Cu -SOD was analyzed by FPLC.

Golden Syrian Hamster and Sprague-Dawley Rat Biodistribution Studies. All animal experiments were carried out according to guidelines specified by the

Washington University Animal Studies Committee and the Jewish Hospital Animal Care Committee.

Six week old male Golden Syrian hamsters (~100 g) were implanted with GW39 human colon carcinoma in the musculature of their right thigh 2 days prior to injection of the radiolabeled conjugates, as described previously (37). Hamster biodistribution experiments were carried out as previously described (16).

Adult, female Sprague–Dawley rats (~185 g) were intravenously injected with 25–50 μ g and 5–10 μ Ci of the ^{64}Cu –antibody conjugates. For each compound, groups of at least three rats were sacrificed at 6, 12, and 24 h post-injection. Samples of blood, lung, liver, spleen, kidney, muscle, bone, ovaries, small intestine, upper large intestine, and lower large intestine were removed, weighed, and activity counted. The percent of injected dose per gram of tissue (%ID/g) and percent of injected dose per organ (%ID/organ) were calculated.

Blood clearance data were obtained from rats at 1, 3, 6, 12, and 24 h time points. Blood was drawn from the rats in ~100 μ L aliquots at the various time points, weighed, and counted to calculate the %ID/g.

In Vivo Metabolism in Sprague–Dawley Rats. Mature, female Sprague–Dawley rats were intravenously injected with 80–250 μ Ci of ^{67}Cu -SCN-TETA-1A3 and ^{67}Cu -SCN-TETA-1A3-F(ab')₂, 100–400 μ Ci of ^{67}Cu -PCBA-1A3 and ^{67}Cu -PCBA-1A3-F(ab')₂, or 400–1100 μ Ci of ^{67}Cu -CPTA-1A3 and ^{67}Cu -CPTA-1A3-F(ab')₂. Livers were removed from rats injected with ^{67}Cu -BFC-1A3 and kidneys were removed from rats injected with ^{67}Cu -BFC-1A3-F(ab')₂ at 1, 3, and 5 day time points. ^{67}Cu acetate was used as a control. The organs were rinsed with water to remove as much blood as possible, homogenized in labeling buffer using a Tekmar tissue homogenizer, and sonicated (3 min, power = 4). The samples were clarified by centrifugation (23500g, 60 min), and the supernatant was passed through a 0.22 μ m spin filter. A 0.2 mL sample was analyzed on a Superose 12 gel filtration column. This column was eluted with 20 mM Hepes and 300 mM NaCl (pH 7.3) buffer at a flow rate of 0.4 mL/min. Fractions were collected every minute and counted on a γ counter. The low molecular weight fractions for the CPTA conjugate were further analyzed by radio-TLC.

RESULTS

The specific activities of the 1A3 conjugates were 2.3–2.6 $\mu\text{Ci}/\mu\text{g}$, while the specific activities of the 1A3-F(ab')₂ conjugates ranged between 1.6 and 3.2 $\mu\text{Ci}/\mu\text{g}$. The specific activities were not optimized to achieve their maximums. All radiolabeled conjugates were purified using either spin column purification or centricon 30 molecular weight membrane filters and shown to be >95% pure by gel filtration chromatography.

The immunoreactivity (IR) values for the $^{64,67}\text{Cu}$ -chelate-1A3 and $^{64,67}\text{Cu}$ -chelate-1A3-F(ab')₂ conjugates ranged between 75% and 90%, with the SCN-TETA conjugates having the lowest IRs of 75% and the PCBA-1A3-F(ab')₂ conjugate having the highest IRs of 90%. The number of chelates conjugated to the mAbs (or the chelate:mAb ratio) for all conjugates were determined using isotopic dilution methods (17). Specific activities, immunoreactivities, and chelate:mAb ratios are summarized in Table 1. The stability of the copper labeled ligands when challenged with a 10-fold excess of non-radioactive copper is summarized in Table 2. Both ^{67}Cu -CPTA and ^{67}Cu -PCBA showed no loss of copper for at least 48 h; ^{67}Cu -TETA shows a rapid initial loss of copper at 2 h and then a slower rate of copper loss over the next 5 days. ^{67}Cu -TETA was used to represent the

Table 1. Specific Activity, Immunoreactivity, and Chelate to Antibody Ratios of the Various Conjugates Labeled with $^{64,67}\text{Cu}$ ($N \geq 3$)

conjugates	specific activity (mCi/mg)	immuno-reactivity (%)	chelate/MAb
BAT-1A3	2.5 $\pm$ 1.1	84.4 $\pm$ 4.1	1.8 $\pm$ 0.2
BAT-1A3-F(ab') ₂	2.7 $\pm$ 0.3	83.3 $\pm$ 3.8	1.4 $\pm$ 0.2
SCN-1A3	2.6 $\pm$ 0.04	75.4 $\pm$ 13.2	0.24 $\pm$ 0.02
SCN-1A3-F(ab') ₂	3.2 $\pm$ 0.8	75.3 $\pm$ 14.1	0.14 $\pm$ 0.02
CPTA-1A3	2.3 $\pm$ 0.9	84.1 $\pm$ 12.7	1.4 $\pm$ 0.2
CPTA-1A3-F(ab') ₂	2.3 $\pm$ 1.1	80.6 $\pm$ 5.1	2.9 $\pm$ 0.5
PCBA-1A3	2.6 $\pm$ 0.1	84.1 $\pm$ 3.7	1.4 $\pm$ 0.2
PCBA-1A3-F(ab') ₂	1.6 $\pm$ 0.7	90.0 $\pm$ 4.2	0.7 $\pm$ 0.1

Table 2. Structural and Thermodynamic Parameters, Lipophilicity, and Charge of the Bifunctional Chelates Evaluated in This Study^a

bifunctional chelate	BAT	SCN-TETA	CPTA	PCBA
type of bond formed	thioether	thiourea	amide	amide
complex charge	2–	2–	1+	1+
Hansch lipophilicity relative to CPTA = 5.00	1.46	1.32	5.00	2.85
$\Delta \log K$ (Cu–Zn)	5.3	5.3	11.7	9.2
$\Delta \log K$ (Cu–Ca)	13.3	13.3		
$\Delta \log K$ (Cu–Mg)	19.6	19.6		
% of $^{64,67}\text{Cu}$ displaced by Cu(II) after 2 days	70	70	15	5

^a The $\Delta \log K$ values are presented for the metal–macrocycle complexes and not the metal–BFC complexes.

stability of ^{67}Cu -BAT and ^{67}Cu -SCN-TETA toward non-radioactive Cu(II) displacement because the α -keto-bromo group of BAT and the isothiocyanato group from SCN-TETA were found to be too reactive for reliable chromatography.

Animal Biodistribution Studies. The ^{64}Cu -labeled mAb conjugates were evaluated and compared in normal rats and in the tumor-bearing hamsters. Rat and hamster biodistributions of the Cu-labeled 1A3 conjugates at 24 h post-injection are shown in Figure 2. Liver and kidney uptake in rats was significantly lower for ^{64}Cu -BAT-2IT-1A3 than for the other conjugates (p values of <0.02 and <0.03, respectively). Tumor uptake in hamsters was high at 24 h for all conjugates except for ^{64}Cu -CPTA-1A3 (p values of <0.04). In the hamsters, the liver and kidney uptake was similar for all of the conjugates.

Rat and hamster biodistributions of 1A3-F(ab')₂ conjugated with the four bifunctional chelates at 24 h post-injection are shown in Figure 3. Liver uptake in rats (%ID/g) was lowest for ^{64}Cu -BAT-2IT-1A3-F(ab')₂ and ^{64}Cu -SCN-TETA-1A3-F(ab')₂, and both were significantly different from ^{64}Cu -CPTA-1A3-F(ab')₂ and ^{64}Cu -PCBA-1A3-F(ab')₂ (p values of <0.02). Kidney uptake in rats (%ID/g) was also lowest for ^{64}Cu -BAT-2IT-1A3-F(ab')₂ (13.57 $\pm$ 2.56) and ^{64}Cu -SCN-TETA-1A3-F(ab')₂ (15.35 $\pm$ 2.55), and both were lower than ^{64}Cu -CPTA-1A3-F(ab')₂ (27.58 $\pm$ 2.25) and ^{64}Cu -PCBA-1A3-F(ab')₂ (23.83 $\pm$ 1.89) (p values of <0.002). Hamsters showed a similar pattern with liver uptake being significantly lower for ^{64}Cu -BAT-2IT-1A3-F(ab')₂ (1.68 $\pm$ 0.31) and ^{64}Cu -SCN-TETA-1A3-F(ab')₂ (1.27 $\pm$ 0.10) than ^{64}Cu -CPTA-1A3-F(ab')₂ (2.71 $\pm$ 0.47) and ^{64}Cu -PCBA-1A3-F(ab')₂ (3.00 $\pm$ 0.67) (p

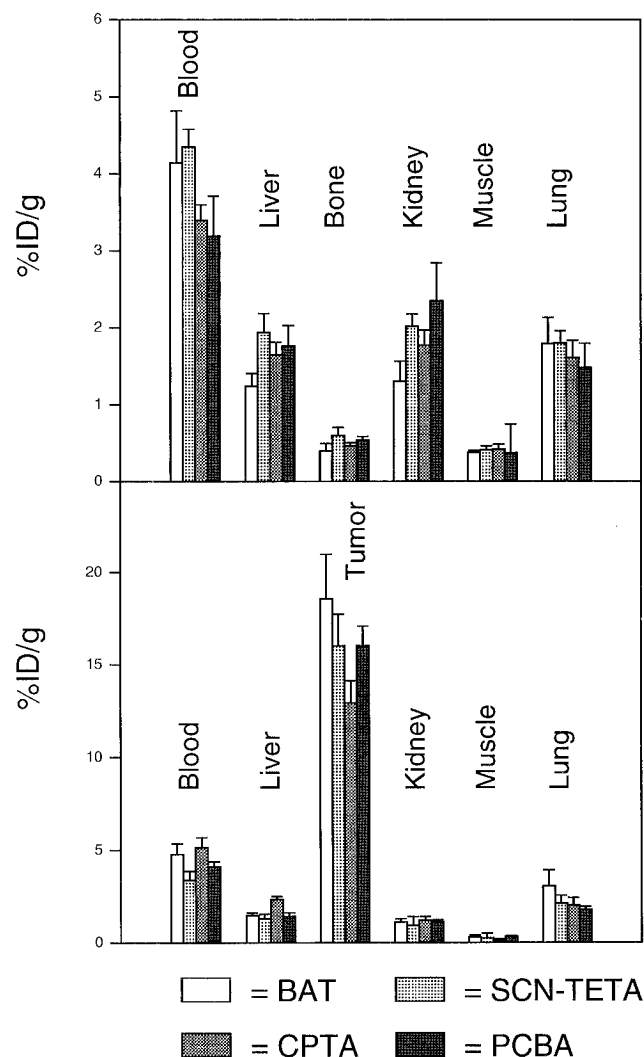


Figure 2. Biodistribution data for ^{64}Cu -BFC-1A3 conjugates in Sprague-Dawley rats (top) and tumor-bearing Golden Syrian hamsters (bottom) at 24 h post-injection. Data are presented as the mean plus or minus the standard deviation of $n \geq 4$.

values of <0.002). In hamsters, kidney uptake of ^{64}Cu -BAT-2IT-1A3-F(ab')₂ (7.99 ± 0.84) and ^{64}Cu -SCN-TETA-1A3-F(ab')₂ (3.35 ± 0.60) was also lower than ^{64}Cu -CPTA-1A3-F(ab')₂ (23.57 ± 2.41) and ^{64}Cu -PCBA-1A3-F(ab')₂ (17.56 ± 2.71) (p values of $<4 \times 10^{-6}$). Furthermore, in hamster liver and kidneys, ^{64}Cu -SCN-TETA-1A3-F(ab')₂ was significantly lower than ^{64}Cu -BAT-2IT-1A3-F(ab')₂ (p values of <0.04 and $<7 \times 10^{-6}$, respectively). Tumor uptake for all of the conjugates was high (between 8.2% and 13.7% ID/g).

Blood clearance data for the ^{64}Cu -labeled 1A3 and 1A3-F(ab')₂ conjugates are shown in Figure 4. The Cu-labeled 1A3-F(ab')₂ conjugates cleared more rapidly than the Cu-labeled 1A3 conjugates. All of the intact conjugates had similar blood clearance patterns; however, ^{64}Cu -PCBA-1A3 had the lowest concentration in the blood at all time points. There were greater differences in blood clearance among the 1A3-F(ab')₂ conjugates; ^{64}Cu -CPTA-1A3-F(ab')₂ and ^{64}Cu -PCBA-1A3-F(ab')₂ cleared more rapidly from the blood than ^{64}Cu -BAT-2IT-1A3-F(ab')₂ and ^{64}Cu -SCN-TETA-1A3-F(ab')₂.

Gel Filtration Chromatography of Liver and Kidney Metabolites. The liver metabolism data for ^{67}Cu -labeled BFC-1A3 conjugates are summarized in Table 3. Liver metabolites from the control rats (injected with ^{67}Cu acetate) showed $>90\%$ of the radioactivity eluted in a peak corresponding to a MW of ~ 35 kDa at

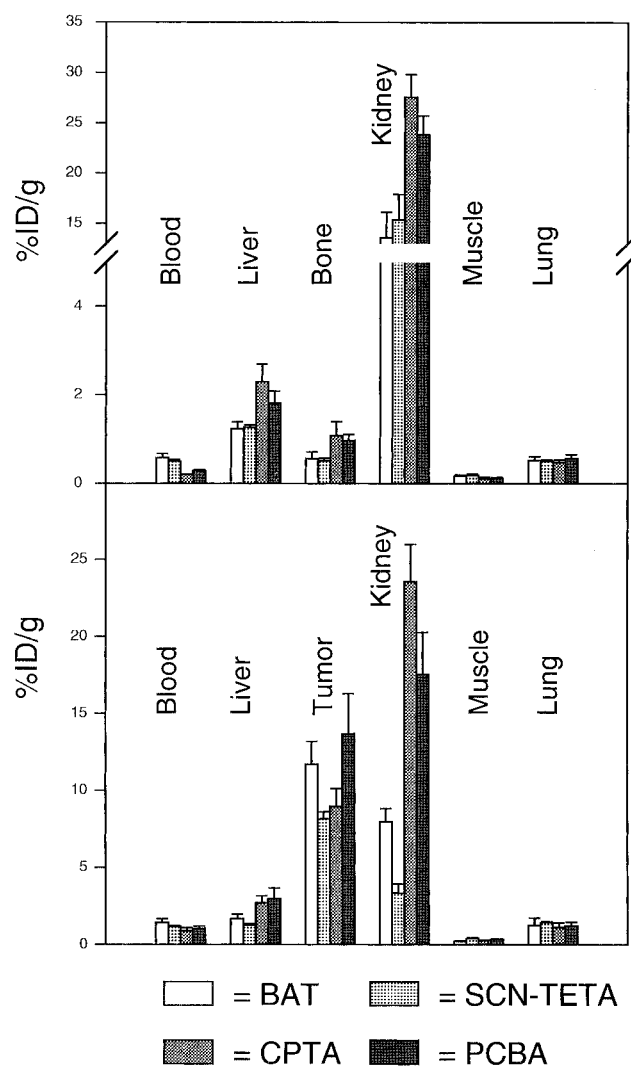


Figure 3. Biodistribution data for ^{64}Cu -BFC-1A3-F(ab')₂ conjugates in Sprague-Dawley rats (top) and tumor-bearing Golden Syrian hamsters (bottom) at 24 h post-injection. Data are presented as the mean plus or minus the standard deviation of $n \geq 3$.

1, 3, and 5 day time points (Figure 5). Analysis of liver homogenates from rats injected with ^{67}Cu -labeled 1A3 conjugates at 1 day post-injection showed that the majority of the ^{67}Cu was associated with intact mAb. For example, at 1 day post-injection, 74% of the radioactivity from rats injected with ^{67}Cu -CPTA-1A3 remained associated with the intact antibody peak (Figure 6). There was also a component that eluted with a MW of 35 kDa (12%) and a low molecular weight component (<5 kDa) (14%). At the later time points, the amount of radioactivity associated with intact antibody decreased (48% at 5 days), while the other two metabolites increased (28% and 24%, respectively). The ^{67}Cu -PCBA-1A3 liver homogenate gel filtration profile at day 1 was similar to ^{67}Cu -CPTA-1A3 at day 1, with 73% of the activity being antibody associated and the rest of the activity being primarily associated with a component having a MW of 35 kDa (26%). At days 3 and 5, the ^{67}Cu -PCBA-1A3 liver metabolite of 35 kDa increased to 59% and 70%, respectively, while the intact mAb peak decreased to 24% by day 5. In the rat liver, the ^{67}Cu -SCN-TETA-1A3 conjugate was $>90\%$ intact at 1 day with the remaining activity eluting with a MW of 35 kDa (8%). By day 5, 43% of the total activity was mAb associated and the remaining metabolites eluted with MWs of 35 kDa (41%) and <5 kDa (16%).

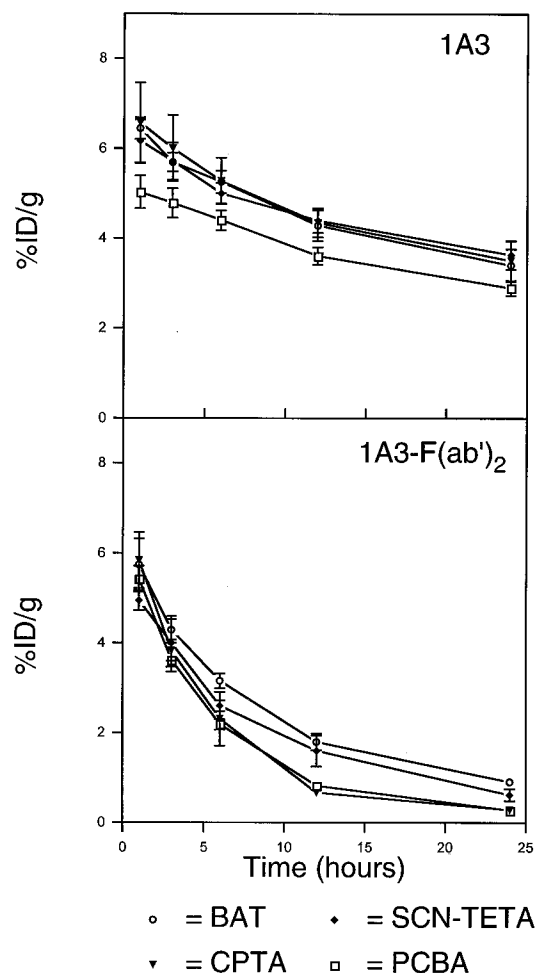


Figure 4. Blood clearance in Sprague-Dawley rats of the various ^{64}Cu -labeled 1A3 conjugates (top) and ^{64}Cu -labeled 1A3-F(ab')₂ conjugates (bottom). Data are presented as the mean plus or minus the standard deviation of $n \geq 3$.

The renal metabolites of Sprague-Dawley rats injected with ^{67}Cu -CPTA-1A3-F(ab')₂, ^{67}Cu -PCBA-1A3-F(ab')₂, ^{67}Cu -SCN-TETA-1A3-F(ab')₂, and ^{67}Cu acetate as a control were analyzed at 1, 3, and 5 days. The rats injected with ^{67}Cu acetate showed two major peaks at day 1, one eluting with a MW of 35 kDa (40%) and one eluting with a MW of 10 kDa (40%). By days 3 and 5, most of the activity was associated with the 35 kDa peak (85% and 90%, respectively), while the rest of the activity eluted with the low molecular weight (<5 kDa) peak (15% and 10%, respectively). ^{67}Cu -CPTA-1A3-F(ab')₂ was completely metabolized to a low molecular weight species (<5 kDa) by 1 day post-injection (Figure 6). Both ^{67}Cu -PCBA-1A3-F(ab')₂ and ^{67}Cu -SCN-TETA-1A3-F(ab')₂ were also >85% converted to a low (<5 kDa) molecular weight species by 1 day post-injection. A peak (<5%) with a MW of 35 kDa appeared at day 5 for both of these conjugates.

Gel Filtration Chromatography of ^{64}Cu -SOD. The gel filtration chromatogram of ^{64}Cu -SOD showed that all of the activity eluted with a MW of 35 kDa (Figure 5). This coincides with the peak from the liver metabolism of rats injected with CuOAc.

Thin Layer Chromatography of Metabolites. The low molecular weight metabolites for the CPTA conjugate at day 1 were collected from the size exclusion chromatography experiment and further analyzed by radio-TLC. Samples were either mixed with ^{67}Cu -CPTA- ϵ -lysine or ^{67}Cu -CPTA standards and applied to glass backed silica gel plates. Both liver and kidney metabolites co-migrated

with the ^{67}Cu -CPTA- ϵ -lysine standard (Figure 7). These metabolites migrated separately from the ^{67}Cu -CPTA standard.

DISCUSSION

CPTA and PCBA were synthesized as previously described (21, 35). The synthesis of BAT was first described by Moi et al. (32); however, an improved synthesis of BAT has been described by Moran et al. that gives the final product in 23% yield (33). A combination of these procedures, along with some minor modifications (38), yielded BAT and SCN-TETA in comparable yields. CPTA- ϵ -lysine is a new compound that was synthesized as a standard for the metabolism studies by an adaptation of the solid-phase method of Franano et al. (29) without the optimization of yield.

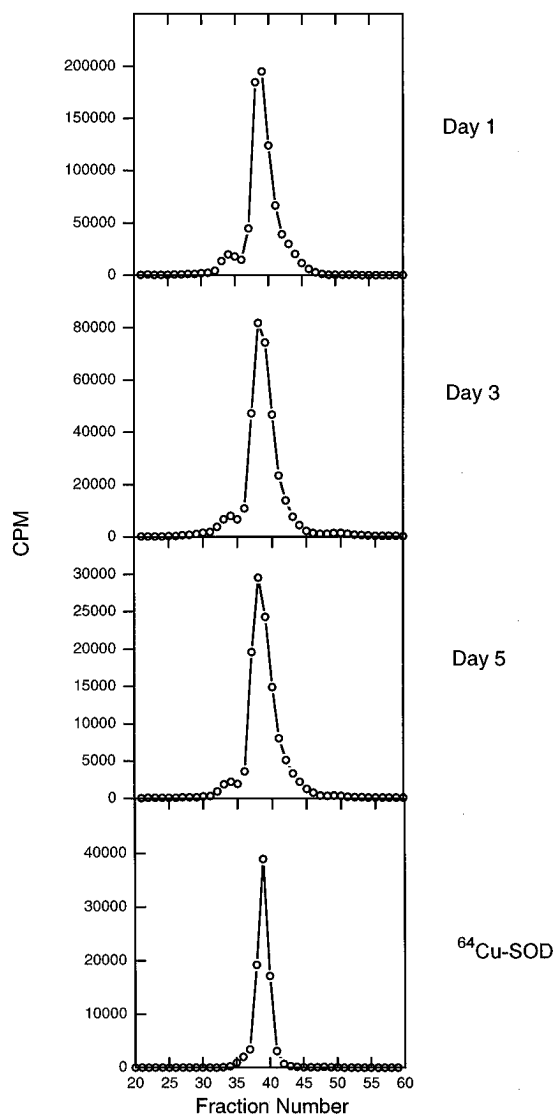
The conjugation and purification of the BAT-2IT-1A3 conjugate was straightforward; however, the presence of 2IT with 1A3-F(ab')₂ fragments reduced disulfide bonds, producing a smaller fragment with a molecular weight of 55 kDa and low IR (17). Therefore, FPLC purification of the BAT-2IT-1A3-F(ab')₂ conjugates was necessary prior to labeling to remove the 55 kDa fragments (17). The conjugation and purification of SCN-TETA to both 1A3 and 1A3-F(ab')₂ was uncomplicated, and size exclusion chromatography showed no breakdown of the intact mAb or fragments; however, the IR of the SCN-TETA conjugates was significantly lower than those of the other conjugates, and only after optimization of the SCN-TETA: mAb ratio and pH were the ^{67}Cu -SCN-TETA conjugates acceptable for *in vivo* studies. The conjugation of CPTA and PCBA to mAbs was straightforward, and the Cu-labeled conjugates had IRs > 80%.

There were differences in biodistribution trends between rats and hamsters. In Sprague-Dawley rats, ^{64}Cu -BAT-2IT-1A3 had the lowest uptake in both the liver and kidneys at 24 h. In hamsters, significantly lower uptake in the kidneys of ^{64}Cu -SCN-TETA 1A3-F(ab')₂ compared to the other conjugates was observed, and this result was not observed in rats. The differences between the two rodent species are not totally understood. A possible explanation may be the different copper binding protein levels in rats and hamsters. The concentrations of these copper binding proteins may affect their biodistribution by transchelation of the copper. Owen showed that different copper binding proteins are present in different concentrations for the same organ in different animal species (41). In most animal species, there are three major copper binding proteins (42): metallothionein (6 kDa), superoxide dismutase (32 kDa), and ceruloplasmin (124 kDa). Protein concentrations also differ between tissues in the same species. Van Hein et al. showed that there is twice as much superoxide dismutase in the liver of a rat than in any other tissue (43).

The differences between ^{64}Cu -BAT-2IT-1A3 and ^{64}Cu -SCN-TETA-1A3 in the liver and kidneys imply that the different linking moieties (thioether vs thiourea) affected the accumulation/retention of activity at these sites. These two bifunctional chelates have the same copper macrocycle, hence the same log *K* and charge, and similar lipophilicities. Therefore, the major difference may be the rate of cleavage of the thiourea bond versus the thioether (44-46). The biodistribution differences between the four ^{64}Cu -BFC-1A3-F(ab')₂ conjugates were notable in both rats and hamsters (Figure 3). The uptake of ^{64}Cu -BAT-2IT-1A3-F(ab')₂ and ^{64}Cu -SCN-TETA-1A3-F(ab')₂ were significantly lower in the liver and kidneys of both rats and hamsters than the uptake of ^{64}Cu -CPTA-1A3-F(ab')₂ and ^{64}Cu -PCBA-1A3-F(ab')₂. This could be

Table 3. Summary of Size Exclusion Chromatography Data for ^{67}Cu -BFC-1A3 Liver Metabolites: Percent of Radioactivity Associated with Three Major Components

	intact antibody (150 kDa)			35 kDa			<5 kDa		
	day 1	day 3	day 5	day 1	day 3	day 5	day 1	day 3	day 5
SCN-TETA	91	48	43	8	40	41	1	12	16
CPTA	74	58	48	12	22	28	14	20	24
PCBA	73	32	24	26	59	70	1	9	6

**Figure 5.** Size exclusion FPLC profiles of ^{67}Cu OAc liver metabolites at 1, 3, and 5 days post-injection and of ^{64}Cu -SOD. Counts per minute (cpm) are plotted versus the fraction number. Note: Fractions 1–20 are the void volume.

due to the lipophilicity and/or charge differences between the BAT/SCN-TETA and the CPTA/PCBA chelates. If the mAbs are metabolized to the Cu(II)-BFC or Cu(II)-BFC-amino acid moieties, the more lipophilic and/or positively charged metabolites are likely to accumulate in the liver and kidneys and not clear as rapidly (47, 48).

Lipophilicities of the copper chelates, as determined by octanol/water partition coefficient ($\log P$) measurements, were not evaluated experimentally due to the low extraction of these complexes into octanol. Since the actual $\log P$ values are likely negative values, due to the charge on the complexes, only relative lipophilicities could be determined by the Hansch method (49). Cu(II)-CPTA was the most lipophilic complex and was assigned an arbitrary $\log P$ value of 5.00. According to Hansch, Cu(II)-BAT and Cu(II)-SCN-TETA are the least lipophilic and Cu(II)-PCBA is intermediate between these com-

plexes and Cu(II)-CPTA (Table 2). At physiologic pH, Cu(II) complexes of BAT and SCN-TETA have a charge of negative two (2^-), while Cu(II) complexes of CPTA and PCBA have a charge of positive one (1^+) (21). The facts that positively charged compounds are known to accumulate in the kidneys (50) and that Cu(II) complexes of CPTA and PCBA are relatively lipophilic may account for their high kidney uptake. Also, differences in the stability of the copper complexes between TETA and CPTA/PCBA may provide an alternate or additional explanation.

The biodistribution differences within a species may be partially due to differences in stability of the Cu-BFC complex. The least stable Cu(II)-BFC complexes are more readily dissociated *in vivo*. One possible mechanism for the release of copper from the bifunctional chelate would be the replacement of copper by other metals present in the body. The difference in stability ($\Delta \log K$) between the Cu(II) complex with that of other metal ions would be a measure of the ease of transchelation. The $\Delta \log K$ values for the various chelates macrocyclic portion (TETA, cyclam, 15aneN5) in Table 2 were derived from literature values (51). All of the Cu(II)-chelate complexes are more stable than the complexes formed with the other biologically related metals. This agrees with an *in vitro* study by Kukis et al. that showed that an antibody-TETA conjugate had a higher selectivity for Cu(II) than for Zn(II), Ca(II), or Mg(II) (52). The Cu-labeled BAT and SCN-TETA conjugates are retained less in the liver and kidneys than those of CPTA and PCBA, yet the $\Delta \log K$ for Cu-Zn is larger for CPTA and PCBA than for BAT and SCN-TETA. These data suggest that displacement of Cu(II) by another metal ion is unlikely. Another explanation is that the $^{64,67}\text{Cu}$ can be displaced by non-radioactive copper. Our data from experiments where the Cu-labeled BFCs were challenged with non-radioactive Cu(II) showed that $\sim 70\%$ of the $^{64,67}\text{Cu}$ in the TETA complex was displaced by cold Cu(II) at 2 days, whereas $<15\%$ of $^{64,67}\text{Cu}$ -CPTA and PCBA were displaced by cold Cu(II) at 2 days. If $^{64,67}\text{Cu}$ was released from the BAT or SCN-TETA conjugates in the liver and kidneys, it would be likely that the $^{64,67}\text{Cu}$ would quickly bind to copper binding proteins and remain trapped in those organs rather than be cleared. Since the $^{64,67}\text{Cu}$ -TETA conjugates have the most rapid clearance from liver and kidneys, it is unlikely that $^{64,67}\text{Cu}$ is being displaced by cold Cu(II) *in vivo*.

The metabolism *in vivo* of ^{111}In -labeled polypeptides and mAbs has been investigated (12, 24, 26–30). Previous studies have shown that ^{111}In -labeled mAbs are degraded to small molecular weight metabolites (24, 26, 30). These studies have indicated that the major small molecular weight metabolite formed was ^{111}In -BFC- ϵ -lysine, a result of proteolysis of the amino acid backbone of the protein. Thus, it is likely that the major metabolite of the Cu-labeled mAbs is $^{64,67}\text{Cu}$ -BFC- ϵ -lysine. By investigating the nature of the metabolite of the different Cu-labeled BFC-mAb conjugates, the cause for the resulting differences in the biodistribution may be elucidated.

The size exclusion data of all ^{67}Cu -BFC-1A3-F(ab')₂ kidney metabolites show that, by 1 day post-injection,

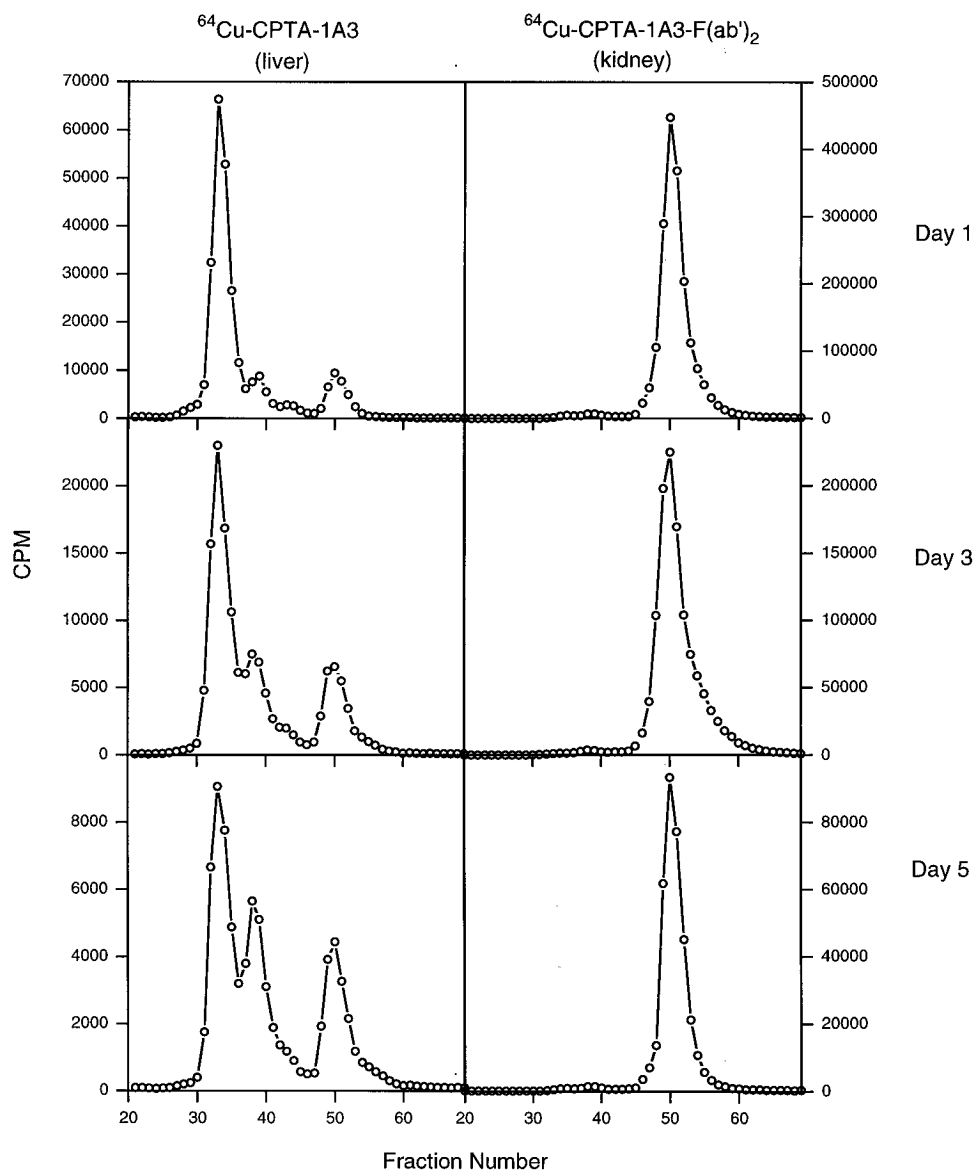


Figure 6. Size exclusion FPLC profiles of ^{67}Cu -CPTA-1A3 liver metabolites (left) and ^{67}Cu -CPTA-1A3-F(ab')₂ kidney metabolites (right) at 1, 3, and 5 days post-injection. Counts per minute (cpm) are plotted versus the fraction number. Note: Fractions 1–20 are the void volume.

>85% of the activity was associated with the low (<5 kDa) molecular weight species. This agrees with the ^{111}In -mAb data (30) and suggests that the $^{64/67}\text{Cu}$ -labeled BFC-1A3-F(ab')₂ conjugates are efficiently deposited within lysosomes by absorptive endocytosis and that subsequent lysosomal degradation was rapid (53). These data suggest that smaller radiolabeled mAbs and peptides may have the same kidney uptake and lysosomal retention as a F(ab')₂ fragment due to the high kidney activity seen with these smaller compounds (54–56). Also, metabolizable linkers may not be a solution to the problem of high renal accumulation unless the radiolabel is prevented from reaching the lysosome or the radiolabeled metabolite is in a form in which it can be rapidly exported from the lysosome.

The ^{67}Cu -PCBA-1A3-F(ab')₂ and ^{67}Cu -SCN-TETA-1A3-F(ab')₂ kidney metabolism data demonstrated the appearance of a minor peak (<10%) with a molecular weight of ~35 kDa at 5 days. This corresponded to the same molecular weight peak observed for the rats injected with ^{67}Cu acetate. This suggests that, after several days, Cu(II) was dissociated from the conjugates and then bound to a 35 kDa protein *in vivo*.

The results of the ^{67}Cu -labeled 1A3 liver homogenates were more complicated than those of the ^{67}Cu -labeled 1A3-F(ab')₂ kidney homogenates. All of the intact 1A3 conjugates showed a similar pattern; at 1 day post-injection, the majority of radioactivity was associated with the intact antibody peak (MW = 150 kDa). At later time points, an increasing amount of activity eluted with a molecular weight of ~35 kDa. In addition, the ^{67}Cu -BFC-1A3 liver homogenates showed a low molecular weight peak that increased over the 5 day period. These results differ from the ^{111}In -labeled glycoprotein data (28) and the ^{111}In - and ^{67}Cu -labeled 1A3-F(ab')₂ data, which showed a rapid conversion to a low molecular weight species; however, the results agree with ^{111}In -labeled 1A3 metabolism data (30). The fact that ^{67}Cu -labeled 1A3 conjugates did not rapidly metabolize to a low molecular weight species (<5 kDa) in the liver suggests that they were not targeted to cell surface receptors and lack an effective uptake mechanism, such as absorptive endocytosis in the kidneys.

At 1 day post-injection, only ^{67}Cu -CPTA-1A3 was partially metabolized in the liver to a low molecular weight species. TLC of the low molecular weight species

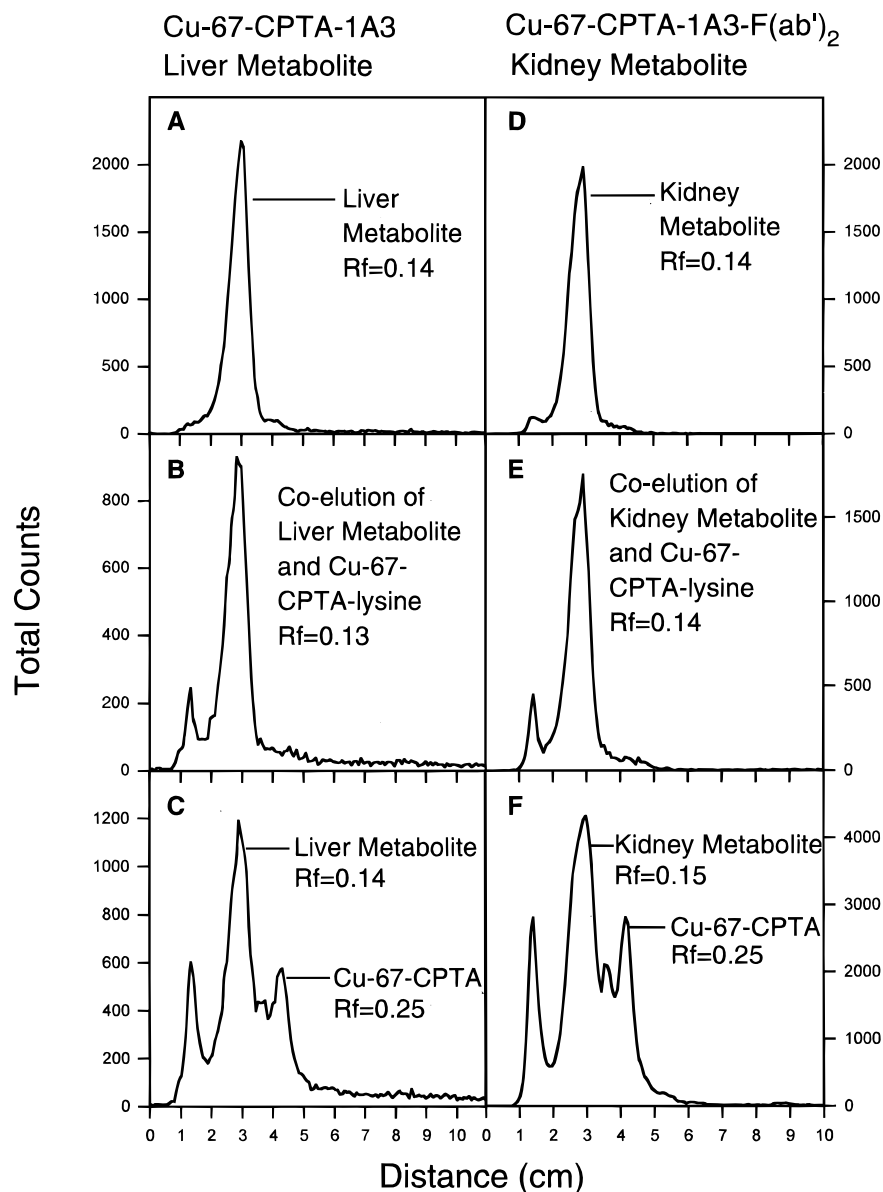


Figure 7. Silica gel radio-TLC of the small molecular weight metabolites for ^{67}Cu -CPTA-1A3 and ^{67}Cu -CPTA-1A3-F(ab')₂ (1 day post-injection) collected by size exclusion chromatography. ^{67}Cu -CPTA-1A3 metabolites are shown alone (A), mixed with ^{67}Cu -CPTA- ϵ -lysine standard (B), and with ^{67}Cu -CPTA standard (C). ^{67}Cu -CPTA-1A3-F(ab')₂ metabolites are shown alone (D), with ^{67}Cu -CPTA- ϵ -lysine standard (E), and with ^{67}Cu -CPTA standard (F). The origin for all plates is 1 cm.

from ^{67}Cu -CPTA-1A3 liver metabolites and ^{67}Cu -CPTA-1A3-F(ab')₂ kidney metabolites identified the species as ^{67}Cu -CPTA- ϵ -lysine (Figure 7). The appearance of the low molecular weight peak for the ^{67}Cu -CPTA-1A3 conjugate, along with the previous data on antibody metabolism (57–60), suggest that the ^{67}Cu -CPTA-1A3 conjugate was partially degraded in the lysosome. The mAb could also localize in extracellular sites and bind non-specifically to surface molecules or be internalized by specific (Fc receptor) or nonspecific (fluid-phase endocytosis) means.

The size exclusion data for ^{67}Cu -PCBA-1A3 and ^{67}Cu -SCN-TETA-1A3 did not show a low molecular weight peak (<1%) at day 1. This might suggest that the radiolabel was not delivered to the lysosome for degradation; however, it is more likely that the ^{67}Cu transchelated to a protein with a larger molecular weight prior to localization in the lysosome.

In the liver, all of the Cu-labeled 1A3 conjugates were partially metabolized to a 35 kDa species. There are two possible explanations for this: first, the radiolabeled antibody may have been partially degraded within en-

dosomes (58–61), giving the intermediate molecular weight peak of 35 kDa; however, this is not in agreement with ^{111}In -labeled mAb studies which show a broadening of the intact mAb peak and the absence of a peak at 35 kDa (30). Alternatively, Cu(II) could be released from the chelate and bind to a 35 kDa protein. The size exclusion data of liver homogenates from rats injected with ^{67}Cu acetate showed that the ^{67}Cu activity elutes at a molecular weight of 35 kDa, which gives credence to the latter argument. The liver contains a 32 kDa enzyme, superoxide dismutase (SOD), which has one Cu(II) ion per active site (42). The concentration of SOD in the rat liver is twice as great as in any other tissue (43). SOD has been labeled with ^{64}Cu and analyzed by size exclusion chromatography and was found to co-migrate with the 35 kDa peak observed in the liver metabolism of ^{67}Cu acetate and ^{67}Cu -BFCs-1A3. Thus, it appears that transchelation of Cu(II) from the 1A3 conjugates to SOD is a reasonable explanation for the 35 kDa metabolite is underway.

The rate at which the radiolabeled intact antibody peak

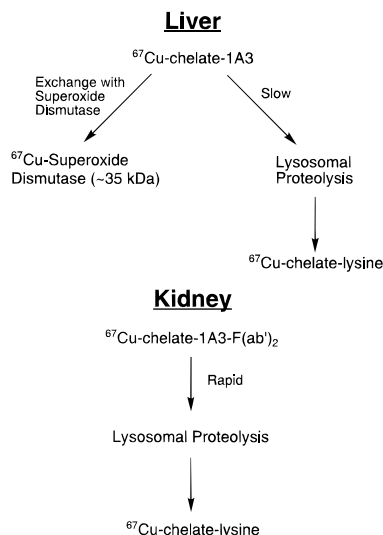


Figure 8. Proposed mechanisms for the uptake and retention of $^{64/67}\text{Cu}$ -labeled 1A3 in the liver (A) and $^{64/67}\text{Cu}$ -labeled 1A3-F(ab')₂ in the kidneys (B).

was converted to the 35 kDa species in the rat liver also differed between the conjugates. For ^{67}Cu -CPTA-1A3 and ^{67}Cu -SCN-TETA-1A3 at day 5, 28% and 41% of the activity was associated with the 35 kDa peak, respectively, while 70% of the activity for ^{67}Cu -PCBA-1A3 was associated with this peak. This suggests that the rate of transchelation of ^{67}Cu from PCBA-1A3 to a 35 kDa protein was more rapid than that of the ^{67}Cu -CPTA-1A3 and ^{67}Cu -SCN-TETA-1A3 conjugates. These data do not correlate with the stability of the complexes, which shows Cu-PCBA as stable a complex as Cu-CPTA and more stable than Cu-TETA (21). We are currently carrying out experiments to elucidate the liver metabolism of other $^{64/67}\text{Cu}$ -BFC-proteins in order to better interpret these results.

It is more difficult to correlate the biodistribution of the Cu-labeled BFC-1A3 conjugates with the metabolite data, since the differences in liver uptake between the four intact mAb conjugates are not as significant as those of the Cu-labeled BFC-1A3-F(ab')₂ uptake in the kidney. Retention of Cu-labeled BFC-1A3 conjugates in the liver is probably a result of transchelation of radioactive Cu(II) to copper binding proteins, such as SOD in the liver (Figure 8). In the kidney, ^{64}Cu -CPTA-1A3-F(ab')₂ and ^{64}Cu -PCBA-1A3-F(ab')₂ had nearly a 2-fold higher uptake over that of ^{64}Cu -SCN-TETA-1A3-F(ab')₂ in rats, and an even greater increased uptake in tumor-bearing hamsters. The kidney metabolism of the ^{67}Cu -BFC-1A3-F(ab')₂ conjugates showed a rapid conversion to small molecular weight metabolites (< 5 kDa) by 1 day post-injection. This suggests that proteolysis of mAb fragments resulted in the small molecular weight lysine or N-terminal amino acid derivatives of the bifunctional chelates (Figure 8). The observed biodistribution differences are most likely due to the resulting charge and/or lipophilicity of these derivatives, which dictate the uptake and clearance from the organ (48).

In summary, the purpose of this study was to correlate biodistribution of $^{64/67}\text{Cu}$ -labeled intact MAb and MAb F(ab')₂ fragments in a rat model to metabolism in nontarget organs such as the liver and kidneys. The liver metabolism of ^{67}Cu -labeled BFC-1A3 conjugates was slow, and it appears that ^{67}Cu was transchelated to a 35 kDa protein (possibly SOD). The metabolism of ^{67}Cu -labeled BFC-1A3-F(ab')₂ conjugates was rapid in the kidneys, and chromatographic results for ^{67}Cu -CPTA-1A3-F(ab')₂ suggest that ^{67}Cu -BFC- ϵ -lysine was the major

metabolite. Therefore, chelate charge and lipophilicity of a $^{64/67}\text{Cu}$ -BFC-amino acid metabolite play a large role in kidney retention, while transchelation of the copper label to a protein appears to be a major factor for liver accumulation.

The understanding of the mechanisms for accumulation of radiometal-labeled BFC-mAb conjugates in nontarget organs is vital for the design of effective imaging and radioimmunotherapy agents. The research presented here is a first step in determining the metabolism of mAbs labeled with copper radioisotopes. The mechanisms of radiolabel accumulation in the liver and kidney that are presented here help to explain the observed biodistribution results; however, more work needs to be done to verify these mechanisms.

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LITERATURE CITED

- (1) Keenan, A. M., Harbert, J. C., and Larson, S. M. (1985) Monoclonal Antibodies in Nuclear Medicine. *J. Nucl. Med.* 26, 531-537.
- (2) Waldmann, T. A. (1991) Monoclonal Antibodies in Diagnosis and Therapy. *Science* 252, 1657-1662.
- (3) DeNardo, S., DeNardo, G., Kukis, D., Mausner, L., Moody, D., and Meares, C. (1991) Pharmacology of Cu-67-TETA-Lym-1 Antibody in Patients with B Cell Lymphoma. *Antibody, Immunoconjugates, Radiopharm.* 4, 36.
- (4) DeNardo, G. L., DeNardo, S. J., Meares, C. F., Salako, Q., Kukis, D. L., Mausner, L. F., Lewis, J. P., O'Grady, L. F., and Srivastava, S. C. (1993) Pilot Therapy of Lymphoma with Fractionated Cu-67-BAT-Lym-1. *J. Nucl. Med.* 34, 93P.
- (5) Delaloye, A. B., Buchegger, F., Smith, A., Schubiger, P. A., Maecke, H. R., Poncioni, L., Gillet, M., Mach, J. P., and Delaloye, B. (1994) Cu-67-Anti-CEA MAb, A Potential Therapeutic Agent: Biokinetic Studies in Patients with Colon Carcinoma. *J. Nucl. Med.* 35, 101P.
- (6) Philpott, G. W., Dehdashti, F., Schwarz, S. W., Connett, J. M., Anderson, C. J., Zinn, K. R., Meares, C. F., Welch, M. J., and Siegel, B. A. (1994) Positron Emission Tomography (PET) with Cu-64-Labeled Monoclonal Antibody (MAb 1A3) in Colorectal Cancer. *J. Nucl. Med.* 35, 12P.
- (7) Philpott, G. W., Dehdashti, F., Schwarz, S. W., Connett, J. M., Anderson, C. J., Zinn, K. R., Cutler, P. D., Welch, M. J., and Siegel, B. A. (1995) RadioimmunoPET (MAB-PET) with Cu-64-Labeled Monoclonal Antibody (MAb 1A3) Fragments [F(ab')₂] in Patients with Colorectal Cancers. *J. Nucl. Med.* 36, 9P.
- (8) Philpott, G. W., Schwarz, S. W., Anderson, C. J., Dehdashti, F., Connett, J. M., Zinn, K. R., Meares, C. F., Cutler, P. D., Welch, M. J., and Siegel, B. A. (1995) RadioimmunoPET: Detection of Colorectal Carcinoma with Positron-Emitting Copper-64-Labeled Monoclonal Antibody. *J. Nucl. Med.* 36, 1818-1824.
- (9) Apelgot, S., Coppey, J., Gaudemer, A., Grisvard, J., Guille, E., Sasaki, I., and Sissoeff, I. (1989) Similar Lethal Effect in Mammalian Cells for Two Radioisotopes of Copper with Different Decay Schemes, Cu-64 and Cu-67. *Int. J. Radiat. Biol.* 55, 365-384.
- (10) Connett, J. M., Anderson, C. J., Baumann, M. L., Schwarz, S. W., Zinn, K. R., Philpott, G. W., and Welch, M. J. (1993) Cu-67 and Cu-64 labeled monoclonal antibody (MAb) as potential agents for radioimmunotherapy. *J. Nucl. Med.* 34, 216P.
- (11) Anderson, C. J., Connett, J. M., Guo, L. W., Schwarz, S. W., Philpott, G. W., Zinn, K. R., and Welch, M. J. (1994) Initial comparison of Cu-67 and Cu-64-labeled anticorectal

- carcinoma MAb 1A3 as agents for radioimmunotherapy in tumor-bearing hamsters. *J. Nucl. Med.* 35, 161P.
- (12) Deshpande, S. V., Subramanian, R., McCall, M. J., DeNardo, S. J., DeNardo, G. L., and Meares, C. F. (1990) Metabolism of Indium Chelates Attached to Monoclonal Antibody: Minimal Transchelation of Indium from Benzyl-EDTA Chelate In Vivo. *J. Nucl. Med.* 31, 218–224.
- (13) Deshpande, S. V., DeNardo, S. J., Kukis, D. L., Moi, M. K., McCall, M. J., DeNardo, G. L., and Meares, C. F. (1990) Yttrium-90-Labeled Monoclonal Antibody for Therapy: Labeling by a New Macrocyclic Bifunctional Chelating Agent. *J. Nucl. Med.* 31, 473–479.
- (14) DeNardo, G. L., Kroger, L. A., DeNardo, S. J., Miers, L. A., Salako, Q., Kukis, D. L., Fand, I., Shen, S., Renn, O., and Meares, C. F. (1994) Comparative Toxicity Studies of Yttrium-90 MX-DTPA and 2-IT-BAD Conjugated Monoclonal Antibody (BrE-3). *Cancer* 73, 1012–1022.
- (15) Anderson, C. J., Rogers, B. E., Connett, J. M., Guo, L. W., Schwarz, S. W., Zinn, K. R., and Welch, M. J. (1994) Comparison of Two Bifunctional Chelates for Labeling Cu-64 to MAb 1A3 and 1A3-F(ab')₂: Chemistry and Animal Biodistribution. *J. Labelled Compd Radiopharm.* 35, 313–315.
- (16) Anderson, C. J., Connett, J. M., Schwarz, S. W., Rocque, P. A., Guo, L. W., Philpott, G. W., Zinn, K. R., Meares, C. F., and Welch, M. J. (1992) Copper-64-Labeled Antibodies for PET Imaging. *J. Nucl. Med.* 33, 1685–1691.
- (17) Anderson, C. J., Schwarz, S. W., Connett, J. M., Cutler, P. D., Guo, L. W., Germain, C. J., Philpott, G. W., Zinn, K. R., Greiner, D. P., Meares, C. F., and Welch, M. J. (1995) Preparation, Biodistribution, and Dosimetry of Copper-64-Labeled Anti-Colorectal Carcinoma Monoclonal Antibody Fragments 1A3-F(ab')₂. *J. Nucl. Med.* 36, 850–858.
- (18) Subramanian, R., and Meares, C. F. (1990) Bifunctional chelating agents for radiometal-labeled monoclonal antibodies. *Cancer Imaging with Radiolabeled Antibodies* (D. M. Goldenberg, Ed.) pp 183–199, Kluwer Academic Publishers, New York.
- (19) McCall, M. J., Diril, H., and Meares, C. F. (1990) Simplified Method for Conjugating Macrocyclic Bifunctional Chelating Agents to Antibodies via 2-Iminothiolane. *Bioconjugate Chem.* 1, 222–226.
- (20) Smith-Jones, P. M., Fridrich, R., Kaden, T. A., Novak-Hofer, I., Siebold, K., Tschudin, D., and Maecke, H. R. (1991) Antibody Labeling with Copper-67 Using the Bifunctional Macrocyclic 4-[(1,4,8,11-Tetraazacyclotetradec-1-yl)methyl]-benzoic Acid. *Bioconjugate Chem.* 2, 415–421.
- (21) Motekaitis, R. J., Rogers, B. E., Reichert, D. E., Martell, A. E., and Welch, M. J. (1996) Stability and Structure of Activated Macrocycles. Ligands with Biological Applications. *Inorg. Chem.*, in press.
- (22) Clarke, E. T., and Martell, A. E. (1991) Stabilities of the alkaline earth and divalent transition metal complexes of the tetraazamacrocyclic tetraacetic acid ligands. *Inorg. Chim. Acta* 190, 27–36.
- (23) Motta-Hennessy, C., Sharkey, R. M., and Goldenberg, D. M. (1990) Metabolism of Indium-111-Labeled Murine Monoclonal Antibody in Tumor and Normal Tissue of the Athymic Mouse. *J. Nucl. Med.* 31, 1510–1519.
- (24) Jones, P. L., Brown, B. A., and Sands, H. (1990) Uptake and Metabolism of ¹¹¹In-labeled Monoclonal Antibody B6.2 by the Rat Liver. *Cancer Res.* 50, 852s–856s.
- (25) Sands, H., and Jones, P. L. (1987) Methods for the Study of the Metabolism of Radiolabeled Monoclonal Antibodies by Liver and Tumor. *J. Nucl. Med.* 28, 390–398.
- (26) Paik, C. H., Sood, V. K., Le, N., Cioloca, L., Carrasquillo, J. A., Reynolds, J. C., Neumann, R. D., and Reba, R. C. (1992) Radiolabeled Products in Rat Liver and Serum after Administration of Antibody-Amide-DTPA-Indium-111. *Nucl. Med. Biol.* 19, 517–522.
- (27) Himmelsbach, M., and Wahl, R. L. (1989) Studies on the Metabolic Fate of ¹¹¹In-labeled Antibodies. *Int. J. Radiat. Appl. Instrum., Part B* 16, 839–845.
- (28) Duncan, J. R., and Welch, M. J. (1993) Intracellular Metabolism of Indium-111-DTPA Labeled Receptor Targeted Proteins. *J. Nucl. Med.* 34, 1728–1738.
- (29) Franano, F. N., Edwards, W. B., Welch, M. J., and Duncan, J. R. (1994) Metabolism of Receptor Targeted ¹¹¹In-DTPA-Glycoproteins: Identification of ¹¹¹In-DTPA-ε-lysine as the Primary Metabolic and Excretory Product. *Nucl. Med. Biol.* 21, 1023–1034.
- (30) Rogers, B. E., Franano, F. N., Duncan, J. R., Edwards, W. B., Anderson, C. J., Connett, J. M., and Welch, M. J. (1995) Identification of Metabolites of In-111-DTPA-Monoclonal Antibodies and Antibody Fragments In Vivo. *Cancer Res.* 55, 5714s–5720s.
- (31) Zinn, K. R., Chaudhuri, T. R., Cheng, T. P., Morris, J. S., and Meyer, W. A. (1994) Production of No-Carrier-Added Cu-64 from Zinc Metal Irradiated under Boron Shielding. *Cancer* 73, 774–778.
- (32) Moi, M. K., Meares, C. F., McCall, M. J., Cole, W. C., and DeNardo, S. J. (1985) Copper Chelates as Probes of Biological Systems: Stable Copper Complexes with a Macrocyclic Bifunctional Chelating Agent. *Anal. Biochem.* 148, 249–253.
- (33) Moran, J. K., Greiner, D. P., and Meares, C. F. (1995) Improved Synthesis of 6-[p-(Bromoacetamido)benzyl]-1,4,8,11-tetraazacyclotetradecane-N,N',N'',N'''-tetraacetic Acid and Development of a Thin-Layer Assay for Thiol-Reactive Bifunctional Chelating Agents. *Bioconj. Chem.* 6, 296–301.
- (34) McMurry, T. J., Brechbiel, M., Kumar, K., and Gansow, O. A. (1992) Convenient Synthesis of Bifunctional Tetraaza Macrocycles. *Bioconjugate Chem.* 3, 108–117.
- (35) Studer, M., and Kaden, T. A. (1986) One-Step Synthesis of Mono-N-substituted Azamacrocycles with a Carboxylic Group in the Side-Chain and their Complexes with Cu²⁺ and Ni²⁺. *Helv. Chim. Acta* 69, 2081–2086.
- (36) Folch, J. R., Lees, M., and Sloane-Stanley, G. H. (1957) Simple method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem.* 226, 497–509.
- (37) Goldenberg, D. M., Witte, S., and Elster, K. (1966) GW-39: A New Human Tumor Serially Transplantable in the Golden Hamster. *Transplantation* 4, 760–763.
- (38) Rogers, B. E. (1995) Synthesis and Investigation of Bifunctional Chelating Agents for Radiolabeling Monoclonal Antibodies. Ph.D. Thesis, Washington University.
- (39) Rana, T. M., and Meares, C. F. (1990) N-Terminal Modification of Immunoglobulin Polypeptide Chains Tagged with Isothiocyanato Chelates. *Bioconjugate Chem.* 1, 357–362.
- (40) Means, G. E., and Feeney, R. E. (1971) *Chemical Modification of Proteins*, pp 11–17, Holden-Day, Inc., San Francisco, CA.
- (41) Owen, C. A. (1982) *Biochemical Aspects of Copper: Copper Proteins, Ceruloplasmin, and Copper Protein Binding*, Noyes Publications, New Jersey.
- (42) Winge, D. R. (1984) Normal Physiology of Copper Metabolism. *Semin. Liver Dis.* 4, 239–251.
- (43) Van Hein, P., Kovacs, K., and Matkovics, B. (1975) Properties of Enzymes II. Comparative study of superoxide dismutase activity in rat tissues. *Enzyme* 19, 1–4.
- (44) Meares, C. F., McCall, M. J., Deshpande, S. V., DeNardo, S. J., and Goodwin, D. A. (1988) Chelate Radiochemistry: Cleavable Linkers Lead to Altered Levels of Radioactivity in the Liver. *Int. J. Cancer* 2, 99–102.
- (45) Arano, Y., Mukai, T., Uezono, T., Wakisaka, K., Motonari, H., Akizawa, H., Taoka, Y., and Yokoyama, A. (1994) A Biological Method to Evaluate Bifunctional Chelating Agents to Label Antibodies with Metallic Radionuclides. *J. Nucl. Med.* 35, 890–898.
- (46) Arano, Y., Mukai, T., Akizawa, H., Uezono, T., Motonari, H., Wakisaka, K., Kairiyama, C., and Yokoyama, A. (1995) Radiolabeled Metabolites of Proteins Play a Critical Role in Radioactivity Elimination from the Liver. *Nucl. Med. Biol.* 22, 555–564.
- (47) Anderson, C. J., Pajean, T. S., Edwards, W. B., Sherman, E. L. C., Rogers, B. E., and Welch, M. J. (1995) In vitro and in vivo Evaluation of Copper-64-Labeled Octreotide Conjugates. *J. Nucl. Med.* 36, 2315–2325.
- (48) Jones-Wilson, T. M. (1995) Preparation and Evaluation of Potential Radiopharmaceuticals labeled with gallium-67, indium-111, and Cu-64,67: Relationships between structure and biological activity. Ph.D. Thesis, Washington University.

- (49) Hansch, C., and Leo, A. (1979) *Substituent Constants for Correlation Analysis in Chemistry and Biology*, Wiley-Interscience Publication, New York.
- (50) Maack, T., and Park, C. H. (1990) Endocytosis and lysosomal hydrolysis of proteins in proximal tubules. *Methods Enzymol.* 191, 340–354.
- (51) Martell, A. E., and Smith, R. M. (1982) *Critical Stability Constants*, Plenum Press, New York.
- (52) Kukis, D. L., Li, M., and Meares, C. F. (1993) Selectivity of Antibody-Chelate Conjugates for Binding Copper in the Presence of Competing Metals. *Inorg. Chem.* 32, 3981–3982.
- (53) Maack, T., Park, C. H., and Camargo, J. F. (1992) Renal Filtration, Transport, and Metabolism of Proteins. *The Kidney: Physiology and Pathophysiology* (D. W. Seldin and G. Giebisch, Eds.) pp 3005–3038, Raven Press, Ltd., New York.
- (54) Yokota, T., Milenic, D. E., Whitlow, M., Wood, J. F., Hubert, S. L., and Schlom, J. (1993) Microautoradiographic Analysis of the Normal Organ Distribution of Radioiodinated Single-Chain Fv and Other Immunoglobulin Forms. *Cancer Res.* 53, 3776–3783.
- (55) Fischman, A. J., Pike, M. C., Kroon, D., Fucello, A. J., Rexinger, D., tenKate, C., Wilkinson, R., Rubin, R. H., and Strauss, H. W. (1991) Imaging Focal Sites of Bacterial Infection in Rats with Indium-111-Labeled Chemotactic Peptide Analogs. *J. Nucl. Med.* 32, 483–491.
- (56) Bakker, W. H., Krenning, E. P., Reubi, J. C., Breeman, W. A. P., Setyono-Han, B., de Jong, M., Kooij, P. P. M., Bruns, C., van Hagen, P. M., Marbach, P., Visser, T. J., Pless, J., and Lamberts, S. W. J. (1991) In Vivo Application of [In-111-DTPA-D-Phe]-Octreotide for Detection of Somatostatin Receptor-Positive Tumors in Rats. *Life Sci.* 49, 1593–1601.
- (57) Geissler, F., Anderson, S. K., and Press, O. (1991) Intracellular Catabolism of Radiolabeled Anti-CD3 Antibodies by Leukemic T Cells. *Cell. Immunol.* 137, 96–110.
- (58) Scharschmidt, B. F., Lake, J. R., Renner, E. L., Licko, V., and Van Dyke, R. W. (1986) Fluid phase endocytosis by cultured rat hepatocytes and perfused rat liver: Implications for plasma membrane turnover and vesicular trafficking of fluid phase markers. *Proc. Natl. Acad. Sci. U.S.A.* 83, 9488–9492.
- (59) Yamazaki, K., and LaRusso, N. F. (1989) The Liver and Intracellular Digestion: How Liver Cells Eat! *Hepatology* 10, 877–886.
- (60) Straus, W. (1964) Cytochemical observations on the relationship between lysosomes and phagosomes in the kidney and liver by combined staining for acid phosphatase and intravenously injected horseradish peroxidase. *J. Cell Biol.* 497–507.
- (61) Schneider, Y.-J., Tulkens, P., de Duve, C., and Trouet, A. (1979) Fate of plasma membrane during endocytosis II. Evidence for recycling (shuttle) of plasma membrane constituents. *J. Cell Biol.* 82, 466–474.

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CORRECTIONS

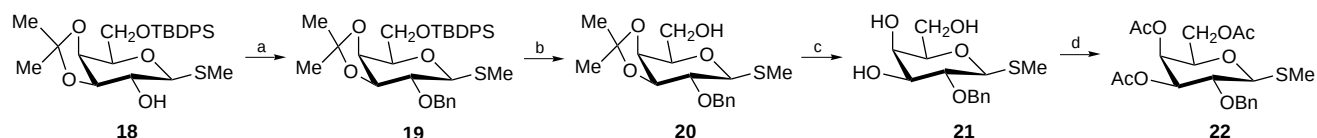
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PURIFICATION OF SUBUNIT B OF SHIGA TOXIN USING A SYNTHETIC TRISACCHARIDE-BASED AFFINITY MATRIX

Page 52. There were several errors in Scheme 3.

Scheme 3^a



^a Abbreviations, reagents, and conditions: Ac = acetyl, Bn = benzyl, Me = methyl, TBDPS = *tert*-butyldiphenylsilyl; (a) 1.9 equiv of BnBr, DMF, NaH, 0 °C, 83%; (b) 0.7 equiv of Bu₄NF, THF, 25 °C, 12 h, 97%; (c) AcOH–H₂O, 80 °C, 2 h, 95%; (d) Ac₂O, C₅H₅N, 0 °C, 1 h, 97%.

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COMMUNICATIONS

Polymers as Surface-Based Tethers with Photolytic Triggers Enabling Laser-Induced Release/Desorption of Covalently Bound Molecules

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A novel design for surface-based macromolecular docking and release is presented together with a strategy to improve and extend biopolymer structure determination capabilities. Polymeric surfaces with arrays of tethers for covalent molecular attachment were designed with photolytic triggers to enable spatially defined, laser-induced uncoupling/desorption of the tethered molecules. Upon photolytic cleavage, a defined portion of the tether ("tail") remains attached to the biomolecule as a probe. Chemically defined memory, determined by the number of reporter tails, reflects the biomolecule interaction with tether-probe devices encountered (i.e., footprint) on the probe surface. To demonstrate function, a surface of poly(4-vinylpyridine) was extended through the pyridinium nitrogens with spacer arms (*N*-ethylsuccinamyl-) producing photolytic pyridinium nitrogen bonds. The photolabile tether was terminated with leaving groups (*N*-hydroxysulfosuccinimide) for covalent attachment of biopolymers. An 18-residue peptide (N terminus of human β -casein) was covalently docked to these tether-probes, irradiated with coherent UV light, and released with two reporter tails of a mass predicted by tether formation at the two primary amine groups and subsequent photolytic cleavage at the intended site. This is the first demonstration of polymeric surface structure enabling the covalent docking and laser-induced uncoupling/desorption of intact macromolecules through the use of photolytic tethers. Surface-based tether-probe devices, operated by coherent light, should advance our ability to explore covalent modifications in biopolymer structure and alterations in conformation, generated either in advance of tethering or through chemical/enzymatic manipulations performed directly *in situ*.

A novel molecular design for surface-based macromolecular docking and release is presented together with a

strategy to improve and extend biopolymer structure determination capabilities. The approach involves a new category of surface chemistries to covalently bind biopolymers that can later be uncoupled/desorbed simultaneously by pulsed laser irradiation.

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Pulsed laser irradiation has already been used to induce the desorption of structurally intact biopolymers from probes with chemically defined and molecular adsorption/desorption surface properties (1). The process

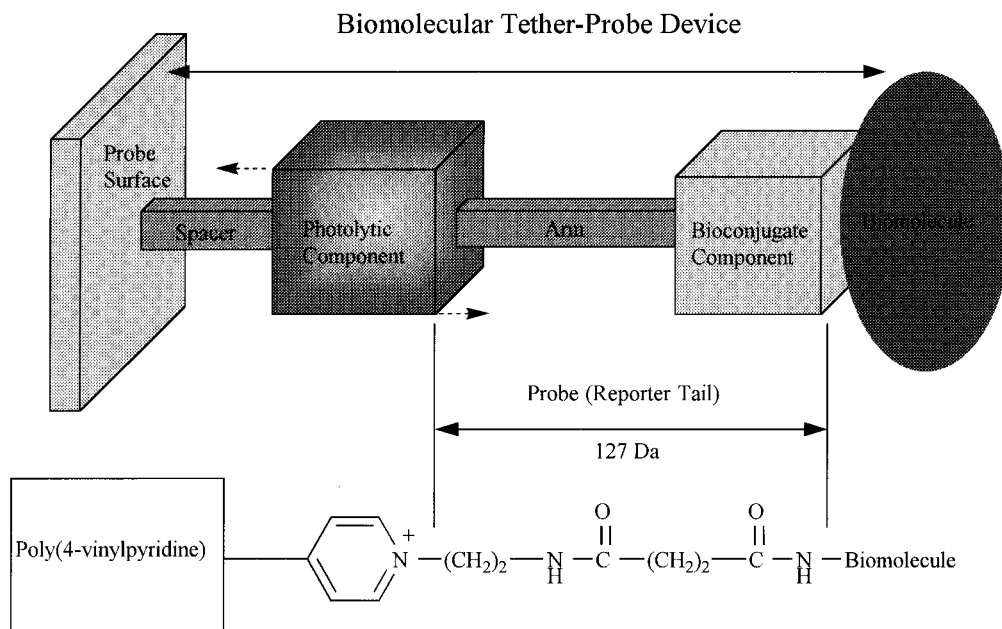


Figure 1. Modular design of biomolecular tether–probe devices. Each device is defined by three components attached to a specified surface architecture. A photolytic component (1) is placed at different relative positions (indicated by dotted arrows) within a variable length spacer arm (2) terminating with a bioconjugation component (3) for covalent attachment through specific sites on the target biomolecule surface. This device is designed so that laser irradiation induces a photolytic event that triggers uncoupling of the tethered biomolecule in a manner that leaves the dissociated biopolymer with one or more reporter tails. The detailed chemical structure of the device used to generate the data shown in Figure 2 is provided.

introduced in 1993, known as surface-enhanced laser desorption/ionization (SELDI) or affinity mass spectrometry (1, 2), was based on use of new probe material compositions and/or defined surface chemistries. In one category of process identified as surface-enhanced affinity capture (SEAC), chemically defined and biologically defined affinity capture devices are covalently bound directly to the modified probe surfaces. Only the captured target biomolecules, docked on the probe surface through *noncovalent* molecular recognition events, are selectively desorbed by laser irradiation.

Inorganic and polymeric probe surfaces have been designed to present even *covalently bound* analyte biomolecules for laser-induced uncoupling/desorption (3–6). Our laboratory was able to accomplish, for the first time, the covalent attachment of biopolymers to a probe surface with tether chemistries enabling single pulses of laser irradiation to initiate subsequent *uncoupling and desorption/ionization* of the bound biomolecules (3, 4). This advance in laser-induced desorption capabilities revealed several new possibilities for the development of solid-phase bioconjugate chemistries for the investigation of biopolymer structure. One goal of this investigation was to determine whether the covalent binding of biomolecules to probe surfaces through more than one point of attachment could be achieved and still generate a homogeneous photolytic reaction product without loss of laser-induced uncoupling/desorption events that are, in effect, simultaneous. A further goal was to determine if polymers could be designed with the necessary photolabile biopolymer attachment and release characteristics. We have now explored a different approach to the design and synthesis of surfaces with the desired chemical reactivities and photolytic tethering capabilities.

Polymeric surface structures with high-density arrays of chemically reactive groups for biopolymer attachment were required to have extremely efficient photolytic triggers to enable laser-induced release (uncoupling/desorption) of the target biomolecules after covalent attachment. The covalent attachment/photolytic release process sought was conceived with the intent of deriving

polymer surface functionality not only to tether target biomolecules but also to probe biopolymer structure. We have now synthesized, within a single chemical construct, a combination tether–probe device and have tested its function both as a covalent photolabile tether and as a probe.

A modular construction approach was adopted to optimize the contribution of three separate tether–probe device components designed to extend outward from a hydrophobic polymer surface (Figure 1). These components included a variable-length spacer arm, a photolytic component located at different positions within this spacer arm, and a zero-length bioconjugate component at the end of the spacer arm for covalent macromolecule attachment. The components within each device were intended to function collectively to accomplish several specific objectives. They must first act as a tether to covalently bind the target molecule at some defined distance from the probe surface. Second, after attachment, the photolytic component within the tether must remain an extremely efficient light-dependent triggering device for release of the tethered biomolecules. Finally, upon laser-induced uncoupling, a defined portion of the tether (“tail”) must be photochemically stable and remain covalently attached to the biomolecule as a probe.

A simple procedure for the light-controlled release of large molecules from covalent anchor sites on a surface was dependent on our ability to demonstrate whether a single laser pulse could be used to induce simultaneously both uncoupling and desorption of covalently bound biomolecules (e.g., peptide) from a polymeric probe surface. More importantly, to implement the structural probe functions outlined, it was essential for us to determine whether simultaneous uncoupling/desorption could be accomplished in cases involving more than one covalent attachment site per macromolecule. To our knowledge, this has never been demonstrated.

Several factors influenced our selection of a polymer as the foundation for the surface-based tether–probe device construction. Poly(vinylpyridine) is a nonconjugated hydrophobic polymer that has attracted attention

primarily as a charge-transfer catalyst with metal ions and as an electroactive polymer (7). For our purposes, however, the 4-vinylpyridine regioisomer (P4VP) presents sterically unhindered pyridyl nitrogen atoms in a ligand configuration which, upon aminoalkylation, should result in the introduction of a homogeneous class of photolytic bonds. Aminoalkylation also provides the chemical basis for extending outward with tether–probe construction. The photolytic site was incorporated at a defined position away from the biopolymer binding site by incorporation of a photoresistant spacer arm (*N*-ethylsuccinamyl-). The spacer arm was terminated with a suitable leaving group (*N*-hydroxysulfosuccinimide) to achieve covalent attachment of the completed tether–probe device to the most reactive nucleophiles available at the target biopolymer surface.

The model peptide chosen to evaluate tether–probe device functions, RETIESLSSEESITEYK (R1–K18), was selected partly because it is a bioactive peptide containing multiple potential phosphorylation sites (consensus sequence) that define important transition metal ion binding properties; the precise structural determinants and chemical basis for specific metal ion binding are yet to be defined (8). Second, this peptide is produced from human β -casein by proteolytic cleavage at the C-terminal side of Lys (K18), a cleavage site common to all peptide fragments produced by tryptic digestion during peptide mapping experiments. Thus, R1–K18 represents other peptides resulting from trypsin cleavage in that it contains two likely covalent attachment sites, the α -amino group of the N terminus and the ϵ -amino group of the lysyl residue at the C terminus.

The R1–K18 peptide was covalently bound to the tether–probe device illustrated in Figure 1. After the tether–probe device served its initial function as a tether, unbound R1–K18 peptide was removed and the second phase of the tether–probe function was initiated by laser irradiation. Laser-induced uncoupling and desorption of the covalently immobilized peptide were documented by time-of-flight mass analysis. These experiments revealed the release of a single macromolecular reaction product with an increase in mass equal to that predicted by a photolytic event occurring at the positively charged pyridinium nitrogen of each bound tether device (Figure 2, bottom). The mass increase observed for the bound peptide upon release (261 Da) was consistent with the formation of two reporter tails (127 Da each); one formed at each of the two available primary amino groups, as predicted. The tail mass of 127 Da was calculated without consideration of the probable addition of a hydrogen atom and/or proton. The precise mechanism of photo-induced bond cleavage at the pyridinium nitrogen is presently unknown. The 2–3-Da difference between the calculated and observed masses of the reporter tail may be due to a time delay or variation in the initial kinetic energy of the uncoupled peptide relative to that of the untethered internal mass standards. Regardless, the dissociated target macromolecule now has a chemically defined “memory” incorporated as a reflection of its interaction with the tether–probe devices on the biopolymer probe surface.

No mass shift was evident for peptides not covalently bound to the tether–probe surface. Laser-induced desorption of the peptide presented on an unmodified polymer platform (i.e., not covalently bound) revealed a mass (2088.2 Da) equal to the mass calculated for this 18-residue peptide (Figure 2, top). The internal molecular mass standard was observed to have the correct molecular mass (2903.0 Da) and is shown as a reference point in both profiles.

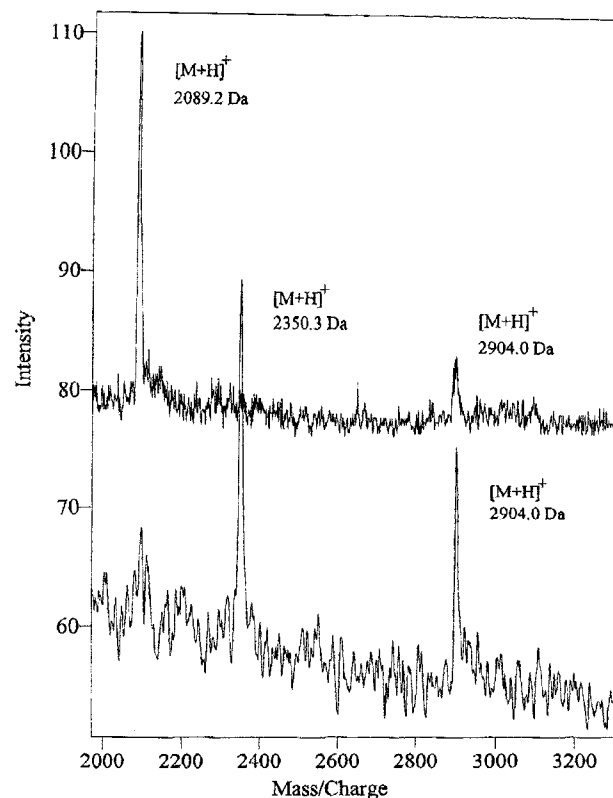


Figure 2. Laser-induced uncoupling, desorption, and mass analysis of the R1–K18 model peptide before (top) and after (bottom) interaction (i.e., covalent binding) and release from the biomolecular tether–probe device. Observed mass values for the protonated pseudomolecular ions $[M + H]^+$ are shown. The mass observed for the R1–K18 model peptide after photolytic cleavage of the covalent tether(s) (2349 Da) is consistent with the generation of two reporter tails resulting from the formation of two tethers followed by photolytic cleavage of each at the site depicted in Figure 1. Peaks labeled $[M + H]^+ = 2904$ Da represent the internal mass calibration standard.

The efficiency and sensitivity of laser-induced desorption/ionization time-of-flight techniques (subfemtomole or attomole detection sensitivity) enable a much more detailed inspection of specific versus nonspecific protein–surface interactions than was possible previously by any other method available. In the present case, it is clear that all traces of noncovalently bound biopolymer have been removed (see Figure 2).

Although we have observed peptides uncoupled from this surface by a photolytic cleavage event involving the urea-type amide bond formed at the site of biomolecule docking, experimental factors associated with the formation of this so-called “0-tail” species are mostly associated with the use of other protocols for protein introduction to the surface. Biopolymer uncoupling/desorption data collected with other new types of tether–probe surface chemistries indicate the formation of tailed species and 0-tail species (5). Thus, surface-enhanced photolabile attachment and release (SEPAR) procedures may be modified or accomplished with surface chemistries constructed to allow macromolecular uncoupling/desorption with or without reporter tails. This capability should accommodate a variety of specific purposes and applications.

The covalent docking of molecules, including biopolymers, in defined surface arrays facilitates remarkably the opportunity to address several broad categories of endeavor at the interface between chemistry and biology (1, 2, 9, 10). First, the ability to identify chemical and structural determinants of important molecular recogni-

tion events is improved through covalent tethering approaches now typified by library screening (e.g., 8, 9). Our efforts to design photolabile bonds enabling simultaneously both covalent ligand uncoupling and desorption with laser irradiation, as an alternative approach to chemical-based cleavage and separation strategies, address objectives and anticipate needs such as those outlined recently by others developing support-bound combinatorial libraries (9, 10). A covalent docking approach also increases greatly the overall sensitivity and efficiency of both chemical and enzymatic digestion methods for the elucidation of biopolymer structure *in situ* (e.g., protein sequence and post-translational modifications).

SEPAR probe functions to be derived from a surface-based *population* of these devices may be anticipated in two ways. First, these functions display a collective ability to covalently anchor a target biopolymer through not just one, but *several*, attachment sites. This would enable enzymatic cleavage of the anchored biopolymer between attachment sites to facilitate subsequent structural evaluations, including internal sequence. Another application under evaluation is the potential of these and other similar tether-probe devices to be used as a tool to evaluate relative alterations in biopolymer size/shape or surface architecture. The light-dependent introduction of one or more well-defined chemical reporter groups (i.e., tails) to the target biomolecule resulting from its laser-induced departure from the surface may allow biopolymer conformations or "footprints" to be probed. Finally, the SEPAR process should, in principle, be applicable to the investigation of a wide variety of molecules, small and large, including not only proteins and other biopolymers such as oligonucleotides and polysaccharides but also synthetic polymers.

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Supporting Information Available: Experimental methods for the tether-probe device and spacer arm (1

page). Ordering information is given on any current masthead page.

LITERATURE CITED

- (1) Hutchens, T. W., and Yip, T. T. (1993) New Desorption Strategies for the Mass Spectrometric Analysis of Macromolecules. *Rapid Commun. Mass Spectrom.* 7, 576–580.
- (2) Hutchens, T. W., and Yip, T. T. (1994) Affinity Mass Spectrometry: Probe Element Surfaces Enhanced for Affinity Capture (SEAC). *Protein Sci.* 3 (Suppl. 1), 81 (Abstract 170-S).
- (3) Hutchens, T. W., and Yip, T. T. (1994) Method and Apparatus for Desorption and Ionization of Analytes. International Patent (PCT) Publication No. WO94/28418.
- (4) Hutchens, T. W., Ching, J., and Yip, T. T. (1995) Design of Mass Spectrometric Probe Surfaces for Photolabile Attachment and Release. *Protein Sci.* 4 (Suppl. 2), 99 (Abstract 204-S).
- (5) Ching, J., Voivodov, K. I., and Hutchens, T. W. (1996) Surface Chemistries Enabling Photo-Induced Uncoupling/Desorption of Covalently Tethered Biomolecules. *J. Org. Chem.* 61, 3582–3583.
- (6) Voivodov, K. I., Ching, J., and Hutchens, T. W. (1996) Surface Arrays of Energy Absorbing Polymers Enabling Covalent Attachment of Biomolecules for Subsequent Laser-Induced Uncoupling/Desorption. *Tetrahedron Lett.* 37, 5669–5672.
- (7) Greg, B. A., and Heller, A. (1991) Redox Polymer Films Containing Enzymes. 1. A Redox-Conducting Epoxy Cement: Synthesis, Characterization, and Electrocatalytic Oxidation of Hydroquinone. *J. Phys. Chem.* 95, 5970–5975.
- (8) Hutchens, T. W., and Yip, T. T. (1992) Model protein surface domains for the investigation of metal ion-dependent macromolecular interactions and metal ion transfer. *Methods (San Diego)* 4, 79–96.
- (9) Egner, B. J., Langley, J. G., and Bradley, M. (1995) Solid Phase Chemistry: Direct Monitoring by Matrix-Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry. A Tool for Combinatorial Chemistry. *J. Org. Chem.* 60, 2652–2653.
- (10) Brummel, C. L., Lee, I. N. W., Zhou, Y., Benkovic, S. J., and Winograd, N. (1994) A Mass Spectrometric Solution to the Address Problem of Combinatorial Libraries. *Science* 264, 399–401.

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Supplemental Information

Preparation of poly(*N*-ethylamine-4-vinylpyridinium bromide) as the photolytic component of a biomolecular tether-probe device. Poly(4-vinylpyridine), P4VP, was derivatized as described previously (6) with the following modifications. P4VP (2 % cross-linked Reillex 402 Polymer with 50000 MW, Aldrich), 1 g, was stirred in 40 mL DMF (Aldrich) containing 2.5 g 2-bromoethylamine hydrobromide (Aldrich) under nitrogen for 72 h at 35°C. The *N*-ethylamine derivative was filtered, washed (twice with 20 vol each) with DMF, methanol, and acetone (Fisher Scientific). The modified polymer was dried under vacuum and stored desiccated.

Extension and addition of spacer arm functionality for coupling of the bioconjugate component. The spacer arm component of poly(*N*-ethylamine-4-vinylpyridinium bromide) (0.258 g) was extended and carboxylated by suspension in a 4 mL aqueous solution of succinic anhydride (1% w/v, pH 7) (Sigma) and incubated for 14 h at room temperature with magnetic stirring. The carboxylated derivative was filtered, washed (20 mL each) with 1 M NaCl, water, and acetone. The product was dried under vacuum and stored in a desiccator (room temperature). The derivatized P4VP was analyzed by laser desorption time-of-flight mass spectrometry and by solid-state ¹³C NMR (Spectral Data Services, Champaign, IL).

Termination of the spacer arm with a bioconjugate component for covalent attachment of the target biomolecule. The carboxylated poly(*N*-ethylamine-4-vinylpyridinium) (0.1 g) was suspended in 3 mL of 0.1 M 2-[*N*-morpholino]ethanesulfonic acid (MES), pH 6, containing 0.5 M NaCl. Then, 1.5 mg of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (Aldrich) and 3.3 mg of *N*-hydroxysulfosuccinimide sodium salt (Fluka) were added and allowed to incubate with stirring at room temperature for 30 min before the activated P4VP was separated by centrifugation (2 min at 4800 rpm, 20°C). The 18-residue model peptide (R1-K18) (representing the N-terminal iron-binding domain of human β -casein) (7), 3 mg, was added to the activated P4VP for coupling for 14 h at room temperature with shaking. Finally, covalently bound R1-K18 peptide was washed sequentially (minimum of 5 times with 5 mL each) with the pH 6 MES buffer, water, 1 M NaCl, water, 0.1 M ammonium citrate, pH 5.0, and 0.1 M ammonium bicarbonate, pH 7.8. The conjugated peptide was suction-dried and stored at 4-5°C.

Photolytic cleavage of biomolecule from the biomolecular tether-probe device for mass analysis. A stainless steel platform was pretreated by sonication/washing in 0.1 M nitric acid, water, acetonitrile/water (50% v/v), and methanol. The completed polymer surface material with peptide covalently coupled through the photolytic biomolecular tether-probe device, (10 μ L of a 50% w/v suspension), was washed with 0.001 N HCl in microcentrifuge tube, centrifuged, and resuspended in methanol. An aliquot (0.5 μ L) of the suspension was deposited on a sample presenting platform together with an aliquot of histidine rich-glycoprotein (HRG) peptide (5-10 pmol/mL) as an internal mass calibration standard. Finally, a saturated solution (65% acetonitrile, 30% methanol, and 5% water containing 0.1% TFA) of 3,5-dimethoxy-4-hydroxycinnamic acid was added as an energy absorbing molecule. The polymeric surfaces with peptide covalently bound were air-dried before insertion into vacuum (10^{-7} torr) for laser irradiation. The spectra shown represent data averaged from 5-25 pulses (3 ns) with a N₂ laser (337 nm) attenuated to a 5-15 μ J output. Mass analysis was performed by time-of-flight measurements in a modified Hewlett-Packard (Palo Alto, CA) Model 1700XP instrument operated at an overall accelerating voltage of 29.5 kV in the positive ion mode. Mass was calculated based on an internal calibrant (2903.0 Da) and by comparison with external calibrants (1046.2 Da, 1771.9 Da, and 2903.0 Da).

Stabilization of DNA Triple-Helix Formation by Appended Cationic Peptides

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We have investigated the impact of appended cationic peptides on the triplex-forming and thermal-stabilizing abilities of an oligonucleotide "third strand" using a homopurine-homopyrimidine 37-mer as the host duplex target. The family of appended cationic peptides studied here contains arginine, lysine, ornithine, and diaminobutyric acid, with the residues being linked through either their α -amino or side chain amino groups. On the basis of optical melting profiles, we find that peptides with different amino acid compositions, but with four positive charges in common, are able to enhance, in a similar manner, the triplex-forming capability of an oligonucleotide. We note the implications of these results for the rational design of third-strand probes.

Triplex-forming oligonucleotides (TFOs) bind to the major groove of double-stranded DNA through Hoogsteen or reverse Hoogsteen hydrogen bonding. Since binding to genomic DNA by a TFO can inhibit transcription, strategic targets for TFOs have included human disease-related genes such as *Ki-ras* (1), *HER-2/neu* (2), *c-erbB* (3), *c-myc* (4), *HIV-1* (5), and the interferon-responsive element (6). In other applications, artificial endonucleases have been created by attaching reactive groups to TFOs (7–9), with sequence-specific cleavage at a unique site in the human genome being mediated by a TFO (10). Targeted mutations also have been achieved by linking a TFO to a photoactivatable group, such as psoralen (11). In addition, TFOs connected to solid supports have been used to achieve affinity purification of double-stranded DNA (12).

To obtain greater TFO binding strengths and sequence specificities, various TFO design strategies have been employed (13, 14). These strategies include the use of modified nucleotides (ref 15 and references therein), the addition of intercalators (16–18) and/or polyamines (ref 19 and references therein) to solution, as well as the direct covalent linking of an intercalator (20, 21), a hydrophobic group (22), or a cross-linking agent (23) to the TFO. We (24) and others (25, 26) have demonstrated that attaching a polyamine to the terminus of a TFO facilitates triplex formation. In other studies, we have shown that a cationic peptide, appended to an antisense strand, will enhance duplex formation when challenged with either DNA (27) or RNA (28) single-stranded targets.

In this report, we show that cationic peptides attached to oligonucleotides also favor triplex formation. We focus here on appended tetrapeptides which possess four positive charges to facilitate comparison with a previous study in which we showed that polyamines with four positive charges enhance triplex formation (24). These tetracationic appended tetrapeptides are shown in the

Table 1. Thermal Stabilities of the Different Cationic Peptide–TFO Conjugates^a

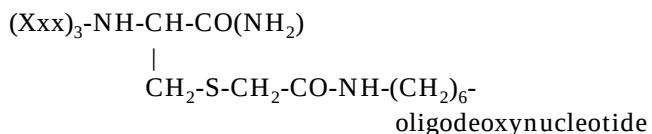
3'	CTCTT TCTTC TCTCT TCTCC TTTCT TCCTC TTTCT CT
5'	GAGAA AGAAG AGAGA AGAGG AAAGA AGGAG AAAGA GA
5'	(Xxx) ₃ -Cys-linker-TC TCTCT TCTCC TTTCT TCCT

Xxx, structure	T _m of triplex
arginine, NH-CH[(CH ₂) ₃ NHC=NH ₂ ⁺ (NH ₂)]-CO	38
lysine, NH-CH[(CH ₂) ₄ NH ₃ ⁺]-CO	40
ornithine, NH-CH[(CH ₂) ₃ NH ₃ ⁺]-CO	39
diaminobutyric acid, NH-CH[(CH ₂) ₂ NH ₃ ⁺]-CO	39
ϵ -lysine, NH-(CH ₂) ₄ -CH(NH ₃ ⁺)-CO	39
δ -ornithine, NH-(CH ₂) ₃ -CH(NH ₃ ⁺)-CO	38

^a Generic TFO is shown in alignment with the duplex target.

first column of Table 1. Note that three of the four charges are associated with basic amino acids, while the fourth charge results from the α -amino group at the N-terminus of the peptide. Further note that the appended cationic peptides contain different positively charged amino acids, with the residues being connected via either their α -amino or side chain amino group. Conjugation to the oligonucleotide is achieved via the side chain of a cysteine residue.

We separately synthesized with a 5'-amino group the TFO strand shown in Table 1, as well as the six amidated tetrapeptides listed. On the basis of the results of previous work (29, 30), we chose to incorporate the 5-methylated analog of cytosine into the TFO to enhance its triplex-forming capacity. After purification, the TFO and the peptide were coupled as previously described (27) using a bifunctional reagent *N*-[(iodoacetyl)oxy]succinimide, to yield the conjugate



where Xxx is a basic amino acid. The TFO–peptide conjugate then was mixed in a 1:1 molar ratio with a solution of the target 37-mer duplex shown at the top of Table 1. Triplex formation was assessed by melting profiles at 260 nm in 10 mM sodium phosphate and 100 mM sodium chloride (pH 6.5) based on well-established characteristic melting behaviors defined in more exten-

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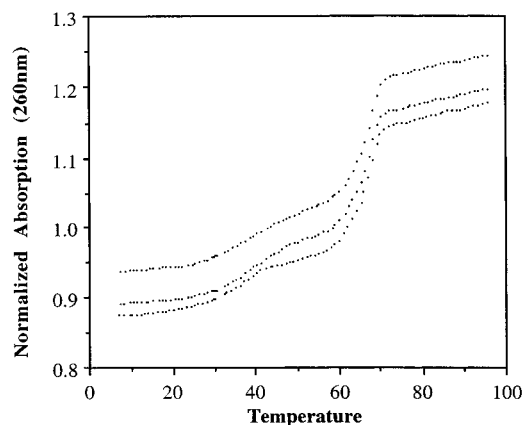


Figure 1. UV melting of the triarginine tetrapeptide-oligonucleotide conjugates. Curves from top to bottom are 21-mer, 18-mer, and 15-mer TFO.

sive studies that used a range of additional techniques (26–30). Simply stated, under appropriate conditions, triplex formation results in the appearance within a melting profile of a second transition at a temperature below the transition of the target duplex. Each sample for melting was prepared by taking a solution containing all three strands at 1 μ M and heating to 90 °C for 15 min, followed by slow cooling to room temperature. The resulting solution was then kept at 4 °C overnight to facilitate proper annealing, followed by heating to 100 °C at a rate of 0.5 °C/min. Melting temperatures were determined from the first derivative of the resulting absorbance versus temperature profiles with an accuracy of ± 0.5 °C.

Inspection of the melting profiles shown in Figure 1 reveals the peptide-conjugated TFOs to exhibit two transitions, the first corresponding to the triplex-to-duplex conversion and the second reflecting the duplex-to-single strand transition at 67 °C. Similar biphasic melting behavior was observed for all other TFO-peptide conjugates studied. The assignment of the second transition to duplex disruption was based on the melting profile of the “free” 37-mer duplex in the absence of the TFO, while the triplex nature of the first transition was confirmed by a negative circular dichroism band at 217 nm (24). Significantly, the “control” unappended third strand does not form a thermally stable triplex under these conditions. Thus, the appended oligopeptide induces the formation of a triplex that otherwise would not form under the conditions of this study. We previously observed a similar influence of appended polyamines (24).

Inspection of the T_m data listed in Table 1 reveals that, for TFO-peptide conjugates with the same oligonucleotide chain length and peptide charge, we observe little difference between the thermal stabilities of the triplexes induced by the various peptide conjugates, with the T_m values clustering between 38 and 40 °C, compared with 42 °C for the corresponding spermine conjugate (24). Thus, the topology/amino acid composition of the appended cationic tetrapeptide is less critical to the triplex-forming capacity, as judged by the thermal stability of the resulting complex, than is the net charge of the peptide portion of the TFO, which is +4 for all the conjugates in Table 1.

To assess the influence of oligonucleotide chain length on TFO capacity, we synthesized a family of conjugates, with a constant peptide component (arginine) and with nucleic acid chain lengths of 15, 18, and 21 nucleotides. The T_m data in Table 2 reveal the triplex-forming capacities of these three conjugates to be nearly independent of chain length. This observation demonstrates

Table 2. Thermal Stabilities of Various Lengths of Peptide and TFO^a

3'	CTCTT	TCTTC	TCTCT	TCTCC	TTTCT	TCCTC	TTTCT	CT
5'	GAGAA	AGAAG	AGAGA	AGAGG	AAAGA	AGGAG	AAAGA	GA
5'	δ -Orn ₃ -Cys-linker-TC							
5'	δ -Orn ₈ -Cys-linker-TC							
5'	Arg ₃ -Cys-linker-TC							
5'	Arg ₃ -Cys-linker-CTCT							
5'	Arg ₃ -Cys-linker-T							

TFO	T_m of triplex
Arg ₃ -21-mer	38
Arg ₃ -18-mer	38
Arg ₃ -15-mer	37
δ -Orn ₃ -21-mer	39
δ -Orn ₈ -21-mer	40

^a The structures of the TFOs are shown in alignment with the duplex target.

that, under the conditions employed here, an oligonucleotide as short as a 15-mer is sufficient for triplex formation when a cationic peptide is attached, even though triplex formation is not observed for the unconjugated 15-mer. Furthermore, the near constancy of the triplex T_m with increasing chain length of the appended TFO suggests that the system is approaching polymer-like behavior in that the thermal stability has become independent of chain length.

We also have assessed preliminarily the influence of peptide chain length on triplex thermal stability by studying two conjugates with constant nucleic acid lengths (21-mers), but varied peptide lengths. As shown by the data in Table 2, we find that in going from three to eight δ -ornithine residues in the peptide little, if any, change in T_m is observed. Thus, for this residue and its linkage to a 21-mer oligonucleotide, the peptide chain length does not impact on the triplex-inducing capacity or the thermal stability of the resulting triplex. Additional studies are required to assess the generality of this observation.

In conclusion, attaching a cationic peptide to the terminus of an oligonucleotide can enhance its capacity to form a triplex with a target duplex. From the limited series of conjugates evaluated in this work, it appears that the nature and length of the cationic peptide is less important than is the charge that the appended peptide imparts to the TFO. We also have shown that it is possible to use shorter oligonucleotides as the triplex-forming strand, if they are appended to cationic peptides. It should be noted that appended cationic peptides may impart additional desirable properties to TFOs, such as enhanced cellular uptake (31) and nuclear localization (32). Recently, free basic oligopeptides have been used to stabilize triplex formation with sequence dependent activity being observed (33). Clearly, future studies on TFOs with varying charge levels which are challenged by a broader range of duplex targets will be needed to define more completely the role of charge on the triplex-forming properties, including sequence specificities.

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LITERATURE CITED

- (1) Mayfield, C., Squibb, M., and Miller, D. (1994) Inhibition of nuclear protein binding to the human Ki-ras promoter by triplex-forming oligonucleotides. *Biochemistry* 33, 3358–3363.

- (2) Ebbinghaus, S. W., Gee, J. E., Rodu, B., Mayfield, C. A., Sanders, G., and Miller, D. M. (1993) Triplex formation inhibits HER-2/neu transcription in vitro. *J. Clin. Invest.* 92, 2433–2439.
- (3) Okada, T., Yamaguchi, K., and Yamshita, J. (1994) Triplex-forming oligonucleotide binding represses transcription of the human c-erbB gene in glioma. *Growth Factors* 11, 259–270.
- (4) Kim, H.-G., and Miller, D. M. (1995) Inhibition of in vitro transcription by a triplex-forming oligonucleotide targeted to human c-myc P2 promoter. *Biochemistry* 34, 8165–8171.
- (5) McShan, W. M., Rossen, R. D., Laughter, A. H., Trial, J., Kessler, D. J., Zengdegui, J. G., Hogan, M. E., and Orson, F. M. (1992) Inhibition of transcription of HIV-1 in infected human cells by oligodeoxynucleotides designed to form DNA triple helices. *J. Biol. Chem.* 267, 5712–5721.
- (6) Roy, C. (1993) Inhibition of gene transcription by purine rich triplex forming oligodeoxyribonucleotides. *Nucleic Acids Res.* 21, 2845–2852.
- (7) Povsic, T. J., and Dervan, P. B. (1990) Sequence-specific alkylation of double-helical DNA by oligonucleotide-directed triple-helix formation. *J. Am. Chem. Soc.* 112, 9428–9430.
- (8) Strobel, S. A., Moser, H. E., and Dervan, P. B. (1988) Double-strand cleavage of genomic DNA at a single site by triple-helix formation. *J. Am. Chem. Soc.* 110, 7927–7929.
- (9) Francois, J.-C., Saison-Behmoaras, T., Barbier, C., Chassignol, M., Thuong, N. T., and Helene, C. (1989) Sequence-specific recognition and cleavage of duplex DNA via triple-helix formation by oligonucleotides covalently linked to a phenanthroline-copper chelate. *Proc. Natl. Acad. Sci. U.S.A.* 86, 9702–9706.
- (10) Strobel, S. A., Doucette-Stamm, L. A., Riba, L., Housman, D. E., and Dervan, P. B. (1991) Site-specific cleavage of human chromosome 4 mediated by triple-helix formation. *Science* 254, 1639–1642.
- (11) Wang, G., Levy, D. D., Seidman, M. M., and Glazer, P. M. (1995) Targeted mutagenesis in mammalian cells mediated by intracellular triple helix formation. *Mol. Cell Biol.* 15, 1759–1768.
- (12) Ito, T., Smith, C. L., and Cantor, C. R. (1992) Sequence-specific DNA purification by triplex affinity capture. *Proc. Natl. Acad. Sci. U.S.A.* 89, 495–498.
- (13) Chubb, J. M., and Hogan, M. E. (1992) Human therapeutics based on triple helix technology. *Trends Biotechnol.* 10, 132–136.
- (14) Thuong, N. T., and Helene, C. (1993) Sequence-specific recognition and modification of double-helical DNA by oligonucleotides. *Angew. Chem., Int. Ed. Engl.* 32, 666–690.
- (15) Priestley, E. S., and Dervan, P. B. (1995) Sequence composition effects on the energetics of triplex helix formation by oligonucleotides containing a designed mimic of protonated cytosine. *J. Am. Chem. Soc.* 117, 4761–4765.
- (16) Mergny, J. L., Duval-Valentin, G., Nguyen, C. H., Perrouault, L., Faucon, B., Rougee, M., Montenay-Garestier, T., Bisagni, E., and Helene, C. (1992) Triple helix-specific ligands. *Science* 256, 1681–1684.
- (17) Wilson, W. D., Tanious, F. A., Mizan, S., Yao, S., Kiselyov, A. S., Zon, G., and Strekowski, L. (1993) DNA triple-helix specific intercalators as antigene enhancers: unfused aromatic cations. *Biochemistry* 32, 10614–10621.
- (18) Pilch, D. S., Martin, M.-T., Nguyen, C. H., Sun, J.-S., Bisagni, E., Garestier, T., and Helene, C. (1993) Self-association and DNA-binding properties of two triple helix-specific ligands: Comparison of a benzo[*e*]pyridoindole and a benzo[*γ*]pyridoindole. *J. Am. Chem. Soc.* 115, 9942–9951.
- (19) Musso, M., and Van Dyke, M. W. (1995) Polyamine effects on purine-purine-pyrimidine triple helix formation by phosphodiester and phosphorothioate oligodeoxyribonucleotides. *Nucleic Acids Res.* 23, 2320–2327.
- (20) Fox, K. R. (1994) Formation of DNA triple helices incorporating blocks of G.GC and T.AT triplets using short acridine-linked oligonucleotides. *Nucleic Acids Res.* 22, 2016–2021.
- (21) Mouscadet, J.-F., Ketterle, C., Goulaouic, H., Carreau, S., Subra, F., Bret, M. L., and Auclair, C. (1994) Triple helix formation with short oligonucleotide-intercalator conjugates matching the HIV-1 U3 LTR end sequence. *Biochemistry* 33, 4187–4196.
- (22) Gryaznov, S. M., and Lloyd, D. H. (1993) Modulation of oligonucleotide duplex and triplex stability via hydrophobic interactions. *Nucleic Acids Res.* 21, 5909–5915.
- (23) Grigoriev, M., Praseuth, D., Guieysse, A. L., Robin, P., Thuong, N. T., Helene, C., and Harel-Bellan, A. (1993) Inhibition of gene expression by triple helix-directed DNA cross-linking at specific sites. *Proc. Natl. Acad. Sci. U.S.A.* 90, 3501–3505.
- (24) Tung, C.-H., Breslauer, K. J., and Stein, S. (1993) Polyamine-linked oligonucleotides for DNA triple helix formation. *Nucleic Acids Res.* 21, 5489–5494.
- (25) Barawkar, D. A., Kumar, V. A., and Ganesh, K. N. (1994) Triplex formation at physiological pH by oligonucleotides incorporating 5-Me-dC-(N4-spermine) *Biochem. Biophys. Res. Commun.* 205, 1665–1670.
- (26) Nara, H., One, A., and Matsuda, A. (1995) Nucleosides and Nucleotides. 135. DNA duplex and triplex formation and resistance to nucleolytic degradation of oligodeoxynucleotides containing syn-norspermidine at the 5-position of 2'-deoxyuridine. *Bioconjugate Chem.* 6, 54–61.
- (27) Zhu, T., Tung, C.-H., Breslauer, K. J., Dickerhof, W. A., and Stein, S. (1993) Preparation and physical properties of conjugates of oligodeoxynucleotides with poly(δ)ornithine peptides. *Antisense Res. Dev.* 3, 349–356.
- (28) Zhu, T., Pooyan, S., Wei, Z., Leibowitz, M. J., and Stein, S. (1996) Gel shift assay: Demonstration of enhanced binding of oligo(δ)-L-ornithine-oligodeoxynucleotide conjugates to complementary DNA and RNA. *Antisense Res. Dev.* 6, 69–74.
- (29) Povsic, T. J., and Dervan, P. B. (1989) Triple helix formation by oligonucleotides on DNA extended to the physiological pH range. *J. Am. Chem. Soc.* 111, 3059–3061.
- (30) Plum, G. E., Park, Y. W., Singleton, S., Dervan, P. B., and Breslauer, K. J. (1990) Thermodynamic characterization of the stability and the melting behavior of a DNA triplex: a spectroscopic and calorimetric study. *Proc. Natl. Acad. Sci. U.S.A.* 87, 9436–9440.
- (31) Leonetti, J.-P., Degols, G., and LeBleu, B. (1990) Biological activity of oligonucleotide-poly(L-lysine) conjugates: mechanism of cell uptake. *Bioconjugate Chem.* 1, 149–153.
- (32) Eritja, R., Pons, A., Esceller, M., Giralt, E., and Albericio, F. (1991) Synthesis of defined peptide-oligonucleotide hybrids containing a nuclear transport signal sequence. *Tetrahedron* 47, 4113–4120.
- (33) Potaman, V. N., and Sinden, R. R. (1995) Stabilization of triple-helical nucleic acids by basic oligopeptides. *Biochemistry* 34, 14885–14892.

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Bispecific Antibody Mediated Targeting of *nido*-Carboranes to Human Colon Carcinoma Cells

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Boron neutron capture therapy, a binary form of cancer treatment, has the potential to deliver potent cytotoxic radiation to tumor cells with minimal collateral damage to normal tissues if methods for the selective accretion of elevated concentrations of boron-10 in tumor can be developed. In this regard, a monoclonal antibody with dual specificity, for both anionic boron cluster compounds (*nido*-carboranes) and a tumor-associated antigen (carcinoembryonic antigen, CEA), was produced. The specific binding of a *nido*-carborane to CEA-expressing tumor cells was achieved using this bispecific antibody. The ability of this bispecific antibody to concentrate selectively at tumor sites *in vivo* has also been demonstrated, thus suggesting its potential for sequestering boron-rich compounds in tumors.

Boron neutron capture therapy (BNCT) is a binary approach to cancer therapy that is based upon the capture of biologically innocuous low-energy neutrons by the nonradioactive isotope boron-10, which results in the formation of energetic, localized, and extremely cytotoxic He and Li nuclei. Successful cancer treatment with BNCT requires the development of boron-containing species that concentrate selectively in tumors, to minimize radiation damage to normal cells. Boron-substituted compounds that may be able to distinguish between normal and neoplastic tissues include boronated porphyrins, nucleosides, amino acids, liposomes, and monoclonal antibodies (MAb) (1).

Immunoproteins have been proposed as a general class of boron-delivery agents. Although we (2–4) and others (5, 6) have successfully synthesized boron-rich immunconjugates that retain immunoreactivity, the *in vivo* delivery of these conjugates to tumor has been poor, primarily as a result of significant nonspecific uptake in liver. This problem is exacerbated by the requirement that each immunoprotein molecule be conjugated to a large number of boron-10 atoms (on the order of 10³) to attain clinically useful tumor-boron concentrations (1). Our own experience has highlighted some of the problems in this area while offering potential solutions to the problem of obtaining high-substitution levels of boron conjugated to antibody molecules (7).

Bispecific antibodies (BsMAb) offer an alternative means for site-directed boron-targeting. This approach to immunoprotein-mediated BNCT requires the creation of an antibody with affinity for both a tumor-associated antigen and a hapten found on a boron-rich macromolecule (Figure 1). The BsMAb is initially pretargeted to

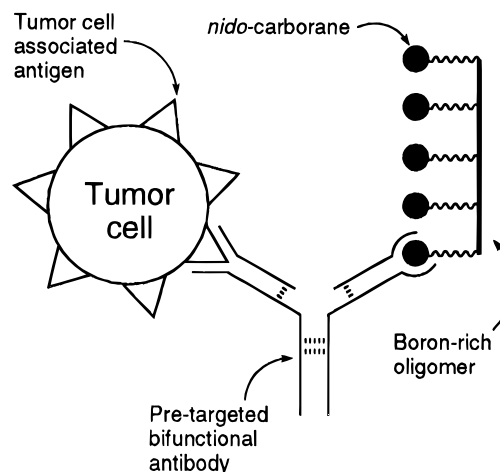


Figure 1. Schematic diagram demonstrating the principle of pretargeting with BsMAbs. The antibody is first pretargeted to the tumor by its reaction with a tumor cell surface antigen. The other antigen combining site is then in position to react with a boron-rich macromolecule that is subsequently administered.

tumor sites by virtue of its tumor antigen specificity. A boron-rich macromolecule is then administered and is retained in the tumor by virtue of its binding to the anti-hapten specificity provided by the tumor-bound BsMAb. The use of BsMAbs for the delivery of diagnostic and therapeutic agents has received heightened interest because of the potential for improving tumor-to-normal tissue differentiation in agent distribution (8, 9). A distinct advantage of BsMAbs when the goal is to deliver elevated quantities of a cytotoxic drug is that modification of antibody caused by direct conjugation methods can be avoided. This feature is of paramount importance in BNCT, since the need to localize high concentrations of boron requires that each antibody molecule deliver hundreds of boron atoms.

We initially turned our attention to the creation of an antibody with binding specificity for derivatives of the readily accessible anionic boron-rich cage, *nido*-7,8-dicarbado-decahydroundecaborate(−1) (**1**, Figure 2). Much of our previous work in this area has utilized derivatives of this *nido*-carborane cage, a stable negatively charged

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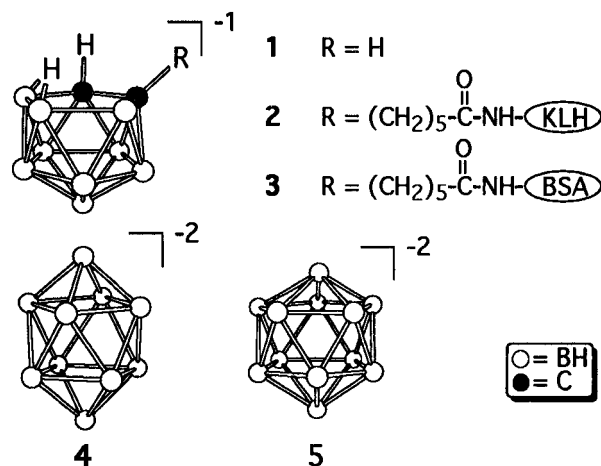


Figure 2. Structures of *nido*-carboranes and *closo*-polyhedral borane anions used in the production and characterization of the *anti-nido*-carborane MABs.

ion composed of nine B–H and two C–H vertices (along with a B–H–B bridging hydrogen) with the structure of an icosahedral fragment. Importantly, this cage structure has been fashioned into designed boron-rich macromolecules, including oligomeric peptides (10, 11) and phosphate diesters (12, 13). We previously reported the production and characterization of two *anti-nido*-carborane MABs, designated HAW101 and HAW102, which were generated by immunization with a *nido*-carborane attached to keyhole limpet hemocyanin (KLH) carrier protein (14) (*nido*-KLH; 2, Figure 2). The affinity constants of HAW101 and HAW102 with a *nido*-carborane attached to bovine serum albumin (*nido*-BSA; 3, Figure 2) were determined by surface plasmon resonance technology to be 1.9×10^9 and 6.8×10^8 M⁻¹, respectively. The HAW101 MAB was selected for further study because of its higher affinity. Its fine specificity was characterized by examining a panel of boron hapten containing compounds in competition assay (14). The parent unsubstituted *nido*-carborane 1 (Figure 2) effectively inhibited HAW101 binding to *nido*-BSA at nanomolar concentrations, demonstrating that the antibody has specificity for the *nido*-carborane cage structure rather than for side chains used for its attachment to protein carriers. Anionic *closo*-polyhedral boranes (4 and 5, Figure 2) were not effective inhibitors, indicating the importance of the open *nido*-carborane cage structure for recognition by the HAW101. By contrast, Liu et al. have produced MABs reactive with anionic *closo*-polyhedral boranes for the development of BsMABs for use in BNCT (15).

We selected the carcinoembryonic antigen (CEA) as the target antigen for the tumor specificity component of BsMABs also possessing *nido*-carborane reactivity. The CEA is a 180 kDa glycoprotein that is produced by a variety of epithelial-derived cancers including colorectal, lung, ovarian, and breast carcinomas (16). MABs against CEA have been under extensive clinical evaluation for their ability to target radionuclides and cytotoxic agents for immunodiagnosis and therapy of cancer (17). Other groups have developed CEA-targeted BsMABs for the tumor-selective delivery of metal chelates (18, 19) or toxins (20). In our studies, the high-affinity *anti*-CEA MAB, T84.66, which has been shown to localize tumors in human patients, was selected for the preparation of BsMABs (21).

Among the various methods for the preparation of BsMABs, we chose to create quadromas via the fusion of two hybridomas each synthesizing MABs with different

specificities (22). Hybridomas producing the T84.66 *anti*-CEA and HAW101 *anti-nido*-carborane MABs were labeled with contrasting lipophilic chromophores and fused with polyethylene glycol.¹ Fused cells, or “quadromas”, were separated by fluorescence-activated cell sorting, and the selected cells were grown in culture and cloned. Supernatants from clones were first tested for reactivity with CEA and *nido*-BSA in separate enzyme immunoassays. In three different cell-sorted fusions, 4–6% (15 total) of the wells positive for cell growth produced supernatants that reacted in both assays. The majority (41–76%) of the clones showed reactivity with only one antigen. A bridging enzyme immunoassay, which assayed for the ability of BsMABs to link a biotinylated *anti*-CEA idiotype antibody to wells coated with *nido*-BSA, was used to identify BsMAB activity in clones reactive with both antigens. Only 3 of the 15 clones positive in both individual antigen assays showed reactivity in the bridging assay (Figure 3A). The reason for the high percentage of clones that displayed no reactivity in the bridging assay is unclear, but it may be due to low levels of BsMAB production or to clones that still contained a mixture of the parental hybridomas. The former explanation seems most likely since antibody purified by protein G chromatography from two of the clones had very modest bispecific activity, suggesting that the parental antibodies were the predominant species synthesized by these clones. Nonetheless, antibody from the third clone, H11, contained substantial BsMAB activity.

BsMABs are monovalent toward a given specificity, while their respective parents are bivalent. Since antibody avidity is influenced by the antibody valency, it was important to establish that the BsMAB, like the *anti*-CEA MAB parent, was capable of localizing to tumor sites *in vivo*. To remove the parental MABs produced by the H11 clone, further purification of BsMAB was carried out by ion exchange chromatography,² as the isoelectric points for the T84.66 and HAW101 parental MABs were quite different (approximately 5.4 and 7.0, respectively). For localization experiments *in vivo*, radioiodinated BsMAB and *anti*-CEA MAB were separately injected into athymic nude mice bearing LS-174T colon carcinoma xenografts. At various time points after injection, tumor as well as normal reference tissues (blood, liver, spleen, kidney, lung) were removed and the antibody uptake was measured. At 24 h the uptake of the BsMAB in tumor was similar to that of the parental *anti*-CEA MAB (Figure 3B). With time, the amount of radioactivity in tumors from animals injected with the BsMAB decreased at a faster rate than that observed in the group administered the *anti*-CEA MAB. The biodistribution and clearance patterns among the reference tissues were similar for both groups at the various time points. Thus, the BsMAB retains the tumor-localizing properties of the parental

¹ The T84.66 *anti*-CEA and HAW101 *anti-nido*-carborane hybridomas were labeled with fluorescent cell linkers PKH26-GL (red) and PKH2-GL (green), respectively, according to the manufacturer's instructions (Zynaxis Cell Sciences, Malvern, PA). The optimal staining concentrations of PKH26-GL and PKH2-GL were 20 and 8 μ M, respectively. After culturing overnight, the labeled hybridoma cells were fused with PEG and then cultured overnight. Prior to cell sorting, dead cells were removed by centrifugation over ficoll-hypaque. Cells were sorted under sterile conditions using a FACS IV. The gating window contained 1.0–1.1% of total cells from fused preparations as compared to 0.2% from a mixture of stained hybridoma cells that were not fused but otherwise treated in the same way. Three sorts were carried out on two separate fusions producing 3000–5000 cells/sort (0.3–0.7% of cells in gated area). Sorted cells were placed in bulk culture for 11–13 days prior to cloning at limiting dilution.

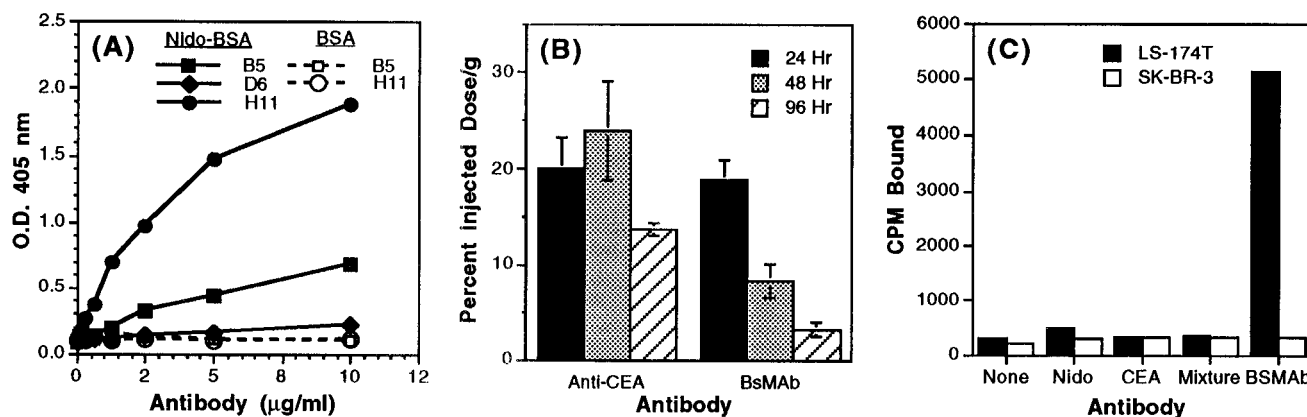


Figure 3. (A) BsMAb activity detected in bridging enzyme immunoassay. Antibody produced by quadromas was purified from ascites by protein G chromatography and then reacted with microtiter wells coated with *nido*-BSA followed by biotinylated *anti*-CEA idiotype MAb. Binding of the anti-idiotype MAb was detected with a streptavidin–enzyme conjugate. The reactions of clones B5, D6, and H11 with *nido*-BSA and of clones B5 and H11 with BSA as negative control are depicted. (B) Localization of radioiodinated BsMAb in LS-174T human colon carcinoma xenografts. Error bars = SEM. (C) Binding of radioiodinated *nido*-carborane ([¹²⁵I]-**6**) to the surface of human colon carcinoma cells mediated by BsMAb. The results shown are the mean of duplicate tubes that were within 5% of one another. This experiment was repeated a second time with identical results.

anti-tumor MAb, although its residence time appears to be somewhat altered. The latter should not have a mitigating effect on the pretargeting properties of the BsMAb since the timing of the administration of the boron-rich macromolecule can be adjusted to the time of optimal antibody accretion in the tumor.

With these data in hand, experiments were performed to determine if the BsMAb could target *nido*-carboranes to cancer cells expressing CEA. CEA-positive human colon carcinoma cells (LS-174T) were sequentially exposed to the ion exchange-purified BsMAb followed by a radioiodinated *nido*-carborane compound [¹²⁵I]-**6** (Figure 4).³ The LS-174T colon carcinoma cells showed enhanced binding of [¹²⁵I]-**6** when previously exposed to the BsMAb as compared to the binding obtained after exposure of the cells to a physical mixture of the respective parental antibodies (Figure 3C). By contrast, only background levels of [¹²⁵I]-**6** were bound to the CEA-negative human breast carcinoma cell line, SK-BR-3, following incubation with either the BsMAb or a mixture of the parental

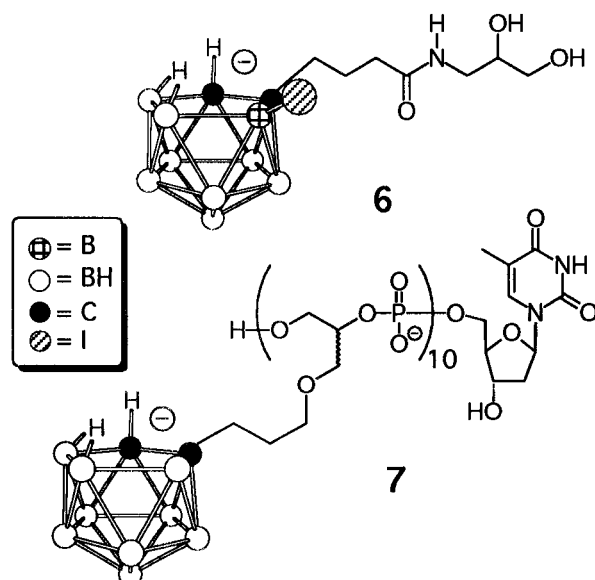


Figure 4. Immunoreactive iodinated (**6**) and oligomeric (**7**) *nido*-carborane derivatives.

antibodies. Of note, the nonspecific binding of [¹²⁵I]-**6** by both tissue-cultured tumor cell lines was very low. These experiments demonstrate for the first time that a BsMAb possessing reactivity with both a human tumor antigen and a boron-containing cluster species can elicit site-directed boron labeling of the appropriate antigen-positive cells.

We have shown that BsMAbs prepared with an *anti*-*nido*-carborane specificity can selectively localize a boron-rich compound to a tumor cell surface. It is anticipated that the high-level boron labeling of tumor cells required *in vivo* for successful BNCT will be accomplished with the use of boron-rich polymers containing appropriate haptenic groups available for specific reaction with BsMAbs. The feasibility of this approach is suggested by the binding of the HAW101 MAb to oligomeric carboranyl phosphate diesters such as the recently synthesized compound **7** (Figure 4). This compound, which contains 90 boron atoms, is bound by the *anti*-*nido*-carborane MAb (**14**) substantially better than any monomeric carborane studied to date (23), and we believe that oligomers of greater length will exhibit similar properties. The availability of BsMAbs with *nido*-carborane binding capacity, coupled with our ability to

² BsMAb was purified from ascites by sequential chromatography over protein G and ABx ion exchanger (J. T. Baker, Phillipsburg, NJ). For ABx chromatography, antibody was equilibrated with 0.01 M MES, pH 6.0, and applied to the ABx using the same buffer. After elution of the parental *anti*-CEA MAb, bound bispecific antibody was eluted using a gradient consisting of 0.01 M NaOAc, pH 6.0, start buffer, and 1.0 M NaOAc, pH 6.0, limit buffer. Parental *anti*-CEA MAb could not be detected in the purified BsMAb preparation as determined by isoelectric focusing. The immunoreactivity of the labeled (Iodogen method) T84.66 *anti*-CEA MAb and BsMAb as determined by binding to solid phase CEA was 80–100% as compared to 4–5% binding to an irrelevant antigen affinity gel. Seven days after LS-174T inoculation into athymic nude mice (NCR-nu), 10 μCi of antibody (1 μg) was administered by tail vein injection. Five to six mice were used for each antibody group at each time point. Animals were euthanized at various time points after injection, and blood, tumor, and normal tissues were removed for determination of radioactivity. All biodistribution studies were carried out under approval of the Institutional Research Animal Care Committee.

³ To tubes precoated with 5% BSA, 2 × 10⁶ LS-174T (CEA-positive human colon carcinoma cell line) or SK-BR-3 (CEA-negative human breast carcinoma cell line) cells were added (in 50 μL of PBS + 0.02% NaN₃) followed by 33.3 pmol of antibody in an equal volume. After incubation for 1 h at 0 °C, unbound antibody was removed and the cells were exposed to *nido*-carborane **6** (Figure 4; 0.03 pmol), which had been radioiodinated by the chloramine-T procedure (2).

design and assemble boron-rich macromolecules that are reactive with these BsMAbs, suggests that this approach holds great potential for the specific *in vivo* loading of tumors with boron. To increase the yield, purity, and targeting properties of the BsMAbs, we have recently cloned the genes for the HAW101 MAb for use in the preparation of engineered BsMAbs.

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LITERATURE CITED

- (1) Hawthorne, M. F. (1993) The role of chemistry in the development of boron neutron capture therapy of cancer. *Angew. Chem., Int. Ed. Engl.* 32, 950.
- (2) Varadarajan, A., Sharkey, R. M., Goldenberg, D. M., and Hawthorne, M. F. (1991) Conjugation of phenyl isothiocyanate derivatives of carborane to antitumor antibody and *in vivo* localization of conjugates in nude mice. *Bioconjugate Chem.* 2, 102.
- (3) Paxton, R. J., Beatty, B. G., Varadarajan, A., and Hawthorne, M. F. (1992) Carboranyl peptide-antibody conjugates for neutron-capture therapy: preparation, characterization, and *in vivo* evaluation. *Bioconjugate Chem.* 3, 241.
- (4) Chen, C.-J., Kane, R. R., Primus, F. J., Szalai, G., Hawthorne, M. F., and Shively, J. E. (1994) Synthesis and characterization of oligomeric nido-carboranyl phosphate diester conjugates to antibody and antibody fragments for potential use in boron neutron capture therapy of solid tumors. *Bioconjugate Chem.* 5, 557.
- (5) Ferro, V. A., Morris, J. H., and Stimson, W. H. (1995) A novel method for boronating antibodies without loss of immunoreactivity, for use in neutron capture therapy. *Drug Des. Discovery* 13, 13.
- (6) Barth, R. F., Adams, D. M., Soloway, A. H., Alam, F., and Darby, M. V. (1994) Boronated starburst dendrimer monoclonal antibody immunoconjugates—evaluation as a potential delivery system for neutron capture therapy. *Bioconjugate Chem.* 5, 58.
- (7) Hawthorne, M. F. (1991) Biochemical applications of boron cluster chemistry. *Pure Appl. Chem.* 63, 327.
- (8) Nolan, O., and O'Kennedy, R. (1990) Bifunctional antibodies: concept, production, and applications. *Biochim. Biophys. Acta* 1040, 1.
- (9) Yuan, F., Baxter, L. T., and Jain, R. K. (1991) Pharmacokinetic analysis of two-step approaches using bifunctional and enzyme-conjugated antibodies. *Cancer Res.* 51, 3119.
- (10) Varadarajan, A., and Hawthorne, M. F. (1991) Novel carboranyl amino acids and peptides: reagents for antibody modification and subsequent neutron-capture studies. *Bioconjugate Chem.* 2, 102.
- (11) Kane, R. R., Pak, R. H., and Hawthorne, M. F. (1993) Solution-phase segment synthesis of boron-rich peptides. *J. Org. Chem.* 58, 991.
- (12) Kane, R. R., Lee, C. S., Drechsel, K., and Hawthorne, M. F. (1993) Solution-phase synthesis of boron-rich oligophosphates. *J. Org. Chem.* 58, 3227.
- (13) Kane, R. R., Drechsel, K., and Hawthorne, M. F. (1993) Automated syntheses of carborane-derived homogeneous oligophosphates. *J. Am. Chem. Soc.* 115, 8853.
- (14) Pak, R. H., Primus, F. J., Rickard-Dickson, K. J., Ng, L. L., Kane, R. R., and Hawthorne, M. F. (1995) Preparation and properties of nido-carborane-specific monoclonal antibodies for potential use in boron neutron capture therapy (BNCT) of cancer. *Proc. Natl. Acad. Sci. U.S.A.* 92, 6986.
- (15) Liu, L., Barth, R. F., Adams, D. M., Soloway, A. H., and Reisfeld, R. A. (1995) Bispecific antibodies as targeting agents for boron neutron capture therapy of brain tumors. *J. Hematother.* 4, 477.
- (16) Sikorska, H., Shuster, J., and Gold, P. (1988) Clinical applications of carcinoembryonic antigen. *Cancer Detect. Prev.* 12, 321.
- (17) Goldenberg, D. M. (1991) Imaging and therapy of gastrointestinal cancers with radiolabeled antibodies. *Am. J. Gastroenterol.* 86, 1392.
- (18) Phelps, J. L., Beilder, D. E., Jue, R. A., Unger, B. W., and Johnson, M. J. (1990) Expression and characterization of a chimeric bifunctional antibody with therapeutic applications. *J. Immunol.* 145 (4), 1200.
- (19) Stickney, D. R., Anderson, L. D., Slater, J. B., Ahem, C. N., Kirk, G. A., Schweighardt, S. A., and Frincke, J. M. (1991) Bifunctional antibody: a binary radiopharmaceutical delivery system for imaging colorectal carcinoma. *Cancer Res.* 51, 6650.
- (20) Corvalan, J. R. F., Smith, W., and Gore, V. A. (1988) Tumour therapy with vinca alkaloids targeted by a hybrid-hybrid monoclonal antibody recognising both CEA and vinca alkaloids. *Int. J. Cancer, Suppl.* 2, 22.
- (21) Beatty, J. D., Williams, L. E., Yamauchi, D., Morton, B. A., Hill, L. R., Beatty, B. G., Paxton, R. J., Merchant, B., and Shively, J. E. (1990) Presurgical imaging with indium-labeled anti-carcinoembryonic antigen antibody for colon cancer staging. *Cancer Res.* 50, 922s.
- (22) Karawajew, L., Mischeel, B., Behrsing, O., and Gaestel, M. (1987) Bispecific antibody-producing hybrid hybridomas selected by a fluorescence activated cell sorter. *J. Immunol. Methods* 96, 265.
- (23) Kane, R. R., Guan, L., Kim, Y. S., Rickard-Dickson, K. J., Primus, F. J., and Hawthorne, M. F. Unpublished results.

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6-Fluoropyridoxal Polymer Conjugates: Novel ^{19}F pH Indicators for Magnetic Resonance Spectroscopy

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Fluorinated macromolecular probes (6-fluoropyridoxal–polymer conjugates) have been synthesized and characterized as potential pH indicators for magnetic resonance spectroscopy and imaging applications. The ^{19}F pH sensor 2-fluoro-5-hydroxy-3-(hydroxymethyl)-6-methyl-4-pyridinecarboxaldehyde (6-fluoropyridoxal; **2**) has been conjugated to carrier molecules (polyamino dextran, polylysine, and albumin) by reductive alkylation for enhanced vascular retention and tissue targeting. The pH indicator polymer conjugates were purified by exhaustive dialysis and isolated in good yields (66–84%). The 6-fluoropyridoxal–polymer conjugates exhibit excellent ^{19}F pH sensitivity and pK_a suitable for *in vivo* studies. The potential application of these polymeric indicators has been demonstrated in whole blood. These novel macromolecular pH probes offer a new approach for studying tissue physiology.

The important role of pH in developmental processes has stimulated the need for better methods of measuring pH in cells, organelles, and microenvironments (1, 2). Deviations of tissue pH and the pH gradient are found to accompany many disease states and may cause dysfunction. In addition, the efficacy of various therapeutic modalities in cancer therapy, notably chemotherapy and hyperthermia, is strongly influenced by pH. Thus, methods of measuring pH could have far-reaching applications in biomedicine, both for physiology research and in the clinic. Consequently, a variety of new methods have been introduced for measurement of cellular pH employing exogenous pH reporter molecules (indicators). Several specific attributes apply to an ideal pH indicator. It should be nontoxic and nonmetabolizable, and the pK_a should be of the same order of magnitude as the pH to be determined. For NMR indicators the chemical shift difference between protonated and deprotonated species should be large to resolve small pH differences (ideally >1 ppm/pH unit) and the pH-dependent chemical shift should be independent of other physiological parameters (e.g., ionic strength, temperature, and plasma proteins). Preferably, the ionized forms are in fast exchange yielding a single narrow resonance.

Traditional techniques of pH measurement are invasive (microelectrodes) or are limited to surface tissue (fluorescence). Methods based on positron emission tomography (PET) or nuclear magnetic resonance (NMR) are much less invasive and more suitable for human studies. PET of radiolabeled compounds (e.g., [^{14}C]CO₂ and [^{14}C]dimethylloxazolidinedione) provides an estimate of aggregate pH (intra- and extracellular compartments), and the measurements may be difficult to interpret (3). In contrast, NMR may provide simultaneous intra- and extracellular pH determinations and can be entirely

noninvasive. ^{31}P NMR is relatively appealing since pH is determined on the basis of endogenous metabolites (relative chemical shift of inorganic phosphate and phosphocreatine) (4). However, measurements of pH using the chemical shift of inorganic phosphate (P_i) require caution because the pK_a is affected by ionic strength, protein solvation, and temperature (5). In addition, ^{31}P NMR suffers from low metabolite concentrations and the crowded overlapping resonances (5). Intracellular pH has also been reported on the basis of the chemical shift of histidine observed by ^1H NMR, but this suffers severely from the intense water signal and multiple overlapping metabolite resonances (6).

Thus, a variety of NMR pH reporter molecules have been synthesized as exogenous indicators possessing enhanced spectral characteristics. Although ^1H (7), ^{13}C (8), and ^{31}P (9) nuclei have been exploited, the NMR characteristics of the ^{19}F nucleus are particularly attractive. ^{19}F is 100% naturally abundant and has NMR sensitivity approaching that of protons. There is a large chemical shift range with minimal background signal *in vivo*. Various fluorinated NMR indicators have been reported; however, early molecules were poorly sensitive to changes in pH (10) and others lacked the appropriate pK_a (11). Recently several new classes of ^{19}F indicators have been reported with significantly improved properties: these include derivatives of fluoroaniline (12), fluoroaminophenol (13), difluoroisobutyric acid (14), and 6-fluoropyridoxal [6-FPOL (**1**); a vitamin B₆ analog] (15). The measurements of pH using NMR indicators generally require ^{19}F concentrations in the range of a millimole (~1 mM).

We are actively pursuing the development of ^{19}F MR agents (15–19) for mapping dynamic functional parameters such as pH, vascular properties, and perfusion with NMR. We have previously demonstrated the use and reliability of 6-FPOL (**1**) as a ^{19}F pH indicator in whole blood (15) and the perfused heart (20). However, for *in vivo* applications it is desirable that the pH indicator undergo tissue-specific or, at least, compartment-specific distribution to reduce the effective dose of the indicator and achieve high temporal and spatial resolution. Enhanced vascular retention and tissue targeting have been

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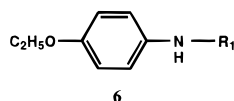
proposed on the basis of conjugating small molecules such as drugs (21, 22) and imaging agents (16, 18, 23–25) to polymers. Indeed, this concept was demonstrated for fluorescent pH indicators (e.g., SNARF-1) coupled to dextran (26). We have now adopted this approach to couple fluorinated NMR pH indicators to carrier molecules. We have previously conjugated a prototype indicator 3-fluorosalicylaldehyde (3-FSA) to polymers and observed an unusual reversal in the titration curve of the ^{19}F signal for 3-FSA conjugates (17). Here, we have investigated the feasibility of conjugating the highly sensitive ^{19}F pH sensing agents “fluorinated vitamin B₆ analogs” to carrier polymers without compromising the pH sensing characteristics. The macromolecular pH indicators could be useful in assessment of disease states such as tumors, ischemia, stroke, and regions of inflammation.

The fluorinated vitamin B₆ analogs are ideally suited for attachment to carrier macromolecules due to their high ^{19}F chemical shift sensitivity to pH (~10 ppm), useful pK_a , and multiple choices of functional groups in the pyridoxine ring for conjugation. The change in ^{19}F chemical shift in vitamin B₆ analog (**1**) with pH is mainly due to the protonation/deprotonation of the 3-position hydroxy group, which influences the electronic environment around fluorine at the 6-position (the effect being more pronounced due to the para location). Assuming that this structure sensitivity relationship holds, the remaining three positions (2, 4, and 5) in the pyridoxine ring could be exploited for conjugation to polymers without sacrificing the pH sensing capability of the pH indicator.

As our first design option, we have conjugated 2-fluoro-5-hydroxy-3-(hydroxymethyl)-6-methyl-pyridinecarboxaldehyde (6-fluoropyridoxal; **2**) to carrier polymers through the aldehyde group at the 4-position in the pyridoxine ring by reductive alkylation. 6-Fluoropyridoxal (**2**) was obtained by selective oxidation (27) of **1** with MnO_2 . We

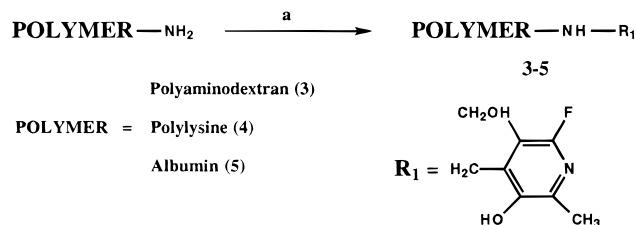


1, 2
R = CH_2OH (**1**)
R = CHO (**2**)



have used three different classes of polymers, namely polyaminodextran (40K) (16), polylysine (98K), and naturally occurring bovine serum albumin. Reaction of 6-fluoropyridoxal (6-FPAL; **2**) with polymer^{1,2} produced a Schiff base, which on subsequent sodium borohydride reduction yielded 6-FPAL–polymer conjugates (**3–5**) (Scheme 1). Attempts to maximize the degree of labeling of polylysine with 6-FPAL (2–3 M excess) resulted in a polymer conjugate that was insoluble in water. By controlling the amounts of reagent (6-FPAL; 0.5 mol) in a reaction mixture, water soluble 6-FPAL–polylysine conjugate (**4**) was obtained. We attribute this to more

Scheme 1^a



^a0.1 M NaHCO_3 , 6-fluoropyridoxal (**2**); NaBH_4 .

Table 1. Characteristics of pH Indicators (1, 2) and Conjugates (3–7)

pH indicator	pH	^{19}F NMR ^a (δ) (ppm)	pH sensitivity ($\Delta\delta$) (ppm)	pK_a	yield (%)
1	2.00	−9.85	9.76	7.9	40
	11.35	−19.61			
2	2.00	−8.65	10.65	8.2	26
	11.40	−19.30			
3	2.00	−8.92	10.52	6.6	72 ^b
	11.60	−19.44			
4	2.00	−8.87	10.88	5.9	62 ^b
	12.00	−19.75			
5	2.00	−8.79	10.35	6.8	84 ^b
	11.00	−19.14			
6	2.00	−9.08	10.30	9.3 ^c	65
	11.50	−19.38			
7	2.00	−8.76	10.72	ND ^d	80 ^b
	11.50	−19.48			

^a Chemical shift w.r.t. NaTFA (0 ppm). ^bBased on amount (mg) of derivatized product recovered in coupling procedure. ^c pK_a of compound **6** was determined in 50% water and dimethyl sulfoxide solution. ^d Precise pK_a of compound **7** could not be determined because this material came out of solution at neutral pH. ND, not determined.

extensive labeling which reduced the availability of free amino groups and hence water solubility. We did not encounter this problem with polyamino dextran and albumin. The pH indicator conjugates (**3–5**) were purified by exhaustive dialysis and isolated in good yields (62–84%, Table 1). A control experiment showed that dialysis successfully removed the reaction byproducts. 6-FPAL (**1**) was dissolved in 0.1 M NaHCO_3 and stirred overnight followed by addition of NaBH_4 . The mixture was stirred for another 4 h and subjected to exhaustive dialysis. Lyophilization of the 6-FPAL dialyzate yielded no visible product. Purity and integrity of the polymers were assessed using gel permeation chromatography (GPC). GPC analyses showed a smooth profile similar to the starting macromolecules without formation of additional low or high molecular weight products. The IR spectra of conjugates (**3–5**) differed from those of starting materials, though there were no distinct well-resolved bands.

The pH indicators (**1, 2**) and conjugates (**3–5**) exhibit a single sharp ^{19}F NMR signal in acid and base as shown in Figure 1. Typical line widths for 6-FPAL (**2**) and the conjugate **3** are 23 and 55 Hz, respectively. We have determined the relative ^{19}F NMR sensitivity of conjugate **4** compared to 6-FPOL (**1**). Relative sensitivity (16) is

² Conjugation Procedure. Reaction of 6-FPAL (**2**) with polymers: the appropriate polymer (polyamino dextran, polylysine, or albumin) (100–200 mg) was dissolved in 0.1 M NaHCO_3 (40–50 mL). 6-FPAL (**2**) was added (0.5–3 M excess) to the polymer solution at room temperature with stirring and allowed to proceed overnight. NaBH_4 (2 M excess) was added to the reaction mixture, and stirring was continued for another 4 h. The conjugate was extensively dialyzed using MWCO 6–8K and 12–14K dialysis tubings (sequentially) against water to remove low molecular weight materials (excess reagents). The solution was lyophilized to yield 6-FPAL–polymer conjugates (**3–5**).

¹ Reagents and Materials. Polyamino dextran (40K) was synthesized as previously reported (16). Polylysine (98K) and bovine serum albumin (BSA) were purchased from Sigma Chemical Co. S-Ethyl trifluoroacetate, sodium trifluoroacetate (CF_3COONa), phenitidine, and sodium bicarbonate (NaHCO_3) were purchased from Aldrich Chemical Co. Spectra Por dialysis tubings (Fisher Scientific) with molecular weight cutoffs (MWCO) of 6–8K and 12–14K were used. All other reagents and solvents were of reagent grade unless otherwise specified.

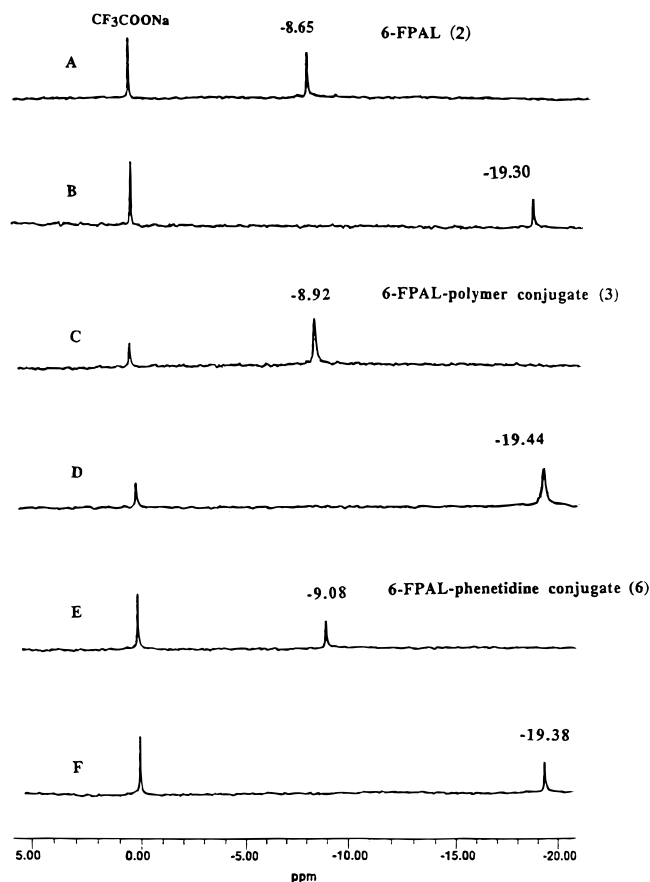


Figure 1. 282.3-MHz ^{19}F NMR spectra of 6-FPAL (2) and conjugates 3 and 6 in acid (A, C, and E) and base (B, D, and F), respectively, with CF_3COONa as an external reference. Conjugates 3 and 6 show a single sharp ^{19}F signal and similar pH sensitivities to the native indicator 6-FPAL (2).

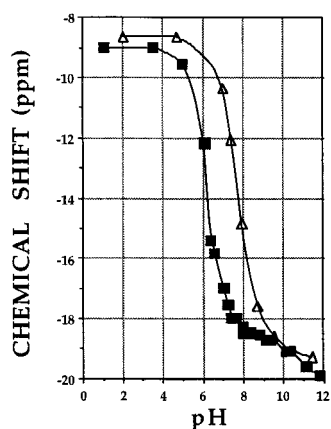


Figure 2. ^{19}F chemical shift of 6-FPAL (2, Δ) and 6-FPAL-polymer conjugate (4, ■) vs pH in water. The pK_a for conjugate is shifted toward the physiological range.

defined here as the number of milligrams of conjugate 4 needed to give the same intensity of ^{19}F NMR signal as that obtained with 1 mg of 6-FPAL (1). The NMR sensitivity provides an estimate of the average degree of molar substitution and *in vivo* NMR detectability of derivatized polymer. The average molar substitution ratio was found to be ~ 25 6-FPAL per carrier molecule in the case of polylysine (4). We have assessed the chemical shift sensitivity and pK_a using ^{19}F NMR spectroscopy for each of the fluorinated vitamin B₆ analogs (1, 2) and conjugates (3–5) over the pH range 2.0–12.0 (Figure 2 and Table 1) using sodium trifluoroacetate (CF_3COONa) as an external reference.^{3,4} The 6-FPAL–

polymer conjugates (3–5) have chemical shift sensitivity in the range 10.35–10.88 ppm, which is similar to that of the starting molecule 6-FPAL (2) (Table 1). This indicates that conjugation at the 4-position of the pyridoxine ring to different classes of polymers with molecular weights in the range 40–98K does not perturb the chemical shift range of the ^{19}F signal in response to pH. The pK_a was found to move toward the physiological range (5.9–6.8) for conjugates, from a more basic starting value ($\text{pK}_a \sim 8.0$) for 6-FPAL (2) (Figure 2 and Table 1).

The ability to conjugate the pH sensor to carrier molecules is an attractive approach in terms of targeting specific tissues, enhancing vascular retention, and differentiating intra- and extracellular pH. However, it is important to investigate the possible effect on pH sensing characteristics of the indicator molecule (6-FPAL, 2) upon coupling to the polymer, *i.e.*, the effect of large molecular size, electric charge, shape, and lypophilicity/hydrophilicity. Thus, 6-FPAL (2) was coupled to a small molecule (*p*-phenetidine)⁵ using linkages similar to those used for polymers, and the pH properties of the 6-FPAL–phenetidine conjugate (6) were investigated. The pH response obtained for 6 was very similar to that of 3–5 (Figure 1; Table 1). These results suggest that polymers do not exert any unusual effect on the pH sensing capability of the indicator (6-FPAL, 1) as compared with monomer (Figure 1; Table 1). However, ^{19}F signal line broadening was observed at certain pH values for the polymer conjugates. The ^{19}F chemical shift sensitivities to pH for 6-FPAL (2) and all of the conjugates (both polymeric and monomeric; 3–6) were found to be very similar, contrasting an unusual reversal observed for conjugates of a prototype indicator 3-fluorosalicylaldehyde (17).

To demonstrate the potential use of these polymeric indicators, we have evaluated the new conjugate (4) in

³ ^{19}F NMR Spectroscopy (15). ^{19}F chemical shift titration curves were established for the samples in distilled water. Sodium trifluoroacetate (CF_3COONa) was used as an external chemical shift reference standard (0 ppm). Typically, 6-FPAL (1), 6-FPAL (2), and 6-FPAL conjugates (2–20 mg) were dissolved in water (500 μL). The pH of the solution in water was altered with aliquots of diluted HCl or NaOH solutions. The pH of the sample was measured in the NMR tube using a combination pH electrode (Wilma, Buena, NJ) attached to a pH meter (Corning 220, Sudbury, U.K.). ^{19}F NMR detection sensitivity was estimated using 6-FPAL (1) solution as an internal standard with relative sensitivity per milligram of material. Shimming was performed on the water signal, and ^{19}F NMR spectra were typically obtained with 4K data points across $\pm 10,000$ Hz. Data were apodized with a 10–20 Hz exponential line broadening prior to Fourier transformation.

⁴ Equipment and Physical Measurements. ^{19}F NMR spectroscopy used a 7 T (282.3 MHz, ^{19}F) Oxford vertical bore magnet under control of a Tecmag console. Infrared spectra (IR) were recorded in KBr on a Mattson Galaxy (2000) FT-IR spectrometer. Gel permeation chromatography was carried out using two Waters ultrahydrogel columns (500 and 250 Å) at 25 °C. Phosphate buffer (2 M) was used for elution at a flow rate of 0.8 mL/min with a Waters 484 (UV-vis) and 410 differential refractometer.

⁵ Reaction of 6-FPAL (2) with Phenetidine. *p*-Phenetidine (55.5 mg, 0.4 M) dissolved in 1 N HCl (1 mL) was added to an aqueous solution (50 mL) of 6-FPAL (2, 75 mg, 0.4 M). The reaction mixture was stirred and brought to pH 8.5 by adding 1 N NaOH. The solution was left in an ice bath for 2 h, and the solid separated out (27) was filtered (40 mg): MS (m/z , FAB) 305 ($M + 1$)⁺. The Schiff base (40 mg, 0.13 M) was dissolved in 20 mL of MeOH followed by sodium borohydride (5 mg, 0.13 M) addition. The resulting mixture was stirred for 4 h. The methanol was evaporated and the residue chromatographed on silica gel to obtain 6 by elution with ethyl acetate and chloroform (26 mg): MS (m/z , FAB) 307 ($M + 1$)⁺.

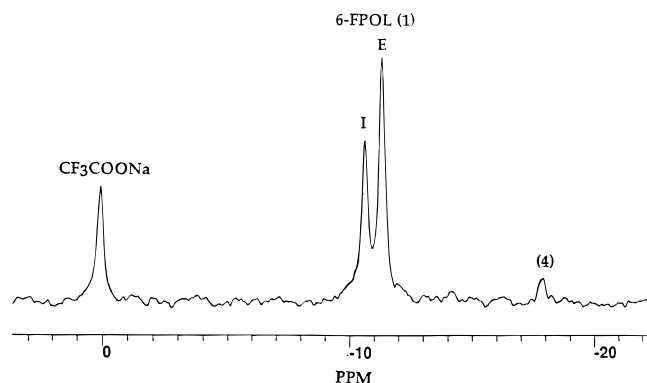
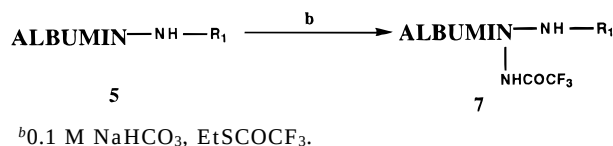


Figure 3. 282.3-MHz ^{19}F NMR spectrum of 6-FPAL-polylysine (**4**) together with 6-FPOL (**1**) in whole blood (pH 7.36) with CF_3COONa as an external reference. Conjugate **4** shows a single resonance (δ -17.89) corresponding to extracellular pH (7.4), while 6-FPOL (**1**) shows two signals (δ -10.7 and -11.4) representing the pH from intracellular (pH 7.18) and extracellular (pH 7.48) compartments. This spectrum was acquired in 20 min from 500 μL of whole pig blood.

Scheme 2^b



whole blood and compared its behavior with that of 6-FPOL (**1**). Conjugate **4** together with **1** were added to whole pig blood, and a capillary of CF_3COONa solution was included as an external chemical shift reference. **4** showed a single signal, whereas 6-FPOL (**1**) showed two signals attributed to intra- and extracellular compartments. When the external pH was altered by addition of acid or base, ^{19}F signals from the indicators moved as expected. Figure 3 shows a representative spectrum of conjugate **4** and **1** using whole blood with extracellular pH 7.36 (electrode). The signals from 6-FPOL (**1**) occurred at δ -10.7 (pH_i 7.18) and δ -11.4 (pH_e 7.48), while conjugate **4** resonated at δ -17.89 (pH 7.4). Thus, the chemical shift indicated essentially the same pH as observed with the electrode and the extracellular resonance of 6-FPOL (**1**). Indeed, centrifugation of the blood sample showed **4** in the supernatant only, verifying restricted distribution (extracellular) of this polymeric indicator (**4**).

Hitherto, we have used an external chemical shift reference (CF_3COONa) to determine relative chemical shift of the pH indicators and hence pH. As a further advance we have now incorporated an intramolecular reference signal ($-\text{COCF}_3$) on the 6-FPAL-albumin conjugate (**5**) to provide direct chemical shift quantitation. This was achieved by selective trifluoroacetylation⁶ of remaining amino groups on **5** with *S*-ethyl trifluorothioacetate (Scheme 2) (**16**). This indicates that there is still scope to enhance the labeling efficiency of 6-FPAL conjugation reaction (*i.e.*, residual free NH_2 groups). The fluorine signal from the intramolecular reference ($-\text{COCF}_3$) was found to be insensitive to changes in pH

⁶ Reaction of Conjugate (**5**) with *S*-ethyl trifluorothioacetate. **5** was dissolved in 0.1 M NaHCO_3 followed by *S*-ethyl trifluorothioacetate addition (5 M excess), while maintaining the pH at ~ 8.0 . The solution was stirred overnight. The reaction mixture was extensively dialyzed using MWCO 6-8K and 12-14K dialysis tubings (sequentially) against water to remove low molecular weight materials. The solution was lyophilized to yield TFA-[6-FPAL-albumin] conjugate (**7**).

and, thus, the assessment of chemical shifts for the resulting conjugate (**7**) no longer required an external reference. The ^{19}F chemical shift sensitivity to pH for **7** was similar to that of the starting conjugate (**5**), suggesting that incorporation of an additional reference fluorine on the indicator may not have any effect on its pH sensing properties. In addition, the stronger CF_3 signal may facilitate ^{19}F MR imaging to reveal the distribution of the molecules and hence "localize" the pH values.

In summary, we have introduced a new class of fluorinated macromolecules for assessment of pH *in vivo* using ^{19}F MRS/MRI. Fluorinated polymeric pH probes were synthesized by conjugation of a ^{19}F pH sensor to carrier polymers. 6-FPAL-polymer conjugates exhibit excellent ^{19}F pH sensitivity and pK_a suitable for assessment of pH *in vivo* with ^{19}F MRI/MRS. The novel macromolecular pH probes open a new line of approach for studying tissue physiology. We believe this approach could serve the dual purpose of restricting the pH sensing molecules to specific biological compartments (*e.g.*, extracellular) and targeting specific tissue (*e.g.*, tumors).

Further ongoing investigations are aimed at (i) application of different chemical linkages or conjugation of the pH sensing moiety at different positions (in the pyridoxine) to polymers for enhancing efficiency of labeling and reduction in ^{19}F signal line width, (ii) exploring active targeting of lesions (labeling antibodies and peptides), as opposed to nonspecific targeting provided by leakage of polymers, and (iii) demonstrating the *in vivo* utility of these fluorinated probes. Besides pH mapping, an immediate extension of the methodology will be to develop other ^{19}F physiological markers, *e.g.*, pO_2 , temperature, redox state, cation concentration, and perfusion using ^{19}F MRI/MRS.

ACKNOWLEDGMENT

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LITERATURE CITED

- (1) Nuccitelli, R. (1982) *Intracellular pH: Its measurement, regulation and utilization in cellular function* (R. Nuccitelli and D. W. Deamer, Eds.) p 161, Liss, New York.
- (2) Roos, A., and Boron, W. P. (1981) Intracellular pH. *Physiol. Rev.* 61, 296.
- (3) Kearfott, K. J., and Junck, L. (1983) C-11 Dimethyloxazolidinedione (DMO): biodistribution, radiation absorbed dose, and potential for PET measurement of regional brain pH: concise communication. *J. Nucl. Med.* 24, 805.
- (4) Moon, R. B., and Richards, J. H. (1973) Determination of intracellular pH by ^{31}P magnetic resonance. *J. Biol. Chem.* 248, 7276.
- (5) Robitaille, P. M. L., Robitaille, P. A., Brown, G. G. J., and Brown, G. G. (1991) An analysis of the pH-dependent chemical-shift behavior of phosphorous-containing metabolites. *J. Magn. Reson.* 92, 73.
- (6) Brown, F. F., Campbell, I. D., Kuchel, P. W., and Rabenstein, D. C. (1977) Human erythrocyte metabolism studies by ^1H spin echo NMR. *FEBS Lett.* 82, 12.
- (7) Gil, M. S., Cruz, F., Cerdan, S., and Ballesteros, P. (1992) Imidazol-1-ylalkanoate esters and their corresponding acids. A novel series of extrinsic ^1H NMR probes for intracellular pH. *Biomed. Chem. Lett.* 2, 1717.
- (8) Chacko, V. P., and Weiss, R. G. (1993) Intracellular pH determination by ^{13}C -NMR spectroscopy. *Am. J. Physiol.* 264, C755.

- (9) Gillies, R. J., Liu, Z., and Bhujwalla, Z. (1994) ^{31}P -MRS measurements of extracellular pH of tumors using 3-amino-propylphosphonate. *Am. J. Physiol.* 267, C195.
- (10) Deutsch, C. J., and Taylor, J. (1987) *NMR spectroscopy of cells and organism* (R. Gupta, Ed.) p 55, CRC Press, Boca Raton, FL.
- (11) Deutsch, C. J., and Taylor, J. S. (1989) New class of ^{19}F pH indicators: fluoroanilines. *Biophys. J.* 55, 799.
- (12) Frenzel, T., Kobler, S., Bauer, H., Niedballa, U., and Weinmann, H. J. (1994) Noninvasive *in vivo* pH measurements using a fluorinated pH probe and fluorine-19 magnetic resonance spectroscopy. *Invest. Radiol.* 29, S220.
- (13) Rhee, C. K., Levy, L. A., and London, R. E. (1995) Fluorinated o-aminophenol derivatives for measurement of intracellular pH. *Bioconjugate Chem.* 6, 77.
- (14) Bental, M., and Deutsch, C. (1994) ^{19}F -NMR study of primary human T lymphocyte activation: effects of mitogen on intracellular pH. *Am. J. Physiol.* 266, C541.
- (15) Mehta, V. D., Kulkarni, P. V., Mason, R. P., Constantinescu, A., Sivasubramanian, A., Goomer, N., and Antich, P. P. (1994) 6-Fluoropyridoxol: a novel probe of cellular pH using ^{19}F NMR spectroscopy. *FEBS Lett.* 349, 234.
- (16) Mehta, V. D., Kulkarni, P. V., Rajur, S. B., Mason, R. P., Babcock, E. E., Constantinescu, A., and Antich, P. P. (1992) Novel molecular probes for ^{19}F magnetic resonance imaging; synthesis and characterization of fluorinated polymers. *Biomed. Chem. Lett.* 2, 527.
- (17) Mehta, V. D., Kulkarni, P. V., Mason, R. P., and Antich, P. P. (1993) Fluorinated macromolecular probes for non-invasive assessment of pH by magnetic resonance spectroscopy. *Biomed. Chem. Lett.* 3, 187.
- (18) Mehta, V. D., Kulkarni, P. V., Mason, R. P., Constantinescu, A., and Antich, P. P. (1994) Fluorinated proteins as potential ^{19}F magnetic resonance imaging and spectroscopy agents. *Bioconjugate Chem.* 5, 257.
- (19) Mehta, V. D., Mason, R. P., Kulkarni, P. V., Lea, P., Constantinescu, A., and Antich, P. P. (1995) ^{19}F MR characterization of fluorinated proteins and relaxation rate enhancement with Gd-DTPA for faster imaging, *Proceedings of the International Symposium on Chemists View of Imaging Centers*, p 305, Plenum Press, New York.
- (20) Hunjan, S., Mason, R. P., Mehta, V. D., Kulkarni, P. V., Sivasubramanian, A., Arora, V., and Antich, P. P. (1995) Intracellular pH measurement in the perfused heart with 6-fluoropyridoxol using ^{19}F NMR. *Proceedings, Third Meeting of the Society of Magnetic Resonance*, Nice, France, Vol. 3, p 1933.
- (21) Maeda, H., Seymour, L. W., and Miyamoto, Y. (1992) Conjugates of anticancer agents and polymers: advantages of macromolecular therapeutics *in vivo*. *Bioconjugate Chem.* 3, 351.
- (22) Sezaki, H., and Hashida, M. (1984) *CRC Crit. Rev. Ther. Drug Carrier Syst.* 1, 1.
- (23) Sinn, H., Schrenk, H. H., Friedrich, E. A., Schilling, U., and Maier-Borst, W. (1990) Design compounds having an enhanced tumor uptake, using serum albumin as a carrier. Part I. *Nucl. Med. Biol.* 17, 819.
- (24) Ogan, M. D., Schmiedl, U., Moseley, M. E., Grodd, W., Paajanen, H., and Brasch, R. C. (1987) Albumin labeled with Gd-DTPA an intravascular contrast-enhancing agent for magnetic resonance blood pool imaging: preparation and characterization. *Invest. Radiol.* 22, 665.
- (25) Weissleder, R., Lee, A. S., and Fischman, A. J. (1991) Polyclonal human immunoglobulin G labeled with polymeric iron oxide: antibody MR imaging. *Radiology* 181, 245.
- (26) Thiebaut, F., Currier, S. J., Whitaker, J., Haughland, R. P., Gottesman, M. M., Pastan, I., and Willingham, M. C. (1990) Activity of the multidrug transporter results in alkalization of the cytosol: measurement of cytosolic pH by microinjection of a pH-sensitive dye. *J. Histochem. Cytochem.* 38, 685.
- (27) Korytnyk, W., and Srivastava, S. C. (1973) Synthesis and physicochemical and biological properties of 6-halogen-substituted vitamin B₆ analogs. *J. Med. Chem.* 16, 638.

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Antisense Sequence-Directed Cross-Linking of DNA Oligonucleotides by Mitomycin C

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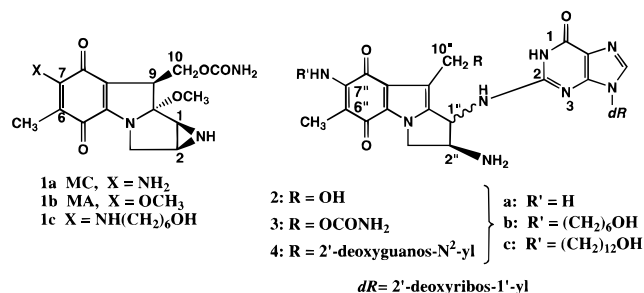
Oligodeoxyribonucleotides (ODNs) conjugated with mitomycin C (MC) via $(-\text{CH}_2-)_n$ tethers of different lengths ($n = 6, 12$) to their terminal 5'-phosphate were synthesized, and their interaction with target complementary single-stranded DNA oligonucleotides was investigated. MC, a clinically used natural anticancer drug, is known to act as a bioreductive alkylating agent of duplex DNA with a remarkable preference for 5'-d(CG) sequences. The usual enzymatic bioreductive techniques known to trigger MC to alkylate DNA were employed in the reaction between the MC-oligonucleotide conjugates and their targets radiolabeled by ^{32}P at their 5'-phosphate. A slow-moving radiolabeled product, detected by polyacrylamide gel electrophoresis using phosphorimaging techniques, was obtained in 15–25% yield with complementary DNA as target. Formation of these products was dependent upon complementary duplex formation. Evidence is presented that the DNA target is alkylated by the mitomycin C moiety of the ODN conjugate at the 2-amino group of a guanine base. These findings suggest that the MC-ODN conjugates may be useful specific inhibitors of cellular or viral gene expression. To our knowledge this is the first report on ODN conjugates of a reductively activated drug of known therapeutic value.

The attachment of DNA-reactive agents to oligodeoxyribonucleotides (ODNs)¹ has led to conjugate molecules capable of inhibiting biosynthesis of specific proteins (1, 2) and blocking of expression of specific genes (3, 4). Examples of such agents are the photoactivatable compounds psoralen (5) and *p*-azidoacetophenone (6), chemically reactive DNA cross-linkers of the N-mustard class (7), α -halocarbonyl compounds (8), and photoactivatable and reductively activatable naphthoquinone derivatives (9). The specificity of biochemical action of a reactive agent-ODN conjugate is based on targeting the reaction to a specific nucleic acid sequence by base complementarity between the target and the agent-ODN conjugate. One of the cross-linking agents not yet exploited in this area is the antitumor antibiotic and clinically used cancer chemotherapeutic drug mitomycin C (MC,¹ **1a**). We report the synthesis of covalent MC-ODN conjugates as potential sequence-specific nucleic acid-inactivating agents. It is shown that MC conjugated to ODNs at their 5'-end specifically alkylates complementary DNA targets.

Reduction of MC activates the C1-aziridine and C10-carbamate positions to alkylate DNA (10). Both monofunctional (**2a** and **3a**) and bifunctional alkylation products of DNA (**4a**) are formed. The single target of alkylation by MC is the 2-amino group of guanine residues in the DNA minor groove. A conspicuous preference is displayed by MC for alkylation and cross-linking of guanines in the CpG sequence (10).

A versatile way of derivatizing the C7-amino substituent of MC is through its analog, mitomycin A (**1b**) (11).

Chart 1



Hence, simple condensation of the latter, with oligonucleotides containing a primary aliphatic amino group tethered to their 5'-phosphate group (5)² yielded MC-ODN conjugates **6–8** (Scheme 1). The products were purified by reversed-phase HPLC; their UV spectra are exemplified in Figure 1. Rigorous proof for the structure of **7** was provided by degradation with snake venom diesterase and alkaline phosphatase to yield **1c**, which was identified by comparison with an authentic synthetic sample (11). The 15-mer MC-ODN conjugates **6–8** used in this study are listed in Chart 2. Molecular modeling of **6** and its shorter $(-\text{CH}_2-)_n$ tether length variants annealed to the complementary 18-mer target ODN **9a** suggested that $n = 10$ gave optimal linker length for cross-linking of conjugates to the targeted guanine G-15.³ We opted for the commercially available amino linker $n = 12$ instead, to synthesize **6**.² In addition, we also synthesized two MC-ODN conjugates **7** and **8** with

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¹ Abbreviations: ODN, oligodeoxyribonucleotide; DPAGE, denaturing polyacrylamide gel electrophoresis; MC, mitomycin C; equiv, equivalent; dG, 2'-deoxyguanosine; dR, 2'-deoxyribose-1'-yl; TBE, Tris-borate-EDTA.

² "5'-amino linker" oligodeoxyribonucleotides (**5**) were synthesized by a DNA synthesizer (ABI Model 380B) using cyanoethyl phosphoramidites (ABI) and "5'-amino linkers" (Glen Research, Sterling, VA) and were purified by reversed-phase HPLC.

³ Personal communication with Dr. David Langley, Bristol-Myers Squibb Co., Wallingford, CT.

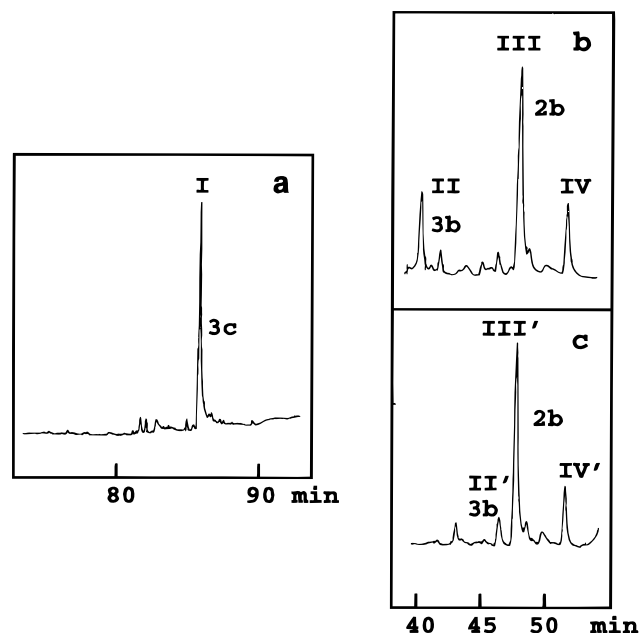


Figure 3. HPLC of enzymatic digests of the reaction products between mitomycin-ODN conjugates and their complementary target ODNs: (a) digest of the cross-linked fraction from the reaction of **6** with **9a**; (b) digest of the cross-linked fraction from the reaction of **7** with **10a**; (c) digest of the non-cross-linked fraction of the same. HPLC: Rainin ODS (C_{18}) column (0.45×15 cm), eluted with 9–54% CH_3CN in 0.03 M NH_4OAc in 90 min for (a) and with 6–36% CH_3CN in 0.03 M NH_4OAc in 90 min for (b) and (c); flow rate = 1 mL/min.

I–IV in parts a and b of Figure 3. Digestion of the isolated faster moving, non-cross-linked electrophoretic bands also yielded adducts, most likely derived from self-strand alkylation of **6** and **7**. These are marked II'–IV' in Figure 3c. Two of them (III' and IV') were identical with III and IV, respectively. All adducts were characterized as adducts of a 7''-(alkyl)aminomitosene and deoxyguanosine, on the basis of their UV spectra, which display (e.g. Figure 1d) a clear combination of these two characteristic chromophores (Figure 1c). The UV spectrum of authentic **3a** ($1''\alpha$; Figure 1b) is essentially identical with the spectra of all of our adducts (see e.g. Figure 1d).⁵ Furthermore, the UV spectra uniquely specify N²- or N¹-substituted dG (15). The latter is discounted, however, since G-N¹ substitution, in contrast to G-N² substitution, has never been observed by mitomycins (10). ESIMS(+)⁶ confirmed the structure of I as monoadduct **3c**, II and II' as monoadducts **3b** ($1''$ -stereoisomers), and III and III' (identical) as monoadduct **2b**. IV (identical with IV') yielded no mass data. It must be a monoadduct since it is isolated from both the cross-linked and non-cross-linked fractions. It may be the $1''$ -stereoisomeric monoadduct **2b**. In summary, it is shown that the cross-linking takes place through monofunctional alkylation of the N² of guanine by the conjugated MC, the same as by free MC (10).

⁴ The cross-linked DNA was isolated preparatively by denaturing gel electrophoresis (20%, 7 M urea, $1 \times$ TBE buffer) and located by UV shadowing technique. This gel slice was crushed and soaked in deionized water for 12 h. The slurry was filtered, and the solution was chromatographed over a Sephadex G-25 column, in 0.02 M NH_4HCO_3 .

⁵ Except for a 6–8 nm red shift of the 314 nm band, due to the change from the 7''-amino to the 7''-alkylamino substituent.

⁶ ESIMS results (m/z): **3c** (I), 755 [(MH)⁺], 678 [(MH – dR)⁺], 253 [(MH₃)³⁺]; **3b** (II), 673 [(MH)⁺ for hydroquinone]; **3b** (II'), 673 [(MH)⁺ for hydroquinone]; **2b** (III, III'), 627 [(MH)⁺].

Site Specificity of Cross-Linking. The cross-linking is dependent on sequence complementarity of the conjugate and its target. No absolute selectivity exists for any of several Gs within the “reach” of the tethered mitomycin, however, as seen by base substitution experiments: Cross-linking yields of **6** + **9a**, **6** + **9b**, and **6** + **9c** systems were 26%, 26%, and 18%, respectively. This indicates that G15 is definitely an alkylation site but that G16 and/or G18 are also alkylated when G15 is replaced by I15. Similarly, **7** + **10a** yielded 15% cross-link, while **7** + **10b** yielded 8%, implicating G14 and/or G18 as additional reaction sites, besides G16.

The results herein demonstrate that the alkylating activity of MC can be targeted to specific DNA via antisense recognition between the target sequence and the MC-oligonucleotide conjugate. The requisite bioreductive activation of MC is accomplished in its ODN-conjugated form by the same reductase systems known to activate free MC *in vitro* and *in vivo* (10).

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LITERATURE CITED

- (1) Pascolo, E., Hudrisier, D., Sproat, B., Thuong, N. T., and Toulmé, J. J. (1994) Relative contribution of photo-addition, helper oligonucleotide and RNase H to the antisense effect of psoralen-oligonucleotide conjugates, on *in vitro* translation of Leishmania mRNAs. *Biochim. Biophys.* 1219, 98–106.
- (2) Levis, J. T., and Miller, P. S. (1994) Properties of exonuclease-resistant, psoralen-conjugated oligodeoxyribonucleotides *in vitro* and in cell culture. *Antisense Res. Dev.* 4, 231–241.
- (3) Grigoriev, M., Praseuth, D., Guieysse, A. L., Robin, P., Thuong, N. T., Hélène, C., and Harel-Bellan, A. (1993) Inhibition of gene expression by triple helix-directed DNA crosslinking of specific sites. *Proc. Natl. Acad. Sci. U.S.A.* 90, 3501–3505.
- (4) Degols, G., Clarenc, J. P., Lebleu, B., and Léonetti, J. P. (1994) Reversible inhibition of gene expression by a psoralen functionalized triple helix forming oligonucleotide in intact cells. *J. Biol. Chem.* 269, 16933–16937.
- (5) Kean, J. M., and Miller, P. S. (1993) Detection of psoralen cross-link sites in DNA modified by psoralen-conjugated oligodeoxyribonucleotide methylphosphonates. *Bioconjugate Chem.* 4, 184–187.
- (6) Praseuth, D., Perrouault, L., Le Doan, T., Chassignol, M., Thuong, N., and Helene, C. (1988) Sequence-specific binding and photocrosslinking of alpha and beta oligodeoxynucleotides to the major groove of DNA via triple-helix formation. *Proc. Natl. Acad. Sci. U.S.A.* 85, 1349–1353.
- (7) Vlassov, V. V., Zarytova, V. F., Kutayvin, I. V., Mamaev, S. V., and Podyminogin, M. A. (1986) Complementary addressed modification and cleavage of a single stranded DNA fragment with alkylating oligonucleotide derivatives. *Nucleic Acids Res.* 14, 4065–4076.
- (8) Meyer, R. B., Tabone, J. C., Hurst, G. D., Smith, T. M., and Gamper, H. J. (1989) Efficient specific cross-linking and cleavage of DNA by stable synthetic complementary oligodeoxyribonucleotides. *J. Am. Chem. Soc.* 111, 8517–8519.
- (9) Chatterjee, M., and Rokita, S. E. (1994) The role of a quinone methide in the sequence specific alkylation of DNA. *J. Am. Chem. Soc.* 116, 1690–1697.

- (10) Tomasz, M. (1994) The mitomycins: Natural cross-linkers of DNA. *Molecular Aspects of Anticancer Drug-DNA Interactions* (S. Neidle and M. Waring, Eds.) Vol. 2, pp 312–349, CRC Press, Boca Raton, FL.
- (11) Iyengar, B. S., Lyn, H.-Y., Cheng, L., and Remers, W. A. (1981) Development of new mitomycin C and porfiromycin analogues. *J. Med. Chem.* 24, 975–981.
- (12) Maniatis, T., Fritsch, E. F., and Sambrook, J. (1989) *Molecular Cloning. A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
- (13) Tomasz, M., Chowdary, D., Lipman, R., Shimotakahara, S., Veiro, D., Walker, V., and Verdine, G. L. (1986) Reaction of DNA with chemically or enzymatically activated mitomycin C: Isolation and structure of the major covalent adduct. *Proc. Natl. Acad. Sci. U.S.A.* 83, 6702–6706.
- (14) Borowy-Borowski, H., Lipman, R., Chowdary, D., and Tomasz, M. (1990) Duplex oligodeoxyribonucleotides cross-linked by mitomycin C at a single site: Synthesis, properties, and cross-link reversibility. *Biochemistry* 29, 2992–2999.
- (15) Singer B., and Grunberger, D. (1983) *Molecular Biology of Mutagens and Carcinogens*, pp 295–334, Plenum Press, New York.

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ARTICLES

Novel Biotinylated Plasmid Expression Vectors Retain Biological Function and Can Bind Streptavidin

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A new method for coupling proteins to plasmid expression vectors is presented. Biotin was covalently attached to a plasmid expression vector containing a chloramphenicol acetyltransferase (CAT) gene. The specific label was one biotin per 100 bp. An electrophoretic mobility shift assay showed that the plasmid was capable of binding multiple streptavidin molecules. When transfected into mouse fibroblasts, the biotinylated plasmid retained 40% of the native plasmid's biological activity, as determined by CAT assay, and was not affected by the binding of streptavidin. The method allows for attachment of any protein to plasmid DNA expression vector while retaining biological function. Hybrid plasmids in which the transcription cassettes were kept free of biotin label were constructed by digesting biotinylated and unbiotinylated plasmids at sites outside the transcription cassette and re-ligating the digestion products. Electron microscopy studies show that the ligation products formed large tangled assemblages of plasmid DNA. When equimolar (with respect to gene number) amounts of these large hybrid biotinylated plasmids were transfected into mouse fibroblasts by means of calcium phosphate precipitation, an increase in CAT expression 25-fold greater than that of original biotinylated plasmid was observed. Slot–blot analysis of total DNA extracted from transfected cells shows that this enhanced activity was not due to increased transfection efficiency. Receptor-mediated delivery could not be shown when a complex comprising biotinylated asialoglycoprotein/streptavidin/biotinylated CAT expression vector was placed in media containing Hep G2 cells.

INTRODUCTION

In the past decade, the field of receptor-mediated gene delivery as a means of achieving gene therapies has attracted a lot of research interest. The strategy takes advantage of the affinity of cell surface receptors for their natural ligands to effect delivery of genes to target tissues *in vivo* (1). Generally, the bioconjugate molecules used to achieve this goal have two components: a gene expression vector and a ligand (usually a protein) with specificity for binding receptors on target cells. The chemistry most often used to join the DNA and ligands is based on the electrostatic attraction between positively charged polylysine and negatively charged DNA (2, 3). The ligand is first covalently bound to polylysine, and the resulting protein conjugate is allowed to interact electrostatically with the nucleic acid component. The complex formed is then used to transfect specific cells *in vivo* by means of receptor-mediated endocytosis.

A number of problems inherent to the use of polylysine have emerged: widespread heterogeneity of the protein conjugate (4), tedious quality control measures (5), insolubility (6), and variability in the efficacy of complexes to effect delivery of genes to tissues possessing the receptors (2, 7). In view of these difficulties, it was

decided to explore alternative methods for attaching protein ligands to expression vectors.

There are a number of methods that allow for the covalent attachment of proteins to nucleic acids (8–11), but these are concerned almost exclusively with the cross-linking of intimate RNA–protein associations and are not useful as general coupling protocols. A more general method that allows for the covalent attachment of any protein to nucleic acids has been reported (12) and describes the covalent coupling of α_2 -macroglobulin to a plasmid DNA chloramphenicol acetyltransferase (CAT) expression vector. However, the authors do not show any data to indicate that the modified expression vector could function in a biological setting. Recently it was speculated that plasmid expression vectors with covalently attached exogenous material would be transcriptionally inactive or give rise to altered transcription (13, 14). While this is somewhat intuitive, there are no experimental results to date to support such speculation. In the present study we revisit this problem and ask the question whether or not it is possible to covalently attach exogenous material to an expression vector and retain transcriptional activity.

To answer this question, biotin was covalently bound to plasmid expression vectors by means of photoactivation (15). The biotinylated plasmid was then tested for two important functions: its ability to bind streptavidin and, following transfection into cells, its ability to function as a template encoding functioning CAT enzyme.

EXPERIMENTAL PROCEDURES

Expression Vector. Our expression vector was a 3871 bp plasmid. The transcription cassette occupies 997

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bp (25.7% of total) and comprised an RNA polymerase II promoter, a CAT gene, and an SV40 polyadenylation signal. Two *NspI* restriction sites flank the transcription cassette, and a unique *BsaI* restriction site also lies outside the transcription cassette. These restriction sites play an important role in making hybrid plasmid constructs (see below).

Biotinylation of Plasmid Expression Vector. Intact plasmid (500 μ g) was prepared in distilled deionized water at a concentration of 1 μ g/ μ L. In a darkroom, 0.5 mg of Immunopure photobiotin (Pierce Chemical Co.) was reconstituted in 500 μ L of distilled deionized water. Equal volumes of DNA and photobiotin were mixed in a 1.5 mL polypropylene microfuge tube. The open-capped sample was then subjected to eight camera flashes from a distance of approximately 15 cm above. The sample was made up to 15 mL with TE buffer (10 mM Tris-Cl, pH 7.4, 1 mM EDTA) and was washed repeatedly with TE buffer in a Centriprep 10 microconcentrator (MW cutoff = 10 000; Amicon, Lexington, MA) to remove unbound photobiotin.

Quantification of Biotinylation. The degree of biotinylation of the plasmid was quantified by means of a fluorescence assay. Fluorescein-labeled avidin (200 μ g, 3.3 nmol; Avidin Neutralite-FL, Molecular Probes Inc., Junction City, OR) was added to 100 μ g (38 pmol) of biotinylated plasmid. The reaction components were allowed to interact for 1 h at 37 °C. The free avidin was separated from the bound on a 10 mL size exclusion chromatography column using Bio-Rad A 1.5 gel equilibrated with TE buffer. Ten fractions of 0.5 mL were collected. The first DNA fraction eluted was quantified. Avidin content was gauged using a fluorescence spectrophotometer. A standard curve of fluorescein-labeled avidin was prepared (range: 0–200 ng/mL.). The excitation beam was set to 490 nm with an 8 nm slit, and the emitted light was detected at 520 nm. There were 40 biotins per plasmid, which represents approximately 1 biotin/100 bp of DNA.

Electrophoretic Mobility Shift Assay (EMSA). The ability of biotinylated plasmid to bind streptavidin was tested. Samples of biotinylated and unbiotinylated plasmid (2 μ g; 0.79 pmol) were prepared in 6 μ L of TE buffer (10 mM Tris-Cl, pH 7.5, 1 mM EDTA). For the biotinylated plasmid, this represented 31.8 pmol of biotin. A 1 μ L aliquot of a 10 mg/mL streptavidin stock (167 pmol, representing a 5.25 molar excess with respect to the biotin) was added to the samples. Samples were mixed and incubated at 37 °C for 1 h. Tracking dye was added, and the electrophoretic migration patterns of the samples were analyzed on 0.8% agarose gels in tris-acetate-EDTA (TAE) buffer.

Transfections. A modified calcium phosphate precipitation method (16) was used to transfect the biotinylated plasmid into mouse fibroblasts (NIH 3T3 cells). Four replicate 100 mm plates of 30–40% confluent cells were transfected with 10 μ g of biotinylated plasmid. Controls plates were transfected with 10 μ g of unbiotinylated plasmid or with buffer alone. The cells were given fresh medium at 24 h after transfection. Cells were harvested at 48 h and cell extracts prepared. When transfection with hybrid concatemer plasmids was performed, 10 μ g of DNA was used. This represented an equimolar amount of gene cassettes.

Preparation of CAT Extracts. Tissue culture plates were drained, and cells were harvested with scrapers in 1 mL of ice-cold phosphate-buffered saline (PBS: 10 mM phosphate, 150 mM NaCl). The cells were pelleted by centrifuging at 2500g for 5 min. The supernatant was removed, and the cells were resuspended and washed

again in 4 mL of PBS. The cells were pelleted again and the supernatants removed. The cells were lysed by resuspending the pellet in 0.5 mL of 0.25 M Tris, pH 7.4/0.5% Triton X-100 and placing on ice for 10 min. The lysate was centrifuged for 2 min at 10000g at 4 °C and then placed in a water bath at 65 °C for 15 min. Samples were centrifuged again for 5 min at 10000g at 4 °C to repellet the lysate debris. The resulting supernatants were the CAT extracts and were reserved on ice. Aliquots of the CAT extracts were reserved for determination of protein concentration using the BCA method (Pierce).

Assay for CAT Activity. CAT reaction buffer was prepared just prior to use: 12.5 mM chloramphenicol, 25 mM Tris-Cl, pH 7.4, 0.25 μ Ci of [³H-acetyl]acetyl-CoA (200 mCi/mmol) or 25 μ M acetyl-CoA. Since chloramphenicol is reconstituted in ethanol, the buffer also contained 10% ethanol. A 50 μ L aliquot of the CAT reaction buffer was placed in a 7 mL scintillation vial. The assay was started by adding 75 μ L of CAT extract to this buffer to give a final reaction volume of 125 μ L. This reaction mixture was overlaid with 3 mL of water-immiscible "Econofluor" scintillant (NEN, Boston, MA). Samples were immediately placed in a liquid scintillation counter. Radiolabeled acylated forms of chloramphenicol diffuse rapidly into the upper phase of scintillant. The radioactivity (c.p.m.) was read at regular intervals over a 2–3 h period. CAT activity was determined from the slopes of the early (linear) portions of these time curves. All CAT activities were normalized for protein concentration.

Plasmid Restriction Digests and Ligations. Restriction enzyme digests were carried out according to the manufacturer's instructions. After digestion with *NspI*, the enzyme was inactivated by incubation at 70 °C for 15 min. Without further treatment, the digests were used in a series of ligation reactions carried out in volumes of 100 μ L, incubated at 16 °C overnight. The ligation reaction components were 100 μ g of *NspI*-digested plasmid (152 pmol-ends), 50 mM Tris-Cl, pH 7.4, 10 mM MgCl₂, 1 mM ATP, 1 mM DTT, and 2 units of T₄ DNA ligase. These high concentrations of pmol-ends and ligase are conducive to intermolecular ligations. Ligation was confirmed by comparing electrophoretic banding patterns of the DNA on 0.8% agarose TAE gels before and after the ligation procedure.

Electron Microscopy. Samples of the CAT plasmid ligation products were prepared for examination by transmission electron microscopy according to the method of Laemmli (17). Formvar-carbon-coated specimen grids were glow-discharged and coated with 1 μ g/mL poly(L-lysine) (MW > 300 000; Sigma Chemical Co.). The grids were then floated on 30 μ L drops of sample on parafilm for 30 min, rinsed for a total of 1 min on 4 drops of distilled water, and then stained on a drop of 2% aqueous uranyl acetate for 1 min. The excess uranyl acetate was wicked off each grid with filter paper, leaving the grid wet with a small amount of stain. After drying, the grids were observed and photographed at 60 kV in a JEOL 100CX TEM. Magnification was calibrated with a diffraction grating replica (2180 lines/mm).

DNA Slot-Blot Analysis. Transfection efficiency was determined by extracting total DNA from lysates of cells that had been transfected either with native plasmid or with a large-sized ligation product derivative of the plasmid. Total DNA was extracted according to a standard phenol/chloroform-based protocol (18). DNA samples (10 μ g) and hybridization buffer were prepared according to the instructions of the manufacturers of Genescreen Plus hybridization membrane (DuPont, Boston, MA). Samples were loaded onto the membrane using

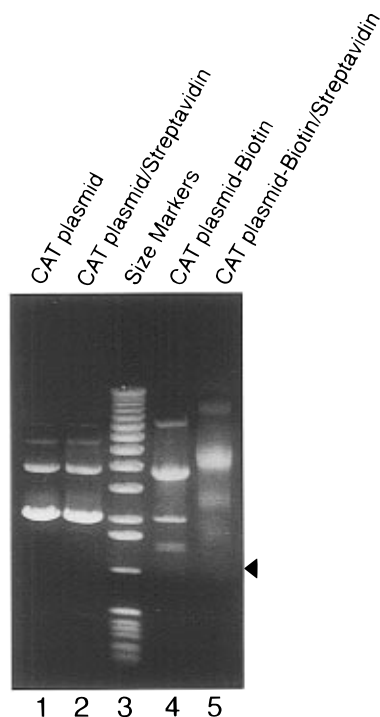


Figure 1. Electrophoresis mobility shift assay confirming the presence of biotin on the plasmid expression vector. Samples of the CAT plasmid (1 μ g) were subjected to electrophoresis on an 0.8% agarose TAE (tris–acetate–EDTA) gel. All samples were preincubated for 15 min at 37 °C in the presence or absence of excess (10 μ g) streptavidin. Lane 1, unbiotinylated plasmid; lane 2, unbiotinylated plasmid with streptavidin; lane 3, 1 kb size markers (BRL); lane 4, biotinylated plasmid; lane 5, biotinylated plasmid with streptavidin. The location of the 1 kb size marker band is indicated by the black triangle.

a Hybri-slot manifold apparatus (BRL). ^{32} P-Labeled CAT ribozyme probe was generated according to methods we have already published in detail elsewhere (19). Prehybridization was for 1 h at 55 °C, and hybridization was allowed to run overnight at 55 °C. Posthybridization washes were carried out in accordance with the instructions of the manufacturers of the membrane. The membrane was monitored between washes with a Geiger counter and, when background signal was sufficiently reduced, was then exposed to Kodak film for 2 h.

RESULTS

Characterization of the Biotinylated Plasmid. A 3871 bp plasmid expression vector for the CAT gene was biotinylated and the unbound photobiotin removed. The specific biotin label was determined by means of a fluorescence assay. The molar ratio of biotin to plasmid was 40. This represents approximately 1 biotin/100 bp of DNA.

The Biotinylated Expression Vector Binds Streptavidin. The ability of the plasmid to bind streptavidin was confirmed using an EMSA. Biotinylated and unbiotinylated plasmids were incubated in the presence of a molar excess of streptavidin. The electrophoretic migration patterns of the DNA were analyzed on 0.8% agarose gels. There was a 100% shift in the banding pattern of the biotinylated plasmid, indicating that all plasmid molecules possessed biotin label. The mobility shift was attributed to the binding of multiple streptavidin molecules (Figure 1, lane 5). The mobility of the native plasmid was not affected by the presence of streptavidin (Figure 1, lane 2).

Biotinylated Expression Vector Remains Biologically Active. The ability of the biotinylated expression

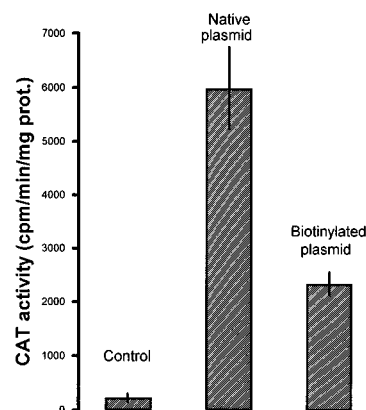


Figure 2. Comparison of biological activity of biotinylated and native plasmid. Replicate 100 mm plates of 30–40% confluent mouse fibroblast (3T3) cells were transfected with 10 μ g of either biotinylated CAT plasmid or unbiotinylated CAT plasmid using a calcium phosphate precipitation method. A control group were transfected with buffer alone ($n = 4$ in all cases). Cells were harvested 48 h after transfection, and the CAT activities of the extracts were determined ($n = 4$ in all cases). The results were normalized for protein content.

vector to function within mammalian cells as a template encoding fully active CAT enzyme was tested. The biotinylated plasmid was transfected into mouse fibroblasts (NIH 3T3 cells) using a calcium phosphate precipitation method. Control groups were transfected with native plasmid or with buffer alone. Cells were assayed for CAT activity 2 days later. The biotinylated plasmid was biologically active, retaining approximately 40% of its original activity (Figure 2). It is improbable that this amount of CAT activity could be attributed to a fraction of the plasmid population being unbiotinylated since the EMSA (Figure 1) shows that all of the plasmids possessed biotin. Further, with 40 randomly attached biotins per plasmid, and a transcription cassette occupying 25.7% of the plasmid, the percentage of expression vectors having biotin-free transcription cassettes is $(1 - 0.257)^{40} \times 100\% = 0.0007\%$. Therefore, the results show that eucaryotic RNA polymerase II was capable of reading through those parts of the duplex DNA bearing the covalently attached biotins. More generally, it is concluded that expression vectors, with multiple covalently attached biotins, are able to continue functioning as templates encoding biologically active proteins.

Biotinylated Expression Vector Remains Biologically Active When Complexed with Streptavidin. To attach proteins, these biotinylated plasmids must be complexed with streptavidin-conjugated ligands. An important question then arises. Is transcription possible from biotinylated template when streptavidin (60 kDa) is complexed? To answer this, our biotinylated expression vector was complexed with streptavidin prior to calcium phosphate transfection into 3T3 cells. The results show no significant difference in biological activity between biotinylated expression vectors either in the presence or in the absence of bound streptavidin (Figure 3).

Construction of Plasmids with Biotin-Free Transcription Cassettes. Clearly, if one could keep the transcription cassette of the expression vector free of biotin, it should be possible to improve the level of expression from the plasmid. A scheme for the production of plasmid constructs with biotin-free transcription cassettes was designed (Figure 4). Biotinylated and unbiotinylated plasmids were digested separately with *NspI* to yield fragments of approximately 2 kb in size. Equimolar amounts of these fragments were cross-linked

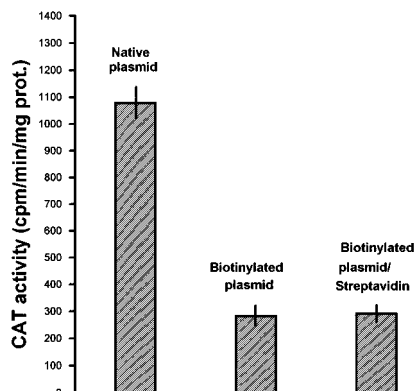


Figure 3. Comparison of biological activity of biotinylated expression vector with and without complexed streptavidin. Biotinylated CAT plasmid was complexed with a 40 times molar excess of streptavidin. Replicate 100 mm plates of mouse fibroblasts were transfected with 10 μ g of the complex using the calcium phosphate precipitation method. Control groups were transfected with unbiotinylated (native) plasmid and with uncomplexed biotinylated plasmid ($n = 4$ in all cases). Cells were harvested 48 h after transfection, and the CAT activities of the extracts were determined ($n = 4$ in all cases). The results were normalized for protein content.

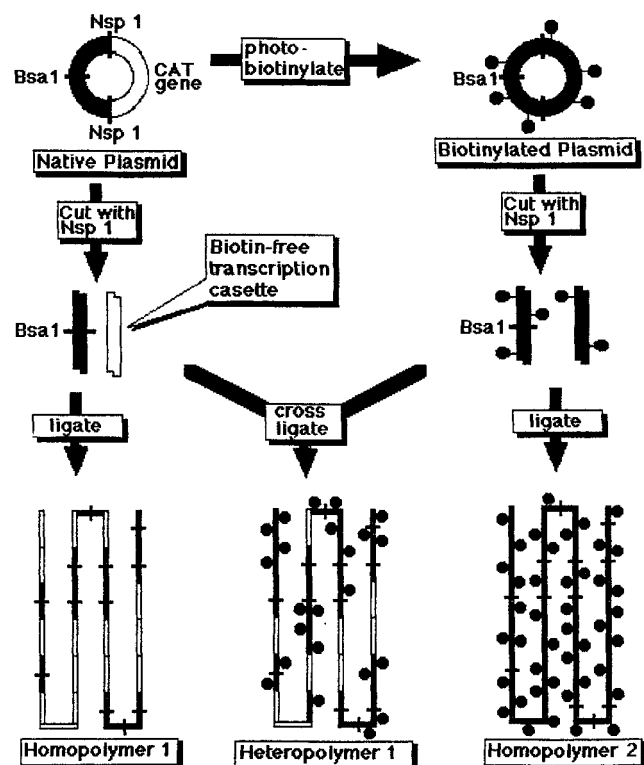


Figure 4. Scheme for the production of biotinylated plasmid expression vectors with biotin-free transcription cassettes. Native and biotinylated CAT plasmid samples are digested with *Nsp*I (Amersham). Equimolar amounts of biotinylated and unbiotinylated fragments are cross-ligated to produce very large concatemers termed "heteropolymer 1". Heteropolymer 1 contains biotin label and many biotin-free transcription cassettes on the same molecule. Homopolymer 1 and homopolymer 2 are produced under similar ligation conditions using only unbiotinylated or biotinylated fragments, respectively.

at high concentration (1 μ g/ μ L DNA or greater) to facilitate intermolecular ligation (20). The electrophoretic migration pattern of cross-ligation products were analyzed on an 0.8% agarose TAE gel. A single broad band migrated to the rear of the largest (12 kb) of the linear DNA size markers (Figure 5A, lane 4). It was not possible to say whether the products were linear or

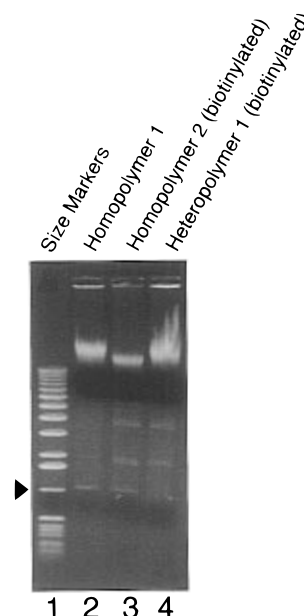


Figure 5. Characterization of the DNA constructs. Samples of DNA constructs (2 μ g) were electrophoresed on an 0.8% agarose TAE gel together with 1 kb size markers (BRL). Lane 1, 1 kb size markers (BRL); lane 2, homopolymer 1; lane 3, homopolymer 2; lane 4, heteropolymer 1. The location of the 1 kb size band is indicated by the black triangle

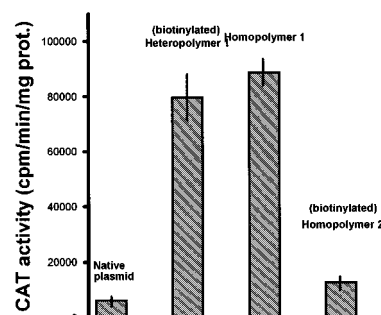


Figure 6. Biological activity of the new plasmid ligation products. Replicate 100 mm plates of mouse fibroblasts were transfected with 10 μ g of heteropolymer 1, homopolymer 1, and homopolymer 2, respectively. A control group was transfected with 10 μ g (an equimolar amount with respect to gene number) of native CAT plasmid. Cells were harvested 48 h after transfection, and the CAT activities of the extracts were determined ($n = 4$ in all cases). The results were normalized for protein content.

circular. The ligation product was termed "heteropolymer 1" to indicate its composition of both biotinylated and unbiotinylated fragments and to reflect its large size. Calcium phosphate transfection of 10 μ g of heteropolymer 1 into 3T3 cells gave unanticipated results. The biological activity was 10 times greater than that achieved with a similar mass (equimolar number of genes) of the native plasmid (Figure 6). This represents an even greater improvement (25-fold) on the biological activity of the original biotinylated plasmid (Figure 2).

Characterizing the Enhanced Biological Activity.

To ascertain whether the increased activity found in heteropolymer 1 was attributable to the presence of biotin, which could possibly interfere with normal cellular exonuclease degradative activity, or to its size and shape, which are known to affect degradation of nucleic acids (4, 21), two separate ligations of unbiotinylated and biotinylated *Nsp*I-generated fragments were made to produce "homopolymer 1" and "homopolymer 2", respectively (Figure 4). The two product sizes were similar to each other and to heteropolymer 1 (Figure 5A, lanes 2

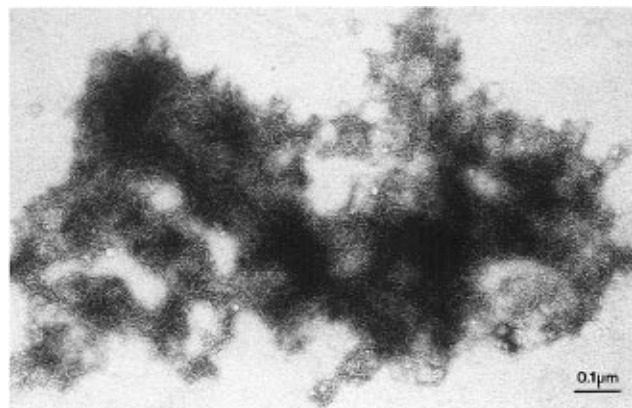


Figure 7. Electron micrograph of homopolymer 1. The native plasmid was digested with *NspI*. The enzyme was heat inactivated, and the digestion products were used in an overnight ligation reaction. See Experimental Procedures for sample preparation.

and 3). When equimolar amounts (10 μ g) of these concatemer products and native plasmid were transfected into 3T3 cells, homopolymer 1 showed the same enhanced activity as heteropolymer 1. Homopolymer 2 (with biotin) had only a fraction of the biological activity shown by homopolymer 1. Since homopolymer 1 lacked biotin but possessed the enhanced activity, it was concluded that enhanced activity was attributable to the size and/or shape of the DNA. The same size enhancement of biological activity was observed when one compared the increased CAT activity of homopolymer 2 (Figure 6) with that of its precursor, biotinylated plasmid (Figure 2). Again there was an approximate 10-fold increase in biological activity.

We also transfected plates of mouse fibroblasts with native and concatemer plasmids using lipofection (data not shown). We did not see the enhanced activity. We feel this may be due to the inherent problems of this particular transfection system: the product literature cautions the user about finding the proper molar ratio of DNA and lipofectin reagent (BRL).

Electron microscopy studies were undertaken to see whether any three-dimensional properties were evident. The DNA appeared as tangled aggregates of plasmid DNA with no other discernible features (Figure 7).

The question arose as to whether the increased activity seen in cells transfected with large-sized ligation products is attributable to an increase in transfection efficiency or to increased levels of transcription from the large polymeric form of the plasmid. To address this question, slot-blot analysis of total DNA extracts of lysates of cells that had been transfected with native plasmid and cells transfected with homopolymer 1 were carried out to quantify the amount of plasmid in the transfected cells. These cells had already been assayed for CAT activity, and the results were 17 507 and 90 093 cpm min⁻¹ (mg of protein)⁻¹, respectively (a 5-fold difference). Our results show there was no difference in transfection efficiency between native plasmid and homopolymer 1 (Figure 8, lanes 3 and 4). By using two positive controls, we also show that the hybridization efficiency of the probe is the same for both forms of the plasmid (Figure 8, lanes 1 and 5). Since transfection efficiency was ruled out, the 5-fold increase in CAT activity observed with cells transfected with homopolymer 1 must be due to another process such as an increase in transcription rate or some differential intracellular distribution of the transfected product leading to greater accumulation of the transfected concatemer plasmid DNA in the nucleus.

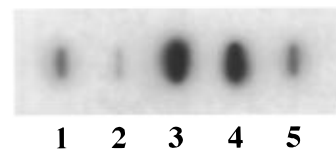


Figure 8. Slot-blot analysis of 10 μ g total DNA extracted from cells transfected with native plasmid and cells transfected with homopolymer 1. Lane 1, positive control (5 ng of native CAT plasmid); lane 2, mock transfection; lane 3, cells transfected with native plasmid; lane 4, cells transfected with homopolymer 1; lane 5, positive control (5 ng homopolymer 1).

Receptor-Mediated Delivery Trials. Some trials were undertaken to assess whether streptavidin bridges linking biotinylated protein ligands and biotinylated CAT expression plasmids could be used to transfect cells by receptor-mediated endocytosis (data not shown). Biotinylated (ratio 1:1) asialo-orosomucoid (AsOR) was added to the streptavidin-biotin-CAT plasmid complex. The theoretical final AsOR/streptavidin/plasmid molar ratio was 120:40:1. The complex (equivalent to 10 μ g of plasmid DNA) was added to 100 mm plates of 30–50% confluent Hep G2 cells. The latter cell type is known to express an abundance of receptors for AsOR on the cell surface. We could not show any delivery. CAT activity was never greater than that found in control plates which received no DNA.

DISCUSSION

The first report of a general method for covalent attachment of any protein to nucleic acids appeared in 1983 (12). α_2 -Macroglobulin was covalently linked to a plasmid expression vector at a protein/DNA molar ratio of 2:1. While the authors claimed that there was no apparent damage to the structure of the DNA, they did not present any data pertaining to this conjugate's ability to function in a biological setting. More recently, it has been speculated that covalent coupling of ligands to the DNA would cause transcriptional inactivation or be mutagenic (13, 14). Again, no data were presented to support these speculations. In this study we have shown that it is possible to get substantial amounts of biological activity (40% of the native activity) from a DNA template with covalently bound biotin. Our data suggest that RNA polymerase II is able to accommodate the presence of these biotins on the plasmid template during transcription.

We have also shown that biotinylated plasmids that have been complexed with streptavidin remain biologically functional when transfected into mouse fibroblasts. It is possible that RNA polymerase II can displace biotin-bound streptavidin from a biotinylated template. In a related report, it has been shown that bacteriophage RNA polymerase had the strength to displace up to 80% of linear DNA template fragments that were attached to streptavidin by means of an end-labeled biotin (22). The ability to bind streptavidin also implies that any biotinylated protein may be added to the conjugate to form a tripartite complex: biotinylated DNA-streptavidin-biotinylated protein.

Making use of avidin-biotin technology to attach protein ligands allows great flexibility in the number of ligands one may attach to the plasmid. Current methods for biotinylation of DNA allow for a great deal of control over the number of ligand binding sites (biotins) that can be placed on a plasmid. We labeled our plasmid expression vector by means of photobiotinylation (Pierce Chemical), which resulted in the covalent attachment of approximately 40 biotins per plasmid. Streptavidin has a valency of four, which means the unoccupied sites may

accommodate as many as 120 biotinylated ligands, making a ligand/DNA molar ratio of 120. Another method using M13 ssDNA, dNTPs—biotin, and Klenow to generate plasmid expression vectors with very high specific biotin label has been reported by us (23).

We also showed that the loss of biological activity incurred by covalent derivatization of a plasmid expression vector may be overcome by the construction of large hybrid plasmids in which a large proportion of the transcription cassettes are kept unmodified. This method worked well for us; it could be used if warranted in the α_2 -macroglobulin situation mentioned earlier (12) and has general applicability in all situations when loss of activity of an expression vector is incurred. The increased transcription rate from the large ligation products was an unanticipated result. The increase may be due to the rearrangement of position-sensitive upstream activation sequences, promoters, or enhancers. There are no precedents for the assemblages presented here. However, Axelrod et al. (24) have shown that tandem repeats of promoters placed upstream of transcription start sites in plasmid expression vectors can produce nonlinear synergistic increases in transcription levels sometimes up to 4 orders of magnitude in excess of the levels in the native plasmid possessing a single promoter. It may be that the increased transcription levels presented here are due to some sort of synergistic effect produced by the chance arrangement of *NspI*-generated fragments and/or the proximity of activation elements in the ligation products.

Our failure to achieve receptor-mediated delivery is difficult to explain. Ligand/DNA molar ratios are unlikely to be the cause. Our ratio of 120:1 is well above what is considered the minimum requirement (10–15:1) and comfortably within the range of recently published ratios for polylysine-based methods (25, 26). The failure to transfect may be due to the packaging of DNA. Polylysine, in addition to binding electrostatically to DNA, also causes condensation of the DNA into particles of definite size and shape (2). It may be that such condensation is a prerequisite for receptor-mediated delivery.

Other applications for biotinylated expression vectors could be manifold. Nuclear localization signals (NLSs) comprising short, highly basic amino acid sequences have been implicated in the import of the SV40 large-T antigen (27) and another protein—nucleoplasmin (28)—into the nucleus. Recently, synthetic peptides containing putative NLSs, conjugated to bovine serum albumin (BSA), were shown to facilitate the translocation of BSA to the nucleus (29). By coupling these same sequences to an expression vector containing a sensitive reporter gene, one would have an assay system capable of providing important information on the relative strengths of NLSs. The cellular fate of transfected expression vectors could also be explored by conjugating biotinylated plasmids with commercially available fluorescein-labeled streptavidin or with antibodies. Subcellular fractionation and/or *in situ* immunohistological stains might then be used to substitute for the more tedious *in situ* hybridizations.

In conclusion, plasmid expression vectors with covalently attached biotins continue to function as templates for transcription in eucaryotic cells. By manipulation of biotinylated and unbiotinylated plasmid fragments, hybrid plasmid constructs of large size were made that are unique in possessing a combination of three valuable features: (1) unhindered transcription cassettes, (2) numerous streptavidin binding sites, and (3) enhanced biological activity.

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LITERATURE CITED

- (1) Mulligan, R. (1993) The basic science of gene therapy. *Science* 260, 926–932.
- (2) Perales, J. C., Ferkol, T., Beegan, H., Ratnoff, O. D., and Hanson, R. W. (1994) Gene transfer *in vivo*: sustained expression and regulation of genes introduced into liver by receptor targeted uptake. *Proc. Natl. Acad. Sci. U.S.A.* 91, 4086–4090.
- (3) Chowdhury, N. R., Wu, C. H., Wu, G. Y., Yerneni, P. C., Bommineni, V. R., and Chowdhury, J. R. (1993) Fate of DNA targeted to the liver by asialoglycoprotein receptor mediated endocytosis *in vivo*. *J. Biol. Chem.* 268, 11265–11271.
- (4) Ferkol, T., Lindberg, G. L., Chen, J., Perales, J. C., Crawford, D. R., Ratnoff, O. D., and Hanson, R. W. (1993) Regulation of phosphoenolpyruvate carboxykinase/human factor IX gene introduced into the livers of adult rats by receptor mediated gene transfer. *FASEB J.* 7, 1081–1091.
- (5) McKee, T. D., DeRome, M. E., Wu, G. Y., and Findeis, M. A. (1994) Preparation of asialoorosomucoid-polylysine conjugates. *Bioconjugate Chem.* 5, 306–311.
- (6) Wu, G. Y., and Wu, C. H. (1988) Evidence for targeted gene delivery to Hep G2 hepatoma cells *in vitro*. *Biochemistry* 27, 887–892.
- (7) Curiel, D. T., Agarwal, S., Wagner, E., and Cotton, M. (1991) Adenovirus enhancement of transferrin-polylysine-mediated gene delivery. *Proc. Natl. Acad. Sci. U.S.A.* 88, 8850–8854.
- (8) Rinke, J., Yuki, A., and Brimacombe, R. (1976) Studies on the environment of protein S7 within the 30-S subunit of *Escherichia coli* ribosomes. *Eur. J. Biochem.* 64, 77–89.
- (9) Sperling, J., and Sperling, R. (1978) Photochemical crosslinking of histones to DNA in nucleosomes. *Nucleic Acids Res.* 8, 2755–2773.
- (10) Vanin, E. F., Burkhard, S. J., and Kaiser, I. I. (1981) *p*-Azidophenylglyoxal: a heterobifunctional photosensitive reagent. *FEBS Lett.* 124, 89–92.
- (11) Baumert, H. G., Skold, S.-E., and Kurland, C. G. (1978) RNA-protein neighbourhoods of the ribosome obtained by crosslinking. *Eur. J. Biochem.* 89, 353–359.
- (12) Cheng, S.-Y., Merlino, G. T., and Pastan, I. H. (1983) A versatile method for the coupling of protein to DNA: synthesis of α_2 -macroglobulin-DNA conjugates. *Nucleic Acids Res.* 11, 659–669.
- (13) Wu, G. Y., and Wu, C. H. (1991) Delivery systems for gene therapy. *Biotherapy* 3, 87–95.
- (14) Chang, A. G. Y., and Wu, G. Y. (1994) Gene therapy: applications to the treatment of gastrointestinal and liver diseases. *Gastroenterology* 106, 1076–1084.
- (15) Forster, A. C., McInnes, J. L., Skingle, D. C., and Symons, R. H. (1985) Non-radioactive hybridization probes prepared by the chemical labelling of DNA and RNA with a novel reagent, photobiotin. *Nucleic Acids Res.* 13, 745–761.
- (16) Chen, C., and Okayama, H. (1987) High efficiency of transformation of mammalian cells by plasmid DNA. *Mol. Cell. Biol.* 7, 2745–2752.
- (17) Laemmli, U. K. (1975) Characterization of DNA condensates induced by poly (ethylene oxide) and polylysine. *Proc. Natl. Acad. Sci. U.S.A.* 72, 4288–4292.
- (18) Strauss, W. M. (1987) Preparation of genomic DNA from mammalian tissue. In *Current Protocols in Molecular Biology*, Section 2.2, Wiley-Interscience, New York.
- (19) Lichter, A., Barrett, N. L., and Carmichael, G. G. (1992) Simple, inexpensive preparation of T1/T2 ribonuclease suitable for use in RNase protection experiments. *BioTechniques* 12, 231–232.

- (20) Struhl, K. (1989) Construction of hybrid DNA molecules. In *Current Protocols in Molecular Biology*, Section 3.16, Wiley-Interscience, New York.
- (21) Wagner, E., Cotten, M., Foisner, R., and Birnstiel, M. L. (1991) Transferrin-polycation-DNA complexes: the effect of polycations on the structure of the complex and DNA delivery to cells. *Proc. Natl. Acad. Sci. U.S.A.* 88, 4255–4259.
- (22) Fujita, K., and Silver, J. (1993) Surprising lability of biotin-streptavidin bond during transcription of biotinylated DNA bound to paramagnetic streptavidin beads. *BioTechniques* 14, 608–617.
- (23) Leahy, P., Carmichael, G. G., and Rossomando, E. F. (1995) Novel plasmid constructs for general and receptor mediated transfections. *FASEB J.* 9, A 1400.
- (24) Axelrod, N. J., Carmichael, G. G., and Farabaugh, P. J. (1990) Enhancer and promoter elements from simian virus 40 and polyomavirus can substitute for an upstream activation sequence in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* 10, 947–957.
- (25) Cotton, M., Wagner, E., and Birnstiel, M. L. (1993) Receptor mediated transport of DNA into eukaryotic cells. *Methods Enzymol.* 217, 618–644.
- (26) Christiano, R. J., Smith, L. C., and Woo, S. L. C. (1993) Hepatic gene therapy: efficient gene delivery and expression in primary hepatocytes utilizing a conjugated adenovirus-DNA complex. *Proc. Natl. Acad. Sci. U.S.A.* 90, 11548–11552.
- (27) Kalderon, D., Richardson, W. D., Markham, A. F., and Smith, A. E. (1984) Sequence requirements for nuclear location of simian virus 40 large-T antigen. *Nature* 311, 33–38.
- (28) Dingwall, C., Sharnick, S. V., and Laskey, R. A. (1982) A polypeptide domain that specifies migration of nucleoplasmin into the nucleus. *Cell* 30, 449–458.
- (29) Bukrinsky, M. I., Haggerty, S., Dempsey, M. P., Sharova, N., Adzhubel, A., Spitz, L., Lewis, P., Goldfarb, D., Emerman, M., and Stevenson, M. (1993) A nuclear localization signal within HIV-1 matrix protein that governs infection of non-dividing cells. *Nature* 365, 666–669.

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Natural Peptides as Building Blocks for the Synthesis of Large Protein-like Molecules with Hydrazone and Oxime Linkages

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Methods are known for the production of synthetic protein-like molecules of nonlinear architecture with molecular masses in the 10–20 kDa range. To synthesize such compounds of higher molecular mass and complexity, chemoselective ligation of natural (as opposed to synthetic) peptide building blocks was studied. In preliminary experiments with model peptides, conditions for the formation of peptide oximes were investigated, and their stability at alkaline pH was examined, to resolve a literature controversy. It was found that low pH (down to 2.1) was suitable for polyoxime formation and that the oxime bond was stable for up to 65 h at pH 8 and for more than 2 h at pH 9. Then, using natural peptides, it was found to be possible to synthesize, and characterize by mass spectrometry, nine-component species with molecular masses >48 kDa. This is about twice the size of homogeneous artificial proteins previously described. Such complex molecules of defined structure are beginning to find applications as vaccine candidates, as radioimmunodiagnostic agents, and as nonviral gene therapy delivery vehicles.

INTRODUCTION

Unprotected peptides, in contrast to fully protected peptides, are generally soluble in aqueous media and easily purified and may be used as building blocks for the synthesis of fairly complex protein-like molecules of high molecular mass (1, 2). Thioether, disulfide, thioester, hydrazone, oxime, thiazolidine, and amide linkages have all been employed for such syntheses, which generally involve the spontaneous ligation of fragments made chemically and lead to well-defined products of branched structure. Linear products involving single or multiple condensations are accessible through chemical (3–5) or enzymic approaches (6).

Until recently, the largest synthetic protein-like molecules made and fully characterized by mass spectrometry have been of branched architecture and have molecular masses in the region of 20–25 kDa (7–9). Rationally synthesized protein-like molecules, as opposed to well-known cross-linked hapten–carrier protein preparations, are beginning to show promise as vaccine candidates against viruses (10, 11) and parasites (malaria, E. Nardin, private communication) and as nonviral gene therapy delivery vehicles (D. Thatcher, private communication). This led us to try to incorporate not just relatively short synthetic peptides but also native proteins with a function, e.g., a tissue-targeting or immunostimulatory role. Our first hybrid consisted of cholera toxin B subunit, which is a mucosal-targeting protein of molecular mass of 11 600 Da, and a synthetic carrier molecule (12) to which five copies of a short synthetic peptide were attached, giving a product with a molecular mass of 20 550 Da (9).

To discover how to make and work with larger and even more complex molecules, we first examined oxime formation as a function of pH. There is some controversy in this area in the literature. Early work with hydra-

zones in aqueous solution (13) had established that the rate of hydrazone formation increased with decreasing pH in the range 3–5 and that an equilibrium was reached which depended particularly on pH (lower yield at lower pH) and on concentration (lower yield at lower concentration). More recent work (1) threw doubt on the earlier study and reported that the reaction rate in aqueous solution increased with increasing pH in the range 4.2–5.7 for the formation of both peptide hydrazones and peptide oximes. Since yields must be optimized when working in dilute solution with native proteins, we first studied this aspect with synthetic peptides. A second important concern is product stability. It has been reported (1) that peptide oximes, while stable in acidic and neutral medium (1, 7), are unstable at pH 9. We therefore exposed peptide oximes to aqueous conditions in the range pH 7–10.

Once these model studies had been completed, we went on to synthesize polyhydrazones and polyoximes of higher molecular mass using native proteins or protein fragments as building blocks. An insulin tetrahydrazone, octahydrazone, tetraoxime, and octaoxime (molecular masses of 24, 25, 48, and 48 kDa, respectively) and an F(ab')₃ trioxime of molecular mass 152 450 Da were made and characterized by mass spectrometry. The interesting biological properties of the F(ab')₃ have been reported elsewhere (14).

This ability to extend the size range of accessible synthetic protein-like molecules augurs well for the fields of vaccination (10) and radioimmunotherapy (14) and also nonviral gene therapy, where it is necessary to bring together several peptides each with a separate function, such as cell targeting, DNA binding, and nuclear location (15).

MATERIALS AND METHODS

Synthesis of Linear Peptides and Templates. Templates carrying four and eight aldehyde groups (Figure 1) were made manually according to standard techniques of peptide chemistry starting with Fmoc¹-Tyr-(tBu)-Sasrin resin (Bachem). After removal of the Fmoc group with 50% piperidine in DMF for 15 min, the resin-bound amino groups were acylated for 1 h with Fmoc-

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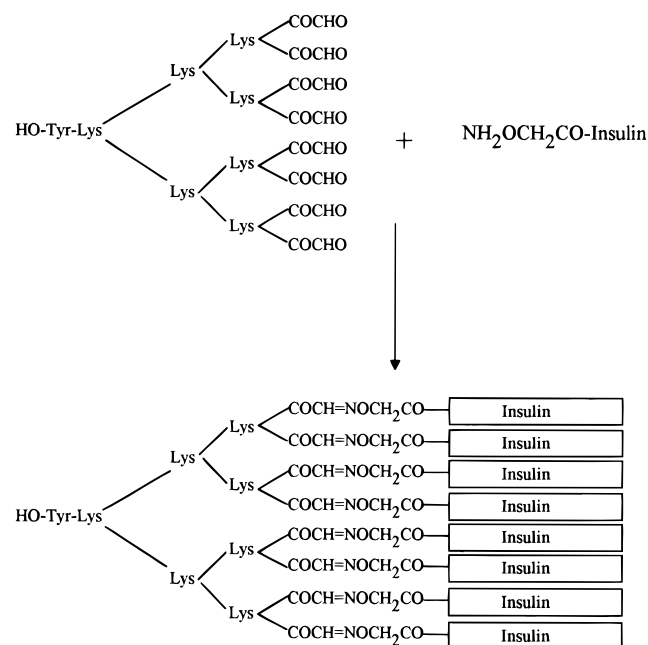


Figure 1. Reaction scheme representing oxime formation between the octaaldehyde template (left) and aminooxyacetyl-insulin to give the octaoxime. The branching Lys residues of the template are acylated on both their alpha and epsilon amino groups and are shown (together with the Tyr residue) unconventionally with their carboxyl groups to the right, for clarity. A tetraaldehyde template, which had one fewer rounds of Lys branching, was also made and used.

Lys(Fmoc) activated with TBTU/HOBT/DIEA, Fmoc-deprotected then reacylated with Fmoc-Lys(Fmoc) on both alpha and epsilon amino groups, to give a resin with four protected amino groups. Acylation of these four amino groups (after Fmoc deprotection) with Boc-Ser(tBu) in similar fashion yielded the tetravalent template, whereas another round of acylation with Fmoc-Lys(Fmoc) prior to coupling of Boc-Ser(tBu) yielded the octavalent template in protected form. The templates were deprotected and cleaved from the resin by vortexing with a mixture consisting of TFA (85%) and thioanisole (15%) for 1 h at room temperature and then precipitating and washing with cold ether. The precipitate was taken up in water, filtered to remove resin, lyophilized, taken up again in water, and purified by HPLC. Pure tetravalent and octavalent templates were characterized by electrospray ionization mass spectrometry (ESI-MS) on the Geneva quadrupole instrument as described earlier (7): found 914.1 and 1776.3, calcd 914.0 and 1775.0, respectively. HPLC was performed using techniques and equipment that has previously been described (7).

Linear peptides were synthesized according to standard automated techniques as previously described (7, 12). The α -melanocyte stimulating hormone (MSH) analog $\text{NH}_2\text{-OCH}_2\text{CO-Nle-Asp-His-(D-Phe)-Arg-Trp-Lys-NH}_2$ has been described previously (16). The tripeptide Ser-Leu-Leu was oxidized with periodate under standard conditions (7) to give glyoxylyl-Leu-Leu, which was isolated by HPLC. All synthetic peptides and templates

were single components on HPLC and had the expected mass spectrum when analyzed by ESI-MS.

Des-Ala^{B30}-insulinylicarbohydrazide, Des-Ala^{B30}-insulinylicarbohydrazide, Des-Ala^{B30}-insulinylicarbohydrazide, and Aminooxyacetyl^{B1}-insulin. Porcine insulin, monocomponent grade, was purchased from Novo-Nordisk, Bagsvaerd, Denmark. Des-Ala^{B30}-insulinylicarbohydrazide was prepared as previously described (12). Des-Ala^{B30}-insulinylicarbohydrazide was prepared similarly by reverse proteolysis in 80% butane-1,4-diol using Phe-NHCH₂CH₂NH-COCH₂ONH₂ as nucleophile [itself prepared by acylation of Boc-NHOCH₂CO-NH-CH₂CH₂-NH₂ (14) with Boc-Phe-OSu followed by deprotection with TFA]. Product was isolated by HPLC (yield 60%) and characterized by ESI-MS: found mass 5968.9 Da, calcd 5968.8 Da. Aminooxyacetyl^{B1}-insulin was prepared according to standard techniques (17). Briefly, A¹,B²⁹-di-Msc-insulin was prepared and acylated quantitatively with Boc-aminooxyacetyl-OSu in DMF. After isolation by HPLC, the Msc groups were quantitatively removed (17) and the product was recovered in Boc-protected form again by HPLC and characterized by ESI-MS on the Geneva quadrupole instrument (7): found mass 5948.1 Da, calcd 5950.6 Da. Just prior to use, the required amount of powder was quantitatively Boc-deprotected by dissolving in neat TFA (10 mg/mL) and incubating for 20 min at room temperature followed by removal of TFA under a stream of nitrogen. The deprotected protein was purified by HPLC, lyophilized to a fluffy white powder, and characterized by ESI-MS in Geneva: found mass 5852.7 Da; calcd 5850.6 Da.

Creation of Aldehyde Groups on the Templates.

The templates in their Ser form, prepared as above, were oxidized with periodate (7, 12). For this, template (200 μL , 15 mM in water for tetravalent template, 7.5 mM for octavalent, i.e. 12 μmol terminal serine) was diluted with 7.8 mL of imidazole buffer (50 mM, pH 6.95, chloride counterion) and then sodium metaperiodate (240 μL , 100 mM in water, i.e. 24 μmol) was added with mixing. After 5 min, the oxidation was quenched by adding 480 μL of ethylene glycol solution (100 mM in water, i.e. 48 μmol). The oxidized templates were isolated by semipreparative HPLC and characterized as the expected tetravalent and octavalent aldehydes (see Figure 1) through the products formed in oximation reactions: no signals were obtainable upon ESI-MS analysis of the polyaldehydes, probably because no positively charged group was present.

Oxime Formation as a Function of pH. Solutions were prepared of the MSH analog $\text{NH}_2\text{-OCH}_2\text{CO-Nle-Asp-His-(D-Phe)-Arg-Trp-Lys-NH}_2$ (5 mM in water) and glyoxylyl-Leu-Leu (10 mM in water), and oxime formation was started by mixing 40 and 60 μL , respectively, of these solutions with 100 μL of buffer: 0.2 M formate (Na), pH 3.0; 0.2 M acetate (Na), pH 4.6; 0.2 M acetate (Na), pH 5.3. The aldehydic peptide was thus initially in a 3-fold excess over the aminooxyacetyl-MSH derivative. At various times (2 min to 24 h), aliquots (10 μL) were analyzed by HPLC using a linear gradient of 1% B/min from 20 to 60%, and yields were calculated from a standard curve obtained with a solution of previously purified oxime of which the concentration had been determined by weight. The oxime was characterized by ESI-MS (found mass 1357.5 Da, calcd 1356.3 Da) and used for oxime stability tests as described just below.

Oxime Stability. The small model peptide oxime obtained by coupling glyoxylyl-Leu-Leu to the MSH analog $\text{NH}_2\text{-OCH}_2\text{CO-Nle-Asp-His-(D-Phe)-Arg-Trp-Lys-NH}_2$ was used to study the stability of the oxime bond under neutral to mildly alkaline conditions. A 5 mM solution of the oxime in water was diluted 10 times in different buffers [0.1 M phosphate (Na), pH 7.0 and 8.0;

¹ Abbreviations: DMF, dimethylformamide; Fmoc, fluorenylmethyloxycarbonyl; TBTU, 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate; HOBT, 1-hydroxybenzotriazole; DIEA, *N,N*-diisopropylethylamine; Boc, *tert*-butoxycarbonyl; TFA, trifluoroacetic acid; HPLC, reversed phase high-pressure liquid chromatography; ESI-MS, electrospray ionization mass spectrometry; OSu, *N*-hydroxysuccinimide ester; MSH, α -melanocyte stimulating hormone; Msc, methylsulfonylmethylloxycarbonyl.

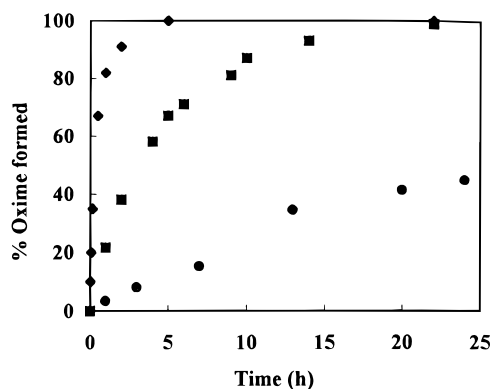


Figure 2. Extent of oxime formation, as a function of time and pH, between glyoxylyl-Leu-Leu (initial concentration 3 mM) and the MSH analog $\text{NH}_2\text{-OCH}_2\text{CO-Nle-Asp-His-(D-Phe)-Arg-Trp-Lys-NH}_2$ (initial concentration of 1 mM). The pH values of the reaction mixtures were 3.0 (◆), 4.6 (■), and 5.3 (●).

0.1 M $\text{NaHCO}_3/\text{Na}_2\text{CO}_3$, pH 9.0 and 10), and the solutions were analyzed by HPLC after 2, 20, and 65 h at room temperature (approximately 22 °C).

Polyoxime Formation as a Function of pH. Solutions were prepared of the hexaaldehyde template $\text{O=CHCO-Gly}_3\text{-[Lys(COCHO)]}_5\text{-Gly-OH}$ (10 mM in water; see ref 7 for preparation) and the aminoxyacetyl peptide $\text{NH}_2\text{-OCH}_2\text{CO-KLEEQRPERVKG-OH}$ (10 mM separately in each of the following: 0.1 M sodium acetate buffer, pH 4.6, 0.1% acetic acid, 0.1% TFA, and 0.1 M sodium phosphate buffer, pH 7.0; see ref 7 for peptide preparation). Oxime formation was initiated by mixing 25 μL (0.25 μmol) of template with 400 μL (4.0 μmol) of peptide derivative. The peptide derivative was thus present in a 2.7-fold excess over each aldehyde group on the template. After incubation at room temperature for various times, the reaction mixture was analyzed by HPLC. The polyoxime product was then isolated by shallow gradient semipreparative HPLC and characterized by mass spectrometry.

Insulin Tetra- and Octahydrazone. Oxidized templates were dissolved (10 mM in acetonitrile/0.1% acetic acid, 4:1 v/v) and then diluted to 1 mM (i.e. 4 or 8 mM in aldehyde groups for the tetra- and octaaldehyde templates, respectively) with 0.1% acetic acid. Sufficient des-Ala^{B30}-insulinylnicarbohydrazide (1 mM in 0.1% acetic acid) was then added to give a molar excess of 1.3 over aldehyde groups on the template. After incubation at room temperature for various times, the reaction mixture was analyzed by HPLC and the polyhydrazone product isolated by shallow gradient semipreparative HPLC and characterized by ESI-MS.

Insulin Octaoxime. Oximation, isolation, and characterization followed the procedure used for insulin hydrazone formation except that, in place of des-Ala^{B30}-insulinylnicarbohydrazide, des-Ala^{B30}-insulinyln-Phe-NHCH₂-CH₂NH-COCH₂ONH₂ and aminoxyacetyl^{B1}-insulin were used with the tetra- and octaaldehyde templates, respectively. After isolation, the insulin octaoxime gave no signal under our standard conditions (7), presumably because its *m/z* values lay beyond the range of our quadrupole instrument (*m/z* < 3000). Therefore, the sample was analyzed on the Manitoba electrospray ionization time-of-flight spectrometer (18), an instrument that, in principle, has an infinite mass range.

RESULTS AND DISCUSSION

Oxime Formation as a Function of pH. Figure 2 shows the progress of the formation of a single oxime bond between glyoxylyl-Leu-Leu and $\text{NH}_2\text{-OCH}_2\text{CO-Nle-Asp-His-(D-Phe)-Arg-Trp-Lys-NH}_2$ in aqueous medium at three pH values. The reaction goes essentially to comple-

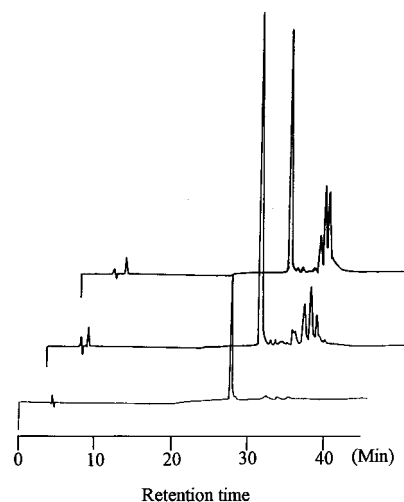


Figure 3. Extent of polyoxime formation after 4 h, monitored at 214 nm: upper trace, pH 2.1; middle trace, pH 4.6; lower trace, pH 7.0. Reaction studied is that between the hexaaldehyde $\text{O=CHCO-Gly}_3\text{-[Lys(COCHO)]}_5\text{-Gly-OH}$ (all consumed) and the aminoxyacetyl peptide $\text{NH}_2\text{-OCH}_2\text{CO-KLEEQRPERVKG-OH}$ (excess elutes at retention time ca. 27 min.). The triplet of reaction products at retention times 30–35 min were characterized as, in order of increasing retention time, tetra-, penta-, and final hexaoxime product (7). The gradient used was 5 min at 0% B, 2% B/min to 20% B, 0.5%/min to 35% B, hold for 5 min, and then return to 0% B over 5 min.

tion at pH 3.0 and 4.6 but is markedly faster at the lower pH: the reaction was still progressing at pH 5.3 after 24 h. These curves resemble those published earlier (13) for hydrazone formation, except that oximation yields are essentially quantitative. To try to understand the increasing rate of reaction with increasing pH reported (1) for both polyhydrazone and polyoxime formation in the range pH 4.1–5.7, we studied the formation of a polyoxime (below).

Polyoxime Formation as a Function of pH. Figure 3 shows the analytical HPLC profiles of the oximation reaction between the hexaaldehyde $\text{O=CHCO-Gly}_3\text{-[Lys(COCHO)]}_5\text{-Gly-OH}$ and the peptide $\text{NH}_2\text{-OCH}_2\text{CO-KLEEQRPERVKG-OH}$ in aqueous medium conducted at pH 2.1, 4.6, and 7.0. In accord with Figure 2, formation of final hexaoxime product is definitely fastest at pH 2.1 and somewhat slower at pH 4.6, and the oximation reaction does not take place at all at pH 7.0. At pH 4.6, hexaoxime formation has been shown to be essentially complete after about 18 h (7). We have carried out many oximation reactions with several tens of different components and have always found faster reaction at lower pH values.

Oxime Stability. The stability of the oxime formed between glyoxylyl-Leu-Leu and $\text{NH}_2\text{-OCH}_2\text{CO-Nle-Asp-His-(D-Phe)-Arg-Trp-Lys-NH}_2$ was studied at room temperature in aqueous medium at pH 7.0, 8.0, 9.0, and 10. Analysis by HPLC showed evidence of instability after prolonged (65 h) exposure to pH 10: Figure 4a (upper trace) shows that under these conditions the oxime (retention time of 26 min) decomposes substantially to give a peak with a retention time of 15 min. (No attempt was made to characterize the decomposition products.) This decomposition is much less marked if the 65 h incubation is performed at pH 9.0 (Figure 4a, middle trace). Decomposition is not detected at shorter times or incubation at less extreme pH: 2 h at pH 9.0, Figure 4a, lower trace; 20 h at pH 8.0, Figure 4b, lower trace; 65 h at pH 7.0, Figure 4b, upper trace. While these results do show evidence of oxime instability at high pH, there are no signs of instability in the physiological range, confirming previous work (7).

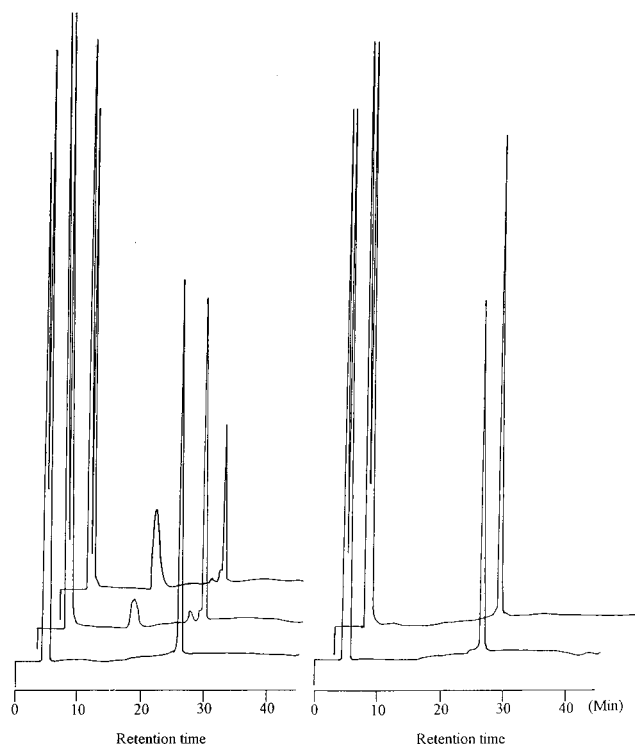


Figure 4. HPLC analysis of the oxime after incubation at room temperature for various times at various pH values, monitored at 214 nm: (a, left) upper trace, pH 10 after 65 h, middle trace, pH 9.0 after 65 h, lower trace, pH 9 after 2 h; (b, right) lower trace, pH 8.0 after 20 h, upper trace, pH 7.0 after 65 h. The gradient used was 5 min at 25% B, 2% B/min to 85% B, and then return to 25% B over 2 min.

Large Polyhydrazones and Polyoximes. Insulin modified enzymatically with carbohydrazide at position B²⁹ was incubated (separately) with a synthetic tetraaldehyde and octaaldehyde in aqueous solution at acidic pH. Formation of the expected polyhydrazone was monitored by HPLC and the product isolated and characterized by ESI-MS. Figure 5 shows the chromatogram obtained with the octaaldehyde reaction after 29 h: excess insulin monomer (retention time of 32 min) is clearly separated from the octahydrazone reaction product (retention time of 49 min). Figure 6 shows the ESI mass spectrum of the insulin octahydrazone: signals appear at higher m/z values than is usual for natural proteins of this size, and many are outside the calibration range (m/z 700–1300) which was established using horse myoglobin. In spite of this, agreement of experimental (47 605 Da) and calculated masses (47 611 Da) is very good. A small amount of heptahydrazone is visible in Figure 6 (responsible for signals at m/z 2620, 2465, 2328, 2205); this may be due to failure to drive hydrazone formation to completion with the 1.3-fold excess of insulin derivative used or possibly to spontaneous hydrolysis under the acidic conditions used to isolate and characterize the product. Using the tetraaldehyde in place of the octaaldehyde, an HPLC profile very similar to that shown in Figure 5 was obtained after 24 h and the corresponding insulin tetrahydrazone was isolated and found to have a mass of 23 834 Da (data not shown), very close to the theoretical value of 23 832 Da.

Using two aminoxy derivatives of insulin in place of the carbohydrazide derivative, HPLC profiles very similar to that shown in Figure 5 were obtained after 24 h and the corresponding insulin tetra- and octaoximes were formed and characterized by ESI-MS. The mass spectrum of the tetraoxime (Figure 7) is that of a single component of mass 24 590 Da, in excellent agreement with the calculated value of 24 593 Da. As pointed out

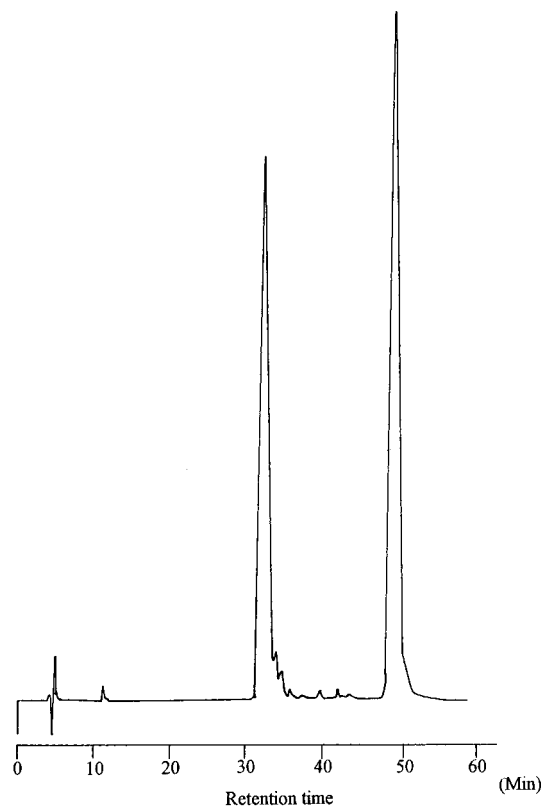


Figure 5. Analytical HPLC of the reaction between des-Ala^{B30}-insulinylnicarbohydrazide and the octaaldehyde template shown in Figure 1, monitored at 214 nm. All template is consumed, and excess insulin derivative elutes (retention time of 32 min) well before the octahydrazone product (retention time of 49 min), which was characterized by ESI-MS. The gradient used was 5 min at 30% B, 0.33% B/min to 50% B, hold for 5 min, and then return to 30% B over 5 min.

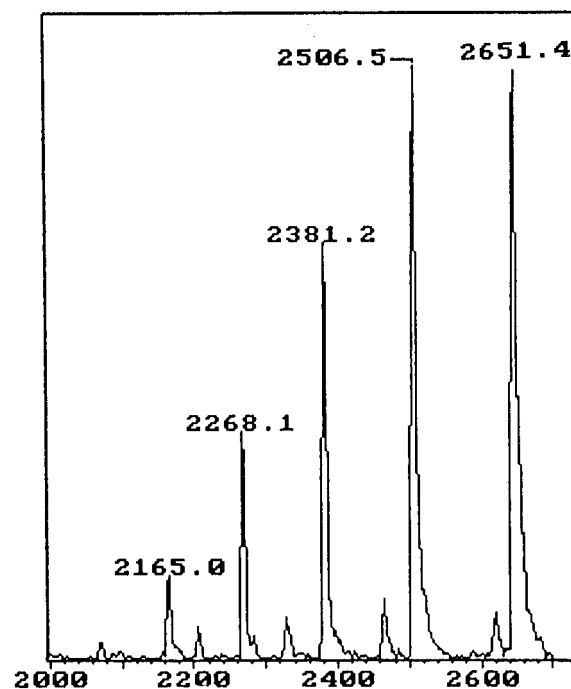


Figure 6. Mass spectrum of the insulin octahydrazone. The series of major signals leads to mass determination of 47 605 Da, calcd 47 611 Da. A few signals of low intensity (m/z 2620, 2465, 2328, 2205) are due to the presence of the heptahydrazone.

above, the octaoxime gave no signal on the Geneva machine, but analysis on the Manitoba time-of-flight instrument was successful in characterizing the reaction

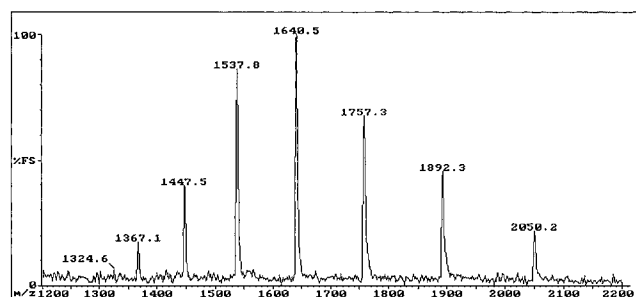


Figure 7. Mass spectrum of the insulin tetraoxime. The spectrum is that of a single component of mass 24 590 Da, in excellent agreement with the calculated mass of 24 593 Da.

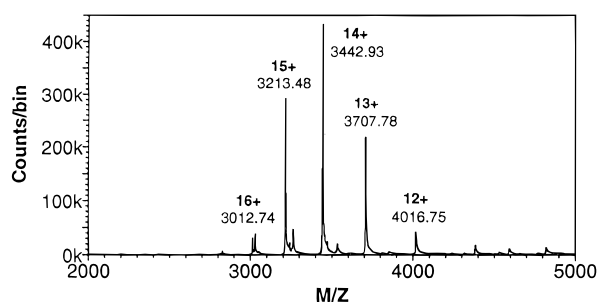


Figure 8. Mass spectrum of the insulin octaoxime obtained on a machine with electrospray ionization and a time-of-flight analyzer (18). The spectrum is that of a major component of mass 48 187 Da, in excellent agreement with the calculated mass of 48 188 Da. As with the octahydrazone, a set of minor signals is consistent with the presence of the hepta derivative (found 42 353 and 42 385 Da, calcd 42 356 and 42 388 Da, for aldehyde and methanol hemiacetal forms, respectively).

product (Figure 8) as the expected octaoxime (mass found, 48 187.1 Da; calcd, 48 188.4 Da).

Production of an $F(ab')_3$ trioxime by enzymatic cleavage and partial reduction of an antibody, alkylation with a heterobifunctional linker, and oximation with a trialdehyde has recently been described (14). The product was characterized by mass spectrometry but, owing to natural partial fucosylation of the Fab' , the product was heterogeneous in this respect: species with no, one, two or three fucosylated fragments were present, and no attempt was made to separate them. It should be emphasized that this slight heterogeneity was due to the nature of the native peptide fragment, not to the chemistry.

CONCLUSIONS

The insulin octaoxime and octahydrazone described above are at least twice as massive as previously described synthetic protein-like molecules that have been made by ligating synthetic peptides (1–5, 7–10). Our synthesis of the $F(ab')_3$ trioxime (14) demonstrates further that very large molecules are accessible through use of native protein building blocks and highly selective chemistry. To increase reaction rates, and provided that the peptide components remain stable, we recommend the use of more acid conditions (e.g. 0.1% acetic acid) than those reported previously (1, 7) for the production of artificial proteins through formation of hydrazone or oxime bonds. Polyoximes are currently showing promise as radioimmunodiagnostic agents, as vaccine candidates, and in the nonviral gene therapy field.

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LITERATURE CITED

- (1) Shao, J., and Tam, J. P. (1995) Unprotected peptides as building blocks for the synthesis of peptide dendrimers with oxime, hydrazone, and thiazolidine linkages. *J. Am. Chem. Soc.* 117, 3893–3899.
- (2) Spetzler, J. C., and Tam, J. P. (1995) Chemoselective approaches to the preparation of peptide dendrimers and branched artificial proteins using unprotected peptides as building blocks. *Biomed. Pept., Proteins Nucl. Acids* 1, 123–132.
- (3) Dawson, P. E., Muir, T. W., Clark-Lewis, I., and Kent, S. B. H. (1994) Synthesis of proteins by native chemical ligation. *Science* 266, 776–779.
- (4) Canne, L. E., Ferré-D'Amaré, A. R., Burley, S. K., and Kent, S. B. H. (1995) Total synthesis of a unique transcription factor-related protein: cMyc-Max. *J. Am. Chem. Soc.* 117, 2998–3007.
- (5) Tam, J. P., Lu, Y.-A., Liu, C.-F., and Shao, J. (1995) Peptide synthesis using unprotected peptides through orthogonal coupling methods. *Proc. Natl. Acad. Sci. U.S.A.* 92, 12485–12489.
- (6) Jackson, D. Y., Burnier, J., Quan, C., Stanley, M., Tom, J., and Wells, J. A. (1994) A designed peptide ligase for total synthesis of ribonuclease A with unnatural catalytic residues. *Science* 266, 243–247.
- (7) Rose, K. (1994) Facile synthesis of homogeneous artificial proteins. *J. Am. Chem. Soc.* 116, 30–33.
- (8) Rao, C., and Tam, J. P. (1994) Synthesis of peptide dendrimer. *J. Am. Chem. Soc.* 116, 6975–6976.
- (9) Rose, K., Zeng, W., Dragovic, M., Brown, L. E., and Jackson, D. C. (1996) Polyoxime artificial proteins as vaccines. In *Peptides: Chemistry and Biology*, Proceedings of the 14th American Peptide Symposium (P. T. P. Kaumaya and R. S. Hodges, Eds.) pp 767–769, Mayflower Worldwide, Birmingham, U.K.
- (10) Rose, K., Zeng, W., Brown, L. E., and Jackson, D. C. (1996) A synthetic peptide based polyoxime vaccine construct of high purity and activity. *Mol. Immunol.* 32, 1031–1037.
- (11) Nardelli, B., Haser, P. B., and Tam, J. P. (1994) Oral administration of an antigenic synthetic lipopeptide (MAP-P3C) evokes salivary antibodies and systemic humoral and cellular responses. *Vaccine* 12, 1335–1339.
- (12) Vilaseca, L. A., Rose, K., Werlen, R., Meunier, A., Offord, R. E., Nichols, C. L., and Scott, W. L. (1993) Protein conjugates of defined structure: synthesis and use of a new carrier molecule. *Bioconjugate Chem.* 4, 515–520.
- (13) King, T. P., Zhao, S. W., and Lam, T. (1986) Preparation of protein conjugates via intermolecular hydrazone linkage. *Biochemistry* 25, 5774–5779.
- (14) Werlen, R. C., Lankinen, M., Offord, R. E., Schubiger, P. A., Smith, A., and Rose, K. (1996) Preparation of a trivalent antigen-binding construct using polyoxime chemistry: improved biodistribution and potential for therapeutic application. *Cancer Res.* 56, 809–815.
- (15) Cotten, M., and Wagner, E. (1993) Non-viral approaches to gene therapy. *Current Opin. Biotechnol.* 4, 705–710.
- (16) Rose, K. (1994) Artificial proteins made by parallel assembly, in *Innovation and Perspectives in Solid Phase Synthesis: Peptides, Proteins and Nucleic Acids, Biological and Biomedical Applications* (R. Epton, Ed.) pp 21–28, Mayflower Worldwide, Birmingham, U.K.
- (17) Offord, R. E. (1980) Semisynthetic proteins, 235 pp, Wiley, Chichester, U.K.
- (18) Verentchikov, A. N., Ens, W., and Standing, K. G. (1994) Reflecting time-of-flight mass spectrometer with an electrospray ion source and orthogonal extraction. *Anal. Chem.* 66, 126–133.

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Characterization of RFB4–*Pseudomonas* Exotoxin A Immunotoxins Targeted to CD22 on B-Cell Malignancies

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To develop an immunotoxin for the treatment of B-cell malignancies, we constructed several candidate conjugates with RFB4, a B-cell specific anti-CD22 IgG₁, and truncated forms of *Pseudomonas* exotoxin (PE). The four versions of PE included PE35 and PE35KDEL, which were linked to RFB4 via a disulfide bond, and PE38 and PE38KDEL, which were linked via a thioether bond. The PE35 truncated forms, which are fully active in ADP ribosylation and lack receptor binding sequences, do not require intracellular proteolytic cleavage in order to be active. PE35KDEL has the consensus endoplasmic reticulum retention signal, KDEL, replacing the wild type PE C-terminal sequence, REDLK. The PE38 forms retain all of domain II and therefore require cleavage to be active within cells. Cytotoxicity experiments on CD22-positive cell lines revealed that the PE35 conjugates were more active than the PE38 versions and the presence of the KDEL sequence generally enhanced toxicity by 5–10-fold compared to that of REDLK. The RFB4–PE35KDEL immunotoxin was most active in cytotoxicity assays against Burkitt's lymphoma cell lines such as Daudi and CA46 (IC₅₀ = 0.2 ng/mL) and displayed little cytotoxicity toward human vascular endothelial cells (IC₅₀ > 20 µg/mL). Results of experiments conducted in nude mice showed that both RFB4–PE35KDEL and RFB4–PE35 could inhibit the development of subcutaneous CA46 tumors.

INTRODUCTION

The difficulty of gaining access to all cancer cells within a solid tumor mass may reduce the effectiveness of immunotoxins targeted to solid tumors. Because antigens and receptors found on the surface of B-cell neoplasms are frequently readily accessible from plasma, they make attractive targets for immunotoxin therapy. A number of different B-cell antigens have been successfully targeted both *in vitro* and *in vivo*, for example CD19, CD22, and CD40 (1–3). Immunotoxins directed at the B-cell specific marker CD22, in particular, have been made using several different plant toxins such as ricin A chain, saporin, gelonin, and pokeweed antiviral protein, e.g. (4–7), and the bacterial toxin *Pseudomonas* exotoxin (PE)¹ (8).

CD22 is a lineage-restricted antigen of the Ig superfamily that is expressed on the surface of normal mature B-lymphocytes and on many malignant B-cells, including chronic B-lymphocytic cells (B-CLL), B-lymphoma cells,

and hairy cell leukemias. Previously, immunotoxins using the anti-CD22 antibody RFB4 chemically conjugated to ricin A chain were shown to be highly effective in killing CD22-expressing cells *in vitro* and in preventing and curing experimental B-cell neoplasms induced in SCID and nude mice (2, 4). *Pseudomonas* exotoxin has been incorporated into immunotoxins targeting a variety of tumor types, and we anticipated it would be comparable to ricin A chain in effectiveness in an anti-CD22 conjugate using RFB4. Because several versions of truncated PE have been produced, we thought that a systematic analysis would be a useful way to optimize the construction of an RFB4 immunotoxin.

PE is a three-domain toxin in which domain I at the N-terminus serves as the cell binding moiety, domain II in the middle of the protein allows translocation of PE across membranes, and domain III at the C-terminus harbors the ADP-ribosylating activity. Full length PE molecules require cleavage between amino acids 279 and 280 (9) and subsequent reduction of a disulfide bond in order to be active as toxins.

PE is particularly attractive for making immunotoxin conjugates because it permits linkage to antibodies via disulfide bonds, thioether bonds, and peptide bonds. Truncated versions of PE have been used to construct immunotoxins of many different specificities. A truncated form of the toxin called PE35 has been constructed, beginning at amino acid residue 280 of full length PE, that contains the translocating and ADP-ribosylating domains and does not require proteolysis for activity (10). PE35 and PE35KDEL [where REDLK is replaced with the consensus ER retention signal KDEL (R. Kreitman, unpublished data)] have been conjugated through disulfide linkages to several IgGs and shown to have greater cytotoxic activity toward targeted cell lines (10; unpublished data) and CLL patient samples (11) than conjugates made with PE38, another truncated form of PE composed of all of domains II and III. PE38 differs from PE35 in that it retains the requirement for intracellular proteolysis.

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¹ Abbreviations: PE, *Pseudomonas* exotoxin A; IgG, immunoglobulin G; CLL, chronic lymphocytic leukemia; IC₅₀, 50% inhibitory concentration; SCID, severe combined immunodeficient; sIg⁺, surface immunoglobulin; EBV, Epstein–Barr virus; dgA, deglycosylated ricin A chain; IPTG, isopropyl β-D-thiogalactopyranoside; SMCC, succinimidyl 4-(N-maleimidomethyl)-cyclohexane-1-carboxylate; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); DTE, dithioerythritol; DMSO, dimethyl sulfoxide; PBS, phosphate-buffered saline; TNB, thiobis(2-nitrobenzoic acid); TE, 10 mM Tris and 1 mM EDTA; HSA, human serum albumin; FBS, fetal bovine serum; HUVEC, human umbilical vein endothelial cells; BSA, bovine serum albumin; NAD, nicotinamide adenine dinucleotide; EF-2, elongation factor-2; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol; TCA, trichloroacetic acid; BES, N,N-bis(2-hydroxyethyl)-2-amino-ethanesulfonic acid; SDS, sodium dodecyl sulfate; Fv, antibody variable region; VLS, vascular leak syndrome; LD₅₀, 50% lethal dose; iv, intravenous; TFR, transferrin receptor.

RFB4 is a murine IgG₁ anti-CD22 monoclonal antibody that reacts exclusively with B-cells in normal human tissues [92–96% of sIg⁺ cells (12)] and with pre-B, Burkitt's, and plasmacytoid cell lines but not with pre-pre-B, EBV-transformed mature B, myeloma, or non-B-cell types in culture (13). Li et al. (13) found that no RFB4-reactive (i.e. CD22) molecules could be detected in normal human sera and sera from patients with CLL, suggesting that this antigen is not shed into the plasma. The immunotoxin RFB4–dgA, in which RFB4 IgG is disulfide-linked to deglycosylated ricin A chain, has been extensively tested in human clinical trials (14–16) and has demonstrated no toxicities except those related to ricin itself. For these reasons, the RFB4 antibody is an excellent candidate for conjugation to PE toxins for treatment of certain B-cell neoplasms. We have examined the efficacy of anti-CD22 immunotoxin conjugates constructed with four different truncated forms of PE.

EXPERIMENTAL PROCEDURES

Antibodies. RFB4 IgG was provided by E. Vitetta (UTSMC, Dallas, TX) and was prepared as described (17).

Toxins. PE35, PE35KDEL, N-LysPE38, and PE38-KDEL were prepared from the periplasm of *Escherichia coli* as described for LysPE38KDEL (8). Expression of recombinant toxin molecules was induced by the addition of IPTG, which served to increase the T7-mediated transcription of the toxin gene.

Cell Lines. HUT102 cells were a gift from T. Waldmann (NCI), and CA46, Raji, and JD38 lines were from I. McGrath (NIH). A431 cells were obtained from G. Todaro (Seattle). Daudi and HL-60 cells were purchased from ATCC (Rockville, MD), and HUVEC were purchased from Clonetics (San Diego, CA).

Reagents. 2-Iminothiolane and SMCC were purchased from Pierce (Rockford, IL). PD-10 desalting columns and Mono-Q anion exchange columns were from Pharmacia Biotech (Piscataway, NJ). DTNB, DTE, and DMSO were purchased from Sigma (St. Louis, MO). Centricon concentrators were from Amicon (Bedford, MA), and TSK G3000SW columns were from Toso-Haas (Montgomeryville, PA).

Conjugation. RFB4 IgG was modified with a 5–7-fold molar excess of 2-iminothiolane in Dulbecco's PBS at pH 7.4 for 1 h at 37 °C. The modified antibody was desalted over a PD-10 column equilibrated in PBS at pH 7.4. For modified antibody used for making disulfide-linked conjugates, the free sulfhydryls were blocked with DTNB added to a final concentration of 1 mM. Moles of free sulfhydryl per mole of protein were calculated as described (18). Excess DTNB and TNB were removed by desalting as above. PE35 and PE35KDEL were reduced with 100 mM DTE at room temperature and desalted over PD-10 columns as above. PE38 and PE38KDEL were modified in PBS at pH 7.4 with a 10-fold molar excess of SMCC at room temperature for 1 h and then desalted over PD-10 columns as above. The antibody and PE were combined and concentrated to 0.5–1 mL in Centricon-30 concentrators (Amicon), incubated for several hours at room temperature, and then refrigerated overnight. Conjugated material was passed over Mono-Q anion exchange resin to separate PE and conjugates from free antibody. The column was eluted with a 0 to 500 mM linear gradient of NaCl in TE. Conjugate (1:1 Ab–PE) eluted at approximately 240 mM NaCl with some contamination from unreacted PE. Gel filtration over a 2.15 × 60 cm G3000SW column equilibrated in PBS at pH 7.4 separated IgG–PE conjugates from unconjugated PE. For the storage of some samples, human serum albumin (HSA) was added to 0.2%, al-

though omission of HSA at immunotoxin concentrations at or above 200 µg/mL did not result in reduced cytotoxic activity. Samples were sterile filtered through Millex-GV filters and frozen at –80 °C.

Stability of Conjugates in Plasma. Plasma was pooled from three healthy donors who did not have neutralizing antibodies against PE. Immunotoxins were diluted 1:10 in plasma and in PBS/0.2% HSA and incubated for different times at 37 °C. Standard cytotoxicity assays were performed to determine the activity of treated immunotoxins.

Cytotoxicity Assays. Cells were maintained in RPMI 1640 containing 10% fetal bovine serum (FBS), 50 u/mL penicillin, 50 µg/mL streptomycin, 1 mM sodium pyruvate, and an additional 2 mM L-glutamine. For cytotoxicity assays, 4 × 10⁴ cells in 50 µL of culture medium were plated in 96-well plates. HUVEC were grown in endothelial cell growth medium (EGM) plus bovine brain extract, both purchased from Clonetics. Cells in the 3rd to 5th passage were seeded in 96-well plates at 4000 cells/well in 200 µL for 3 days at 37 °C. For Daudi, CA46, Raji, JD38, HL60, and HUT 102 cytotoxicity experiments, immunotoxins were serially diluted in RPMI + 10% FBS and 50 µL was added to cells. For HUVEC experiments, immunotoxins were diluted in PBS/0.2% bovine serum albumin (BSA) and 10 µL was added to cells. Plates were incubated for 20–24 h at 37 °C and then pulsed with 1 µCi/well [³H]leucine in 50 µL of normal or leucine-free RPMI for 4–5 h at 37 °C (Daudi, CA46, HL60, and HUT 102) or 1 µCi/well in 10 µL of PBS for 7 h (HUVEC). Radiolabeled material was captured on filter mats and counted in a Betaplate scintillation counter (Pharmacia, Gaithersburg, MD). Quadruplicate sample values were averaged, and inhibition of protein synthesis was determined by calculating percent incorporation compared to control wells without added toxin.

In Vivo Toxicity. Female BALB/c 6–8-week-old mice were obtained from the National Cancer Institute (Frederick, MD). Various amounts of immunotoxins diluted in 200 µL of PBS/0.2% HSA were injected into the tail vein. Two or more mice were injected for each point and were observed over a period of 7 days for deaths or severe toxicity.

Inhibition of Tumor Development. Female 6–8-week-old athymic nude mice were obtained from the National Cancer Institute. Mice were irradiated with 300 rad of total body irradiation 3 days prior to injection of tumor cells. CA46 human Burkitt's lymphoma cells were seeded at 1.8 × 10⁵/mL 2 days prior to injection in mice. Cells were washed and suspended in serum-free RPMI, and 8 × 10⁶ to 1 × 10⁷ cells in 200 µL were injected subcutaneously on the dorsal flank on day 0. Mice were injected with immunotoxin or PBS/0.2% HSA diluent in the tail vein every day for four doses starting on day 1 (24 h after injection of tumor cells). Tumor size was measured at 1–3 day intervals with precision calipers, and the volumes in mm³ were calculated by the equation $v = 0.4w^2l$, where w is width and l is length.

ADP-Ribosylating Activity. ADP ribosylation activity of RFB4–PE35, RFB4–PE35KDEL, and RFB4–PE38 was determined by measuring transfer of ADP-ribose from [¹⁴C]NAD to EF-2 by the method of Collier and Kandel. Immunotoxins were diluted to 205 µL in 50 mM Tris (pH 8), 1 mM EDTA, 0.1% BSA, and 40 mM DTT and incubated with wheat germ extract in the presence of 2.4 µM [¹⁴C]NAD (6 × 10⁵ cpm) for 15 min at 22 °C. Protein was precipitated with TCA and pelleted. Pellets were washed once in 6% TCA, solubilized, and counted in a scintillation counter.

Binding Studies. Daudi cells grown to >10⁶/mL were

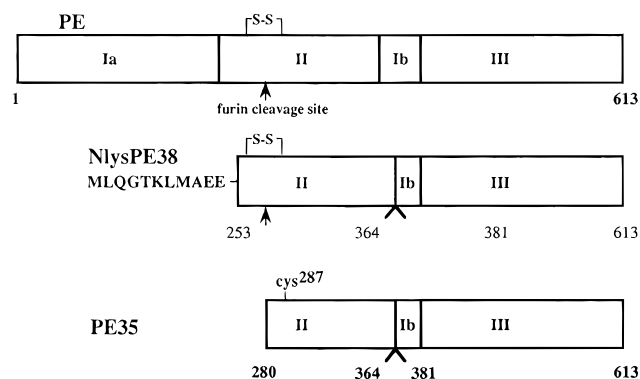


Figure 1. Schematic model of forms of PE used to make RFB4 conjugates. Full length PE comprises domains I, II, Ib, and III. The furin cleavage site is within the domain II disulfide loop. N-LysPE38 is deleted for domain I. An 11-amino acid sequence has been added to provide a lysine residue for modification with SMCC. PE35 has a deletion of domain I and a part of domain II up to amino acid 280. No disulfide loops remain. Ser²⁸⁷ is replaced with Cys to provide a free sulfhydryl for conjugation.

washed twice in ice cold binding buffer [RPMI, 50 mM BES (pH 6.8), and 1% BSA] and plated at 10^6 cells per 150 μ L of binding buffer per well on 96-well plates on ice. To the cells were added 0.672 ng of [¹²⁵I]RFB4 (7.3×10^8 cpm/nmol) in binding buffer and varying concentrations of RFB4-PE35, RFB4-PE35KDEL, and RFB4-PE38. Cells were incubated for 3 h on ice, washed twice in cold binding buffer, and solubilized in 200 μ L of 0.5% SDS/TE. Bound [¹²⁵I]RFB4 was quantitated on a Wallac 1470 Wizard γ counter. The means of duplicate samples were used for calculations.

RESULTS

Conjugation of RFB4 with Truncated Forms of PE. To evaluate the utility of PE for treating B-cell tumors, several different immunotoxins were made with the anti-CD22 antibody RFB4. RFB4 IgG was chemically conjugated to two truncated forms of PE called PE38 and PE35 (Figure 1). The KDEL C-terminal variants PE35-KDEL and PE38KDEL were also tested. Because PE38 and PE38KDEL do not have free sulfhydryls, a reactive maleimide was introduced using the heterobifunctional cross-linking agent SMCC. Reduced PE35 and PE35-KDEL each contain a single free sulfhydryl which can be used for conjugation by formation of a stable disulfide bond. This approach causes no loss of toxin activity. Prior to conjugation, RFB4 IgG was reacted with 2-iminothiolane to introduce an average of one free sulfhydryl per IgG and then mixed with either SMCC-modified PE38 or reduced PE35. The resulting conjugates were purified free of unreacted antibody and PE by sequential Mono-Q anion exchange and TSK gel filtration chromatography. RFB4-PE38, RFB4-PE38KDEL, RFB4-PE35, and RFB4-PE35KDEL were indistinguishable in their Mono-Q and TSK elution profiles. The 1:1 and 1:2 RFB4-PE conjugates eluted sequentially from Mono-Q in separate peaks, and the 1:2 conjugates were discarded. RFB4-PE38 and RFB4-PE38KDEL are thioether-linked conjugates in which the active PE moiety cannot be released without proteolysis. In contrast, RFB4-PE35 and RFB4-PE35KDEL and disulfide-linked conjugates, and the active toxin component can be released by reduction without proteolysis.

Cytotoxicity. RFB4 conjugates with PE38, PE38-KDEL, PE35, and PE35KDEL were tested for their ability to inhibit protein synthesis against the CD22-positive Burkitt's lymphoma cell lines Daudi, CA46, JD-38, and Raji and the non-B, CD22-negative lines HL-60

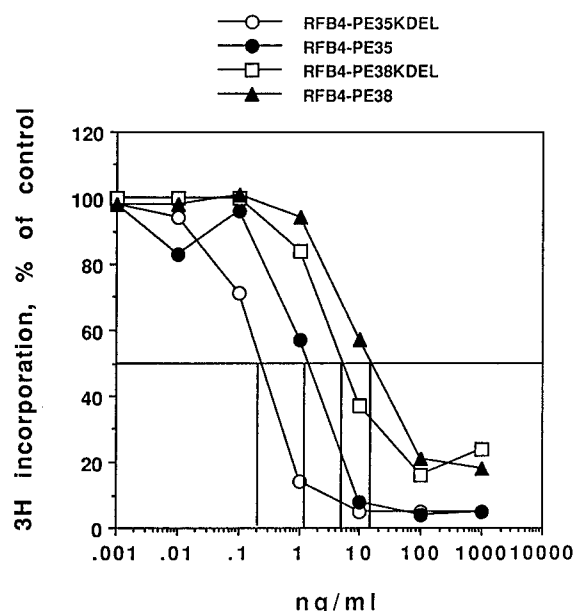


Figure 2. Cytotoxicity of RFB4-PE conjugates on Daudi cells. Cytotoxicity of RFB4 monoclonal antibody conjugates containing PE35, PE35KDEL, PE38, and PE38KDEL after 24 h of incubation. Values are representative of at least three experiments for each conjugate.

(promyelocytic leukemia) and HUT 102 (T-cell lymphoma). RFB4-PE35 and RFB4-PE35KDEL were very active on CD22-positive lines [e.g. IC₅₀ values of 1 ng/mL for RFB4-PE35 and 0.2 ng/mL for RFB4-PE35KDEL on Daudi (Figure 2) and CA46 cells], while RFB4-PE38KDEL and RFB4-PE38 had only moderate cytotoxic activity toward the same lines [IC₅₀ values of 10 and 15 ng/mL on Daudi (Figure 2) and CA46 cells]. The CD22-negative lines were insensitive to all RFB4 immunotoxins [IC₅₀ values of >1000 ng/mL for all RFB4 conjugates on HL-60 and HUT 102 (not shown)]. Unconjugated RFB4 IgG, PE35, PE35KDEL, and PE38 were not cytotoxic toward any of the cell lines at concentrations of up to 1000 ng/mL (not shown). PE38KDEL was not tested on these cell lines.

Specificity. To confirm that the RFB4 conjugates bound specifically to CD22, competition assays were performed in which excess unconjugated RFB4 IgG was mixed with RFB4-PE35 and RFB4-PE35KDEL and was added to CA46 and Daudi cells. Cytotoxicity caused by either of the conjugates was reduced more than 100-fold by addition of 10–30 μ g/mL unconjugated RFB4 IgG (values for Daudi cells, which are similar to those for CA46, are shown in Figure 3). No competition occurred when a 10–30 μ g/mL mAb directed toward an unrelated antigen was added. The possibility that toxicity could be caused by nonspecific internalization of immunotoxin was excluded by assays showing that a nonrelevant immunotoxin conjugate analogous to RFB4-PE35KDEL did not cause inhibition of protein synthesis in Daudi (Figure 3) and CA46 cells, which do not display the targeted antigen.

Cytotoxicity against Endothelial Cells. Cytotoxicity against human vascular endothelial cells (HUVEC) has been suggested as a determinant of the propensity of immunotoxins to cause dose-limiting vascular leak syndrome when administered to human patients (19). Several ricin-containing immunotoxins, in particular RFB4-dgA, have been observed to cause HUVEC toxicity (19) and vascular leak syndrome (VLS) (14–16). We therefore tested RFB4-PE35KDEL for its cytotoxic activity against primary cultures of HUVEC, which arise

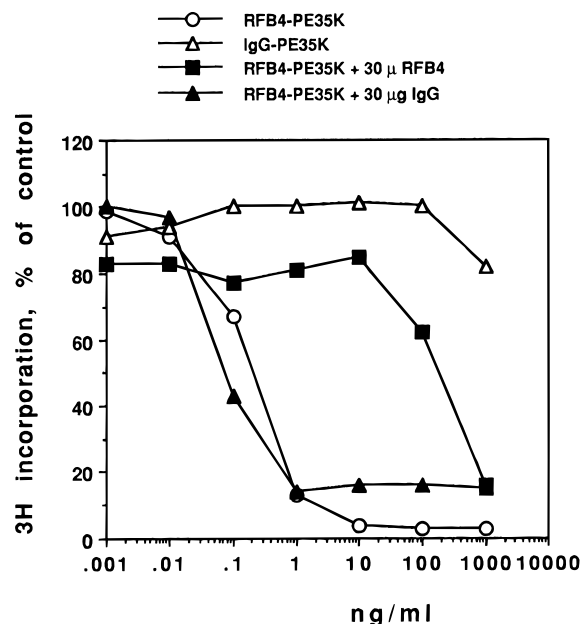


Figure 3. Specific cytotoxicity of the RFB4-PE35KDEL conjugate toward Daudi cells. RFB4 antibody (30 μ g) competes >100-fold of RFB4-PE35KDEL cytotoxicity. Nonrelevant PE35KDEL conjugate is not cytotoxic on Daudi cells at concentrations which are toxic for RFB4-PE35KDEL. Values represent two experiments which gave identical results.

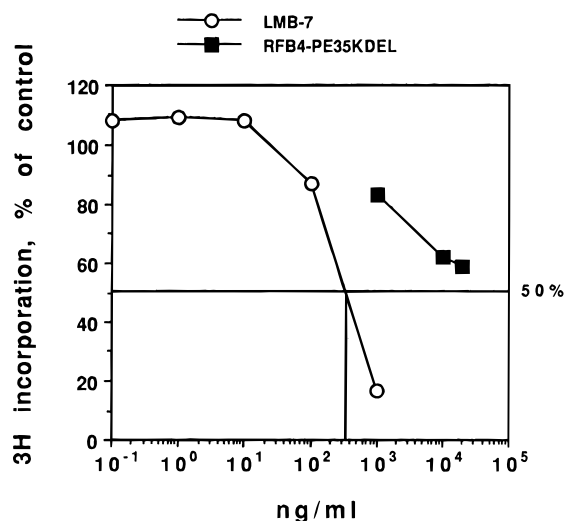


Figure 4. Cytotoxicity of RFB4-PE35KDEL on HUVECs. RFB4-PE35KDEL has an IC_{50} of >20 000 ng/mL on HUVECs after 24 h of exposure. LMB-7, another PE-containing immunotoxin, is more than 10 times more cytotoxic than RFB4-PE35KDEL.

outside of the hematopoietic lineage and thus are not expected to express CD22. The IC_{50} of RFB4-PE35KDEL was greater than 20 000 ng/mL (Figure 4), suggesting that, if HUVEC cytotoxicity can be correlated with VLS, this immunotoxin would not likely cause VLS in humans by killing endothelial cells.

ADP-Ribosylating Activity of Conjugates. The relative ADP-ribosylating activity of each conjugate was measured by *in vitro* assay, and the activities of different RFB4 immunotoxins were compared. RFB4-PE35, RFB4-PE35KDEL, RFB4-PE38, and RFB4-PE38KDEL differed from one another in ADP-ribosylating activity by not more than 2-fold (data not shown), demonstrating that the difference in cytotoxic activities of these conjugates was not due to a reduction in the enzymatic capacity of the PE portion of the molecules.

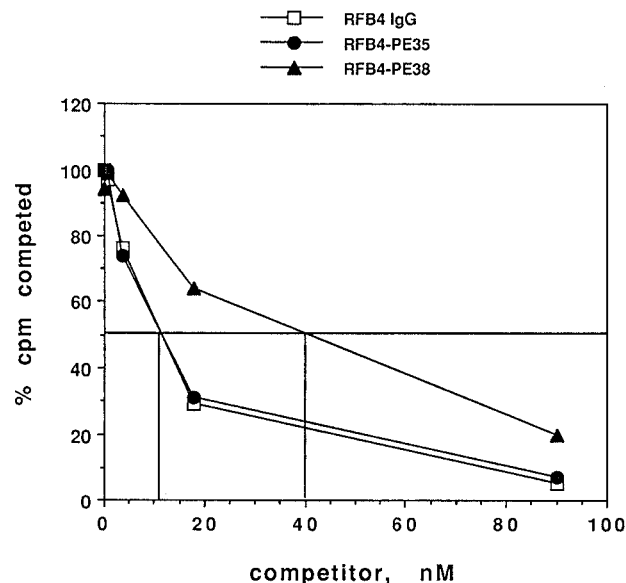


Figure 5. Binding of RFB4-PE conjugates to Daudi cells. [^{125} I]-RFB4 antibody binding is competed 50% by 12 nM RFB4 IgG and RFB4-PE35. RFB4-PE38 competes 50% of counts at 40 nM. Competition was performed under equilibrium binding conditions at 4 $^{\circ}$ C. Binding values were determined in two experiments with nearly identical results.

Table 1. Multiple-Dose LD_{50} Values

immunotoxin ^a	dose (μ g $\times$ 4) ^b	survivors/total
RFB4-PE35	20	2/2
	30	0/2
	40	0/2
RFB4-PE35KDEL	2	2/2
	4	2/2
	5	1/2
	7.5	0/2

^a Immunotoxins were diluted in PBS/0.2% HSA and administered iv in a volume of 200 μ L. ^b Doses shown were given at 24 h intervals for 4 consecutive days. Mice were observed for toxicity for 7 days after last administration in immunotoxin.

Binding of RFB4 Immunotoxins to Daudi Cells. To ensure that conjugation of RFB4 to PE35 and PE38 did not cause a reduction in binding to the CD22 antigen, the capacity of the immunotoxins to displace [^{125} I]RFB4 IgG on Daudi cells was examined. Daudi cells grown to >10⁶ cells/mL (10⁶ cells/well, 0.2 mL) were incubated with 0.0225 nM [^{125} I]RFB4 alone and 0.0225 nM [^{125} I]RFB4 plus 0.036–22.5 nM RFB4-PE35, RFB4-PE38, and unlabeled RFB4 IgG at 4 $^{\circ}$ C. Both RFB4-PE35 and RFB4 IgG displaced 50% of [^{125} I]RFB4 at 12 nM, demonstrating that conjugation of PE35 to RFB4 through a disulfide linkage resulted in no loss of binding activity (Figure 5). RFB4-PE38 competed 50% of bound [^{125} I]-RFB4 at 40 nM.

Toxicity of RFB4-PE35KDEL and RFB4-PE35 in Mice. Because RFB4-PE35 and RFB4-PE35KDEL were the most active of the conjugates in tissue culture cytotoxicity experiments, we evaluated their usefulness as *in vivo* reagents. To select the maximum tolerated doses of RFB4-PE35KDEL and RFB4-PE35 for anti-tumor experiments, LD_{50} values were determined by multiple-dose iv injection of varying amounts of immunotoxin into the tail veins of BALB/c mice (Table 1). Two mice were tested for each concentration of immunotoxin, and at each dose level, immunotoxin was administered daily for 4 consecutive days. At doses of 4 $\times$ 4 μ g of RFB4-PE35KDEL, all mice survived without obvious toxicity. At 4 $\times$ 5 μ g, one mouse died after the fourth dose. Doses greater than 4 $\times$ 5 μ g of RFB4-PE35KDEL

were rapidly lethal (death occurred 2 days after first administration of immunotoxin) to all of the mice. The multiple-dose LD₅₀ of RFB4-PE35 was between 4×20 and $4 \times 30 \mu\text{g}$, because no mice in the group receiving the $4 \times 20 \mu\text{g}$ dose but both mice in the $4 \times 30 \mu\text{g}$ dose group died.

Stability of RFB4-PE35 and RFB4-PE35KDEL in Plasma. In order for a disulfide-linked immunotoxin to be effective as an antitumor agent *in vivo*, the disulfide bond must be stable to reduction in the bloodstream. The stability of the disulfide-linked immunotoxins RFB4-PE35 and RFB4-PE35KDEL was examined by incubating these immunotoxins in human plasma at 37 °C for times ranging from 30 min to 24 h. Cytotoxic activity of treated samples on CA46 cells was compared to that of samples that were diluted in plasma and added to cells immediately without incubation at 37 °C. No change in activity compared to unincubated controls was seen in samples incubated for up to 24 h (data not shown), indicating that inactivation of RFB4 disulfide-linked immunotoxins in blood is not rapid.

Inhibition of CA46 Tumor Development with RFB4-PE35KDEL. The excellent cytotoxic activities of RFB4-PE35KDEL and RFB4-PE35 *in vitro* indicated that they should be evaluated for *in vivo* activity. Therefore, the ability of RFB4-PE35 immunotoxins to prevent or delay formation of CA46 tumors in irradiated nude mice was assessed (Figure 6). Mice that had been inoculated with CA46 cells were randomly assigned to treatment groups, with four or five mice in each group. Mice were administered four iv doses each of RFB4-PE35KDEL (1–4 μg) or RFB4-PE35 (1–10 μg), RFB4 IgG (1–5 μg), IgG-PE35KDEL (nonrelevant immunotoxin control), or PBS/0.2% HSA diluent control. Treatment was begun 24 h after injection of cells and administered at 24 h intervals for 4 days. By day 21, tumors in mice receiving diluent, 1 μg of RFB4 IgG or IgG-PE35K, grew to a size averaging 1700 mm³. Treatment with 5 μg of RFB4 IgG resulted in tumors averaging 1560 mm³ on day 21. Ninety-one percent of mice that had received $4 \times 1\text{--}4 \mu\text{g}$ of RFB4-PE35KDEL had no detectable tumors and did not develop tumors in the subsequent 90 days in which they were observed. One mouse out of 10 treated with $4 \times 2 \mu\text{g}$ RFB4-PE35KDEL developed a slow-growing tumor that was 12% (205 mm³) of the average size of tumors in the control group on day 21. Additionally, two of 14 mice treated with $4 \times 1 \mu\text{g}$ of RFB4-PE35KDEL developed tumors (44 and 8% of average control tumor size), while cage mates remained tumor-free. Two of eight surviving mice treated with $4 \times 1 \mu\text{g}$ of RFB4-PE35 did not develop tumors; four of five mice treated with 2 μg of RFB4-PE35 grew tumors that were on average 38% of the size of those of control-treated mice on day 21. Treatment with 5 μg of RFB4-PE35 prevented tumor growth in one of five mice, and the average size of tumors on day 21 was 12% of control. Two of five mice receiving 10 μg of RFB4-PE35 developed tumors that were detectable on day 18 and by day 21 were 2% the size of control tumors. Table 2 lists antitumor and toxicity data for all experiments. We conclude that RFB4-PE35KDEL and RFB4-PE35 were very effective in preventing or slowing the growth of CD22-positive tumors at nontoxic doses.

DISCUSSION

Up to 60% of B-cell malignancies are potentially treatable with an immunotoxin targeted to CD22 (20). The murine anti-CD22 monoclonal antibody RFB4 has been extensively tested to confirm its B-cell specificity (13) and its ability to localize at CD22-positive tumor

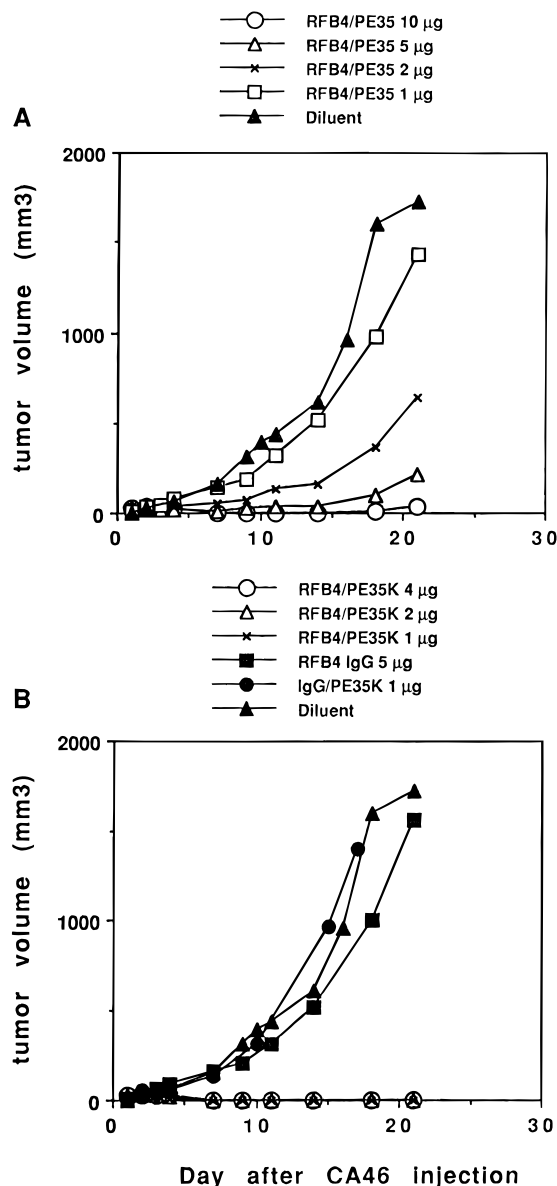


Figure 6. Antitumor activity of RFB4-PE35 and RFB4-PE35KDEL on CA46 subcutaneous tumors in nude mice. (A) Formation of tumors in mice inoculated with CA46 cells was inhibited by four doses of 10, 5, and 2 μg of RFB4-PE35. (B) Formation of CA46 tumors was prevented by four doses of 4, 2, or 1 μg of RFB4-PE35KDEL. See Table 2 for details on numbers of mice used.

Table 2. Prevention of CA46 Tumors in Nude Mice

immunotoxin ^a	dose ($\mu\text{g} \times 4$) ^b	survivors	tumor-free day 21
RFB4-PE35	10	5/5	3/5
	5	5/5	1/5
	2	5/5	1/5
	1	8/9	2/8
RFB4-PE35KDEL	4	9/10	9/9
	2	10/10	9/10
	1	14/14	12/14

^a Immunotoxins were diluted in PBS/0.2% HSA and administered iv in a volume of 200 μL . ^b Doses shown were given at 24 h intervals for 4 consecutive days.

sites (21). Furthermore, RFB4-ricin A chain immunotoxins have shown promise *in vitro*, in murine tumor models, and, to some extent, in the human clinical setting (14–16). However, the dose-limiting toxicity in humans of ricin A chain-based immunotoxins, including RFB4-dgA, is a vascular leak syndrome. Trials using RFB4-

dgA have shown maximum tolerated dose levels of 28.8 mg/m² per 192 h when continuously infused (16) and 75 mg/m² when administered in two to six doses at 48 h intervals (14). Signs of vascular leak were apparent at doses as low as 9.6 mg/m² per 192 h (continuous infusion) and 12.5 mg/m² (when doses were given at 48 h intervals) (14, 16). To achieve antitumor effects consistently, higher dose levels are probably needed.

Pseudomonas exotoxin itself is not believed to cause VLS. This toxicity has been apparent and dose-limiting with only one PE-based immunotoxin (LMB-1, directed at the Lewis^y antigen) (22) but not others (23–25). In *in vitro* studies (26), LMB-1 and LMB-7 (a recombinant Fv version of LMB-1) were directly toxic to HUVEC in 20 h assays and also caused visible rounding of the cells, while PE38 (a recombinant form of PE that has no cell-binding domains) neither was cytotoxic nor caused cell rounding. Two other PE-based recombinant immunotoxins, ATac(Fv)PE38 and e23(dsFv)PE38 directed at the p55 subunit of IL-2R and the erbB-2 receptor, respectively, were also not cytotoxic to HUVEC.

Inhibition of HUVEC protein synthesis by LMB-1 and LMB-7 preceded cell rounding. In the same study, ricin A chain (RTA) caused rapid cell rounding which, in contrast to LMB-1 and LMB-7, apparently precedes inhibition of protein synthesis (19). LMB-1 and LMB-7 cytotoxicity on HUVEC could be prevented by competition with excess B3 antibody, the parent antibody used to make LMB-1 conjugate, demonstrating that binding of these immunotoxins is specific, whereas RTA appears to bind HUVEC in a nonsaturable manner. Finally, dgRTA (also called dgA) inhibited protein synthesis to a similar degree as non-HUVEC-directed immunotoxin IT-dgRTA, indicating that toxicity is due to ricin binding and not to binding mediated by the antibody portion of the immunotoxin.

Because RFB4-dgRTA immunotoxins have caused VLS in human patients, we examined the ability of RFB4-PE immunotoxins to exert cytotoxic effects on HUVEC by measuring inhibition of protein synthesis. RFB4-PE immunotoxins are at least 20-fold less toxic to HUVEC than LMB-1 and LMB-7. Because we believe that the only PE-based immunotoxin to cause VLS (LMB-1) exerts this toxicity through the antibody moiety of the molecule and not the PE itself, we predict that VLS will not occur in patients treated with RFB4-PE immunotoxins since CD22 is not present on endothelial cells.

We have shown that, by exchanging *Pseudomonas* toxin for ricin in an RFB4 immunotoxin, we can obtain specific *in vitro* toxicities comparable to those reported for the RFB4-dgA immunotoxin. Also, RFB4-PE35 and RFB4-PE35KDEL immunotoxin conjugates are remarkably effective in their ability to inhibit the establishment of lymphoma tumors in nude mice inoculated with CA46 cells. The CA46 tumors that form in untreated inoculated mice are not grossly vascularized and appear macroscopically and microscopically similar to human lymphoma masses (A. Wellmann, unpublished observations). RFB4-PE immunotoxins, therefore, seem promising as therapeutic agents in circumstances of minimal residual disease.

Cytotoxicity of RFB4-PE immunotoxins toward B-cells is particularly dependent on the molecular form of PE that is used. In this study, RFB4 coupled to PE35 or PE35KDEL was quite toxic to cultured CD22-positive B-cells, while RFB4 conjugated to PE38 was at least 10-fold less active. This difference probably represents a reduced ability of this cell type to proteolytically process PE, although some of the reduction in cytotoxicity must be attributed to reduced binding of RFB4-PE38 to CD22.

Recently, native PE was shown to require furin-mediated cleavage to produce the C-terminal fragment that translocates to the cell cytosol (27). Failure to generate this fragment results in reduced or no toxicity. If cells lack furin or furin is present only in subcellular locations separate from the endocytic pathway of the toxin, one would expect reduced toxicity compared to immunotoxins in which PE does not need to be cleaved by furin.

Conjugation of RFB4 IgG to the PE mutants PE35 and PE35KDEL through disulfide bonds is efficient and results in immunotoxin molecules that are very active against CD22-positive cells both *in vitro* and *in vivo*. Recombinant immunotoxins made of PE and RFB4 are currently being developed. Previous results have indicated that recombinant immunotoxins are often more active than their corresponding chemical conjugates (28–30). In addition, recombinant immunotoxins are 25% of the size of chemical conjugates using whole antibody and thus are more likely to penetrate tumor masses.

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LITERATURE CITED

- Uckun, F. M., Manivel, C., Arthur, D., Chelstrom, L. M., Finnegan, D., Tuel-Ahlgren, L., Irvin, J. D., Myers, D. E., and Gunther, R. (1992) *In vivo* efficacy of B43 (anti-CD19)-pokeweed antiviral protein immunotoxin against human pre-B cell acute lymphoblastic leukemia in mice with severe combined immunodeficiency. *Blood* 79, 2201–2214.
- Ghetie, M.-A., Richardson, J., Tucker, T., Jones, D., Uhr, J. W., and Vitetta, E. S. (1991) Antitumor activity of Fab' and IgG-anti-CD22 immunotoxins in disseminated human B lymphoma grown in mice with severe combined immunodeficiency disease: Effect on tumor cells in extranodal sites. *Cancer Res.* 51, 5876–5880.
- Francisco, J. A., Gilliland, L. K., Stebbins, M. R., Norris, N. A., Ledbetter, J. A., and Siegall, C. B. (1995) Activity of a single-chain immunotoxin that selectively kills lymphoma and other B-lineage cells expressing the CD40 antigen. *Cancer Res.* 55, 3099–3104.
- Ghetie, M.-A., May, R. D., Till, M., Uhr, J. W., Ghetie, V., Knowles, P. P., Relf, M., Brown, A., Wallace, P. M., Janossy, G., Amlot, P., Vitetta, E. S., and Thorpe, P. E. (1988) Evaluation of ricin A chain-containing immunotoxins directed against CD19 and CD22 antigens on normal and malignant human B-cells as potential reagents for *in vivo* therapy. *Cancer Res.* 48, 2610–2617.
- Bonardi, M. A., French, R. R., Amlot, P., Gromo, G., Modena, D., and Glennie, M. J. (1993) Delivery of saporin to human B-cell lymphoma using bispecific antibody: Targeting via CD22 but not CD19, CD37, or immunoglobulin results in efficient killing. *Cancer Res.* 53, 3015–3021.
- French, R. R., Penney, C. A., Browning, A. C., Stirpe, F., George, A. J. T., and Glennie, M. J. (1995) Delivery of the ribosome-inactivating protein, gelonin, to lymphoma cells via CD22 and CD38 using bispecific antibodies. *Br. J. Cancer* 71, 986–994.
- Uckun, F. M., Ramakrishnan, S., and Houston, L. L. (1985) Immunotoxin-mediated elimination of clonogenic tumor cells in the presence of human bone marrow. *J. Immunol.* 134, 2010–2016.
- Kreitman, R. J., Hansen, H. J., Jones, A. L., FitzGerald, D. J. P., Goldenberg, D. M., and Pastan, I. (1993) *Pseudomonas* exotoxin-based immunotoxins containing the antibody LL2 or LL2-Fab' induce regression of subcutaneous human B-cell lymphoma in mice. *Cancer Res.* 53, 819–825.
- Ogata, M., Fryling, C. M., Pastan, I., and FitzGerald, D. J. (1992) Cell-mediated cleavage of *Pseudomonas* exotoxin between Arg²⁷⁹ and Gly²⁸⁰ generates the enzymatically active

- fragment which translocates to the cytosol. *J. Biol. Chem.* 267, 25396–25401.
- (10) Theuer, C. P., Kreitman, R. J., FitzGerald, D. J., and Pastan, I. (1993) Immunotoxins made with a recombinant form of *Pseudomonas* exotoxin A that do not require proteolysis for activity. *Cancer Res.* 53, 340–347.
- (11) Kreitman, R. J., Chaudhary, V. K., Kozak, R. W., FitzGerald, D. J. P., Waldmann, T. A., and Pastan, I. (1992) Recombinant toxins containing the variable domains of the anti-Tac monoclonal antibody to the interleukin-2 receptor kill malignant cells from patients with chronic lymphocytic leukemia. *Blood* 80, 2344–2352.
- (12) Campana, D., Janossy, G., Bofill, M., Trejdosiewicz, L. K., Ma, D., Hoffbrand, V., Mason, D. Y., Lebacqz, A.-M., and Forster, H. K. (1985) Human B cell development. I. Phenotypic differences of B lymphocytes in the bone marrow and peripheral lymphoid tissue. *J. Immunol.* 134, 1524–1530.
- (13) Li, J.-L., Shen, G.-L., Ghetie, M.-A., May, R. D., Till, M., Ghetie, V., Uhr, J. W., Janossy, G., Thorpe, P. E., Amlot, P., and Vitetta, E. S. (1989) The epitope specificity and tissue reactivity of four murine monoclonal anti-CD22 antibodies. *Cell. Immunol.* 118, 85–99.
- (14) Vitetta, E. S., Stone, M., Amlot, P., Fay, J., May, R., Till, M., Newman, J., Clark, P., Collins, R., Cunningham, D., Ghetie, V., Uhr, J. W., and Thorpe, P. E. (1991) Phase I immunotoxin trial in patients with B cell lymphoma. *Cancer Res.* 51, 4052–4058.
- (15) Amlot, P., Stone, M. J., Cunningham, D., Fay, J., Newman, J., Collins, R., May, R., McCarthy, M., Richardson, J., Ghetie, V., Ramilo, O., Thorpe, P. E., Uhr, J. W., and Vitetta, E. S. (1993) A phase I study of anti-CD22 deglycosylated ricin A chain immunotoxin in the treatment of B cell lymphomas resistant to conventional therapy. *Blood* 82, 2624.
- (16) Sausville, E. A., Headlee, D., Stetler-Stevenson, M., Jaffe, E. S., Solomon, D., Figg, W. D., Herdt, J., Kopp, W. C., Rager, H., Steinberg, S. M., Ghetie, V., Schindler, J., Uhr, J., Wittes, R. E., and Vitetta, E. S. (1995) Continuous infusion of the anti-CD22 immunotoxin IgG-RFB4-SMPT-dgA in patients with B-cell lymphoma: A phase I study. *Blood* 85, 3457–3465.
- (17) Ghetie, V., Thorpe, P., Ghetie, M. A., Knowles, P., Uhr, J. W., and Vitetta, E. S. (1991) The GLP large scale preparation of immunotoxins containing deglycosylated ricin A chain and a hindered disulfide bond. *J. Immunol. Methods* 142, 223–230.
- (18) FitzGerald, D. J. P. (1987) Construction of immunotoxins using *Pseudomonas* exotoxin A. *Methods Enzymol.* 151, 139–145.
- (19) Soler-Rodriguez, A.-M., Ghetie, M.-A., Oppenheimer-Marks, N., Uhr, J. W., and Vitetta, E. S. (1993) Ricin A-chain and ricin A-chain immunotoxins rapidly damage human endothelial cells: Implications for vascular leak syndrome. *Exp. Cell. Res.* 206, 227–234.
- (20) Anderson, K. C., Bates, M. P., Slaughenhaupt, B. L., Pinkus, G. S., Schlossman, S. F., and Nadler, L. M. (1984) Expression of human B cell-associated antigens on leukemias and lymphomas: A model of human B cell differentiation. *Blood* 63, 1424–1433.
- (21) Ghetie, M.-A., Richardson, J., Tucker, T., Jones, D., Uhr, J. W., and Vitetta, E. S. (1990) Disseminated or localized growth of a human B-cell tumor (Daudi) in SCID mice. *Int. J. Cancer* 45, 481–485.
- (22) Pai, L. H., Wittes, R. E., Setser, A., Goldspiel, B., FitzGerald, D., Willingham, M. C., and Pastan, I. (1994) Phase I study of the immunotoxin LMB-1, an anti-cancer murine MAb B3, coupled to a recombinant form of *Pseudomonas* exotoxin. *Proc. Am. Assoc. Cancer Res.* 35, 507.
- (23) Davey, R. T., Boenning, C. M., Herpin, B. R., Batts, D. H., Metcalf, J. A., Wathen, L., Cox, S. R., Polis, M. A., Kovacs, J. A., and Falloon, J. (1994) Use of recombinant soluble CD4 *Pseudomonas* exotoxin, a novel immunotoxin, for treatment of persons infected with human immunodeficiency virus. *J. Infect. Dis.* 170, 1180–1188.
- (24) Ramachandran, R. V., Katzenstein, D. A., Wood, R., Batts, D. H., and Merigan, T. C. (1994) Failure of short-term CD4-PE40 infusions to reduce virus load in human immunodeficiency virus-infected persons. *J. Infect. Dis.* 170, 1009–1013.
- (25) Pai, L. H., Bookman, M. A., Ozols, R. F., Young, R. C., Smith, J. W., II, Longo, D. L., Gould, B., Frankel, A., McClay, E. F., Howell, S., Reed, E., Willingham, M. C., FitzGerald, D. J., and Pastan, I. (1991) Clinical evaluation of intraperitoneal *Pseudomonas* exotoxin immunoconjugate OVB3-PE in patients with ovarian cancer. *J. Clin. Oncol.* 9, 2095–2103.
- (26) Kuan, C.-T., Pai, L. H., and Pastan, I. (1995) Immunotoxins containing *Pseudomonas* exotoxin that target Le^y damage human endothelial cells in an antibody-specific mode: relevance to vascular leak syndrome. *Clin. Cancer Res.* 1, 1589–1594.
- (27) Chiron, M. F., Fryling, C. M., and FitzGerald, D. F. (1994) Cleavage of pseudomonas exotoxin and diphtheria toxin by a furin-like enzyme prepared from beef liver. *J. Biol. Chem.* 269, 18167–18176.
- (28) Batra, J. K., Jinno, Y., Chaudhary, V. K., Kondo, T., Willingham, M. C., FitzGerald, D. J., and Pastan, I. (1989) Antitumor activity in mice of an immunotoxin made with anti-transferrin receptor and a recombinant form of *Pseudomonas* exotoxin. *Proc. Natl. Acad. Sci. U.S.A.* 86, 8545–8549.
- (29) Chaudhary, V. K., Queen, C., Junghans, R. P., Waldmann, T. A., FitzGerald, D. J., and Pastan, I. (1989) A recombinant immunotoxin consisting of two antibody variable domains fused to *Pseudomonas* exotoxin. *Nature* 339, 394–397.
- (30) Brinkmann, U., Pai, L. H., FitzGerald, D. J., Willingham, M., and Pastan, I. (1991) B3(Fv)-PE38KDEL, a single-chain immunotoxin that causes complete regression of a human carcinoma in mice. *Proc. Natl. Acad. Sci. U.S.A.* 88, 8616–8620.

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Direct, Solid Phase Assembly of Dihydropyrroloindole Peptides with Conjugated Oligonucleotides

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A new controlled pore glass (CPG) support is described that allows for the direct synthesis of oligonucleotide derivatives carrying a minor groove binding (MGB) agent at the 3'-terminus. The MGB consisted of three repeating 1,2-dihydro-3*H*-pyrrolo[2,3-*e*]indole-7-carboxylate (DPI) subunits. The DPI trimer (DPI₃) was prepared directly on the CPG support using repeated addition of the DPI subunit. The subunit was protected at the *N*-3-position with *tert*-butyloxycarbonyl residue and activated at the 7-carboxy residue by esterification with the 2,3,5,6-tetrafluorophenyl group. A linker, which provided the starting point for oligonucleotide synthesis, was introduced by reaction of the terminal *N*-3 with *p*-nitrophenyl 4-[bis(4-methoxyphenyl)phenylmethoxy]butyrate. When used as a support for oligonucleotide synthesis, this modified CPG gave the desired 3'-DPI₃-octathymidylate [(dTp)₈-DPI₃] conjugate in good yield. This conjugate formed hyperstabilized complexes with complementary polyribo- (*T*_{max} = 35 °C) and polydeoxyriboadenylic (*T*_{max} = 69 °C) acids. In contrast to the *N*-carbamoyl derivative reported earlier by us, it demonstrated higher cooperativity of melting transitions.

INTRODUCTION

Oligonucleotides conjugated to specific functional groups are important research tools in the molecular biology and chemistry of nucleic acids. These functional groups can be markers for diagnostic applications or reactive residues for therapeutic purposes. Some of these functional groups are helix-stabilizing agents. For example, covalent attachment of intercalators to the ends of oligonucleotides is a powerful way to enhance oligonucleotide hybridization properties (1–4). Recently, we have developed an alternative hybrid stabilization method based on covalent attachment of a minor groove binding agent (MGB)¹ (5, 6). 1-Methylpyrrole-2-carboxamide (MPC) (5) and *N*-3-carbamoyl 1,2-dihydro-3*H*-pyrrolo[3,2-*e*]indole-7-carboxylate (CDPI, Figure 1) (6) oligomers were prepared and their hybridization properties examined. Conjugation of the CDPI₃ group to either terminus of octathymidylate stabilized its duplex with polydeoxyriboadenylic acid by as much as 44 °C. A similar but lesser effect was observed for d(pT)₈ + poly(rA) duplexes. The MPC-oligothymidylate conjugates possessed similar properties in a duplex with poly(dA) but failed to stabilize the (dTp)₈ + poly(rA) hybrid, as well as G,C-rich DNA–DNA duplexes (5).

The ODN–CDPI₃ conjugates that we reported previously (6) were prepared according to two methods. In the first, the ODN was modified postsynthetically with

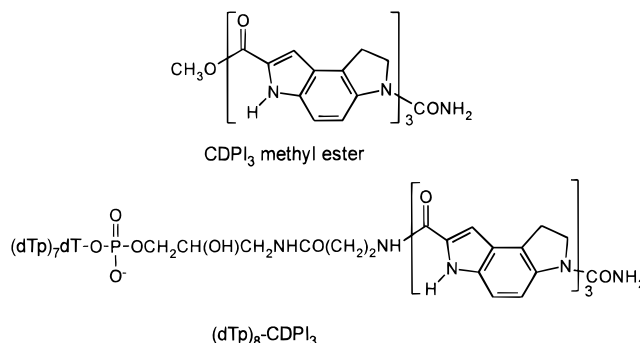


Figure 1. Structure of methyl *N*-3 carbamoyl 1,2-dihydro-3*H*-pyrrolo[3,2-*e*]indole-7-carboxylate trimer (7) and its 3'-octathymidylate conjugate prepared by approach described in ref 6.

an amine linker at one terminus. Alternatively, a controlled pore glass (CPG) solid support bearing the CDPI₃ residue was employed, with the desired conjugate obtained directly after oligonucleotide synthesis. Both approaches require utilization of the 2,3,5,6-tetrafluorophenyl ester of CDPI₃ (TFP-CDPI₃) (6), an acylating derivative of the MGB. Assembly of this trimeric MGB requires several steps, and preparation of the activated acylating reagent is complicated by its low solubility. Herein, we describe a new, more versatile solid phase approach that avoids the preparation of the complex CDPI₃ analog. The approach is based on a combination of solid phase peptide synthesis for assembling the DPI₃ segment followed by phosphoramidite chemistry for incorporation of the oligonucleotide sequence. A solid phase method for the preparation of polyamide–oligonucleotide conjugates, previously developed by Haralam-bidis et al. (8), bears similarities.

EXPERIMENTAL PROCEDURES

Reagents. All air- and water-sensitive reactions were carried out under a slight positive pressure of argon. Anhydrous solvents were obtained from Aldrich (Milwaukee, WI). Standard reagents for the β -cyanoethylphosphoramidite coupling chemistry were purchased from Glen Research (Sterling, VA). Enzymes were

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¹ Abbreviations: ODN, oligodeoxyribonucleotide; MPC, 1-methylpyrrole-2-carboxamide; DPI, 1,2-dihydro-3*H*-pyrrolo[3,2-*e*]indole-7-carboxylate; CDPI, *N*-3 carbamoyl 1,2-dihydro-3*H*-pyrrolo[3,2-*e*]indole-7-carboxylate; TFP, 2,3,5,6-tetrafluorophenyl; CPG, controlled pore glass; *T*_{max}, melting temperature for an ODN complex, determined at the maximum of derivative of the melting curve; MGB, minor groove binder; DMF, *N,N*-dimethylformamide; DMSO, dimethyl sulfoxide; MMTr and DMTr, 4-monomethoxytrityl and 4,4'-dimethoxytrityl, respectively; tBOC, *tert*-butyloxycarbonyl; HOBt, 1-hydroxybenzotriazole; placement of the MGB to the right of the ODN denotes 3'-conjugation.

obtained from Sigma (St. Louis, MO). Flash chromatography was performed on 230–400 mesh silica gel. UV–visible absorption spectra were recorded in the 200–400 nm range on a Lambda 2 (Perkin-Elmer) spectrophotometer. ^1H NMR spectra were run at 20 °C on a Varian Gemini 300 spectrometer; chemical shifts are reported in parts per million downfield from $(\text{CH}_3)_4\text{Si}$.

2,3,5,6-Tetrafluorophenyl 3-[[[(4-Methoxyphenyl)-diphenyl]methyl]aminol]propyl Butanedioate (1). To a solution of 3-aminopropanol (5.0 g, 67 mmol) in 100 mL of dry CH_2Cl_2 was added 4-monomethoxytrityl chloride (10.0 g, 33 mmol). After 30 min, the mixture was diluted with 900 mL of CH_2Cl_2 and washed twice with 1 L of water. The organic phase was dried over Na_2SO_4 and then concentrated to an oil. The residue was purified by flash chromatography (hexane/ethyl acetate, 1:1) to give 8.2 g (70%) of the desired *N*-(4-monomethoxytrityl)-3-aminopropan-1-ol as a pale yellow syrup: ^1H NMR (CDCl_3 , 300 MHz, ppm) 7.43–7.19 (m, 12H, MMTr), 6.82 (d, 2H, $J = 9$ Hz, MMTr), 3.88 (t, 2H, $J = 5$ Hz, CH_2O), 3.78 (s, 3H, OCH_3), 2.39 (t, 2H, $J = 6$ Hz, CH_2N), 1.70 (m, 2H, CH_2).

To a solution of the above MMTr derivative (4.0 g, 11.8 mmol) in 20 mL of anhydrous CH_2Cl_2 was added 1.18 g (11.8 mmol) of succinic anhydride, 2 mL (14 mmol) of triethylamine, and 50 μL of 1-methylimidazole. The mixture was stirred for 3 days. 2,3,5,6-Tetrafluorophenyl trifluoroacetate (10) (3.0 g, 11.5 mmol) was added dropwise. After 30 min, the mixture was concentrated and redissolved in 30 mL of hexane/ethyl acetate (3:1). The product was purified by flash chromatography using the same solvent as eluent. The fractions containing pure product were combined and evaporated *in vacuo* to give 5.7 g (81%) of the title compound as colorless syrup: ^1H NMR (CDCl_3 , 300 MHz, ppm) 7.50–7.15 (m, 12H, MMTr), 6.82 (m, 2H, MMTr), 4.28 (t, 2H, $J = 7$ Hz, CH_2O), 3.78 (s, 3H, OCH_3), 2.96 (t, 2H, $J = 7$ Hz, COCH_2), 2.72 (t, 2H, $J = 7$ Hz, COCH_2), 2.21 (t, 2H, $J = 7$ Hz, CH_2N), 1.82 (m, 2H, CH_2).

Preparation of DPI_3 –CPG (6). *Derivatization of the CPG.* To long-chain aminoalkyl controlled pore glass (Sigma, 500 Å, 4.0 g) were added **1** (1.5 g, 2.5 mmol) and 1.5 mL of triethylamine in 15 mL of anhydrous DMF. After being shaken for 20 h, the CPG was washed with DMF (100 mL), acetone (200 mL), and ether (50 mL) and dried under vacuum. The degree of functionalization was quantitated by spectrophotometric assay of the monomethoxytrityl cation ($\lambda = 472$, $\epsilon = 60 \text{ mM}^{-1}\text{cm}^{-1}$) released upon acid treatment (60% HClO_4 /methanol, 1:1) of a small amount of CPG. MMTr loading was found to be 41 $\mu\text{mol/g}$. The residual amino groups were capped with a mixture of pyridine (20 mL) and acetic anhydride (2 mL) for 30 min. The CPG (**2**) was then washed with acetone (200 mL) and ether (100 mL) and dried under vacuum overnight.

DPI_3 –CPG Synthesis. The CPG (**2**) (2.0 g) was treated with trifluoroacetic acid (2×10 mL), washed with CH_2Cl_2 , neutralized with 20% triethylamine in CH_2Cl_2 , washed with CH_2Cl_2 again, and dried *in vacuo*. It was then reacted with a solution of 2,3,5,6-tetrafluorophenyl 3-(*tert*-butyloxycarbonyl)-1,2-dihydro-3H-pyrrolo[3,2-*e*]indole-7-carboxylate (**3**) (**6**) (78 mg, 0.33 mmol), triethylamine (0.6 mL), and 1-hydroxybenzotriazole (44 mg, 0.33 mmol) in 6 mL of anhydrous DMSO for 24 h. After washings with 100 mL portions of DMSO, acetone, and ether, the CPG was capped as described above and dried. The deprotection, coupling, and capping steps were repeated two more times, giving rise to the CPG-coupled 3-(*tert*-butyloxycarbonyl)-1,2-dihydro-3H-pyrrolo[3,2-*e*]indole-7-carboxylate trimer (**4**).

Addition of Linker. After deprotection of the terminal amino group with trifluoroacetic acid, the CPG was treated with *p*-nitrophenyl 4-[bis(4-methoxyphenyl)phenylmethoxy]butyrate (**5**) (**8**) (0.9 g, 1.7 mmol), triethylamine (0.6 mL), and 1-hydroxybenzotriazole in 6 mL of anhydrous DMSO for 6 h. After washing and drying, the CPG was analyzed for DMTr content and found to have a loading of 29 $\mu\text{mol/g}$. Residual amino groups were acetylated as above and the CPG (**6**) was used for DNA synthesis.

Oligonucleotide Synthesis and Molar Extinction Coefficients. Trityl-off 3'- DPI_3 octathymidylate [(dTp) $_8$ –CD PI_3] was prepared in 1 μmol scale using the modified CPG support (**6**) (~50 mg) on an ABI 394 according to the protocol supplied by the manufacturer. For comparison, an octathymidylate bearing a 3'-CD PI_3 [(dTp) $_8$ –CD PI_3] was prepared according to our previously described method (**6**). The concentration of both conjugates was determined in water using the extinction coefficient of $110.1 \text{ mM}^{-1}\text{cm}^{-1}$ (**6**).

Thermal Denaturation Studies. Hybrids formed between MGB-tailed (dTp) $_8$ conjugates and complementary polyadenylates were melted in 140 mM KCl, 10 mM MgCl_2 , and 20 mM HEPES–HCl (pH 7.2) using a Lambda 2 (Perkin-Elmer) spectrophotometer equipped with a PTP-6 automatic multicell temperature programmer. Each conjugate (2 μM) was mixed with sufficient polymer to give a T:A ratio of 1:1. To determine the concentrations of poly(dA) and poly(rA), ϵ_{260} values of 8.4 and $10.3 \text{ mM}^{-1}\text{cm}^{-1}$ were used (**9**). Thermal dissociation curves were obtained by heating samples from 0 to 100 °C with a temperature increase of 0.5 °C/min. Before melting, the samples were preheated at 100 °C and cooled to the starting temperature over a 10 min period. The melting temperatures (T_{max} values) of complexes were determined from the derivative maxima.

Nuclease Digest of (dTp) $_8$ – DPI_3 Conjugate. To a solution of (dTp) $_8$ – DPI_3 (~15 μg) in 50 μL of a buffer containing 50 mM Tris–HCl (pH 8.5) and 10 mM MgCl_2 were added RQ1 DNase (5 μL , 1000 U/mL), phosphodiesterase I (5 μL , 100 U/mL), and calf intestine alkaline phosphatase (1 μL , 650 U/mL). The solution was incubated at 37 °C for 30 h. Ten microliter aliquots were taken during this period to monitor the progress of the reaction by HPLC analysis.

RESULTS AND DISCUSSION

We have previously prepared a CPG with an attached CD PI_3 residue for synthesis of ODNs with this residue on the 3'-terminus and have shown that the residue is stable to the treatments used in automated phosphoramidite chemistry (**6**). In that approach, the CD PI_3 residue was bound to the solid support through a specially designed aminodiol linker which provided attachment points for (a) the CD PI_3 residue, (b) the solid support, and (c) the initial 3'-nucleic acid residue. Although this method was efficient for the preparation of the conjugates, drawbacks included the necessity for CD PI_3 reagent (TFP–CD PI_3) and the complexity of the aminodiol linker. We describe here an alternative approach that combines solid support synthesis of both the trimeric minor groove binding moiety and its attached ODN. The DPI trimer, which is chemically stable to ODN synthesis conditions, is assembled first on the CPG, followed by ODN extension. This approach also provides for conjugation of the ODN through the *N*–3 amino function of the DPI moiety, as opposed to the *C*–7 carboxy group in our previously published approach (**6**).

The preparation of the new MGB-bound CPG support is shown on Figure 2. Long-chain alkylamine CPG was

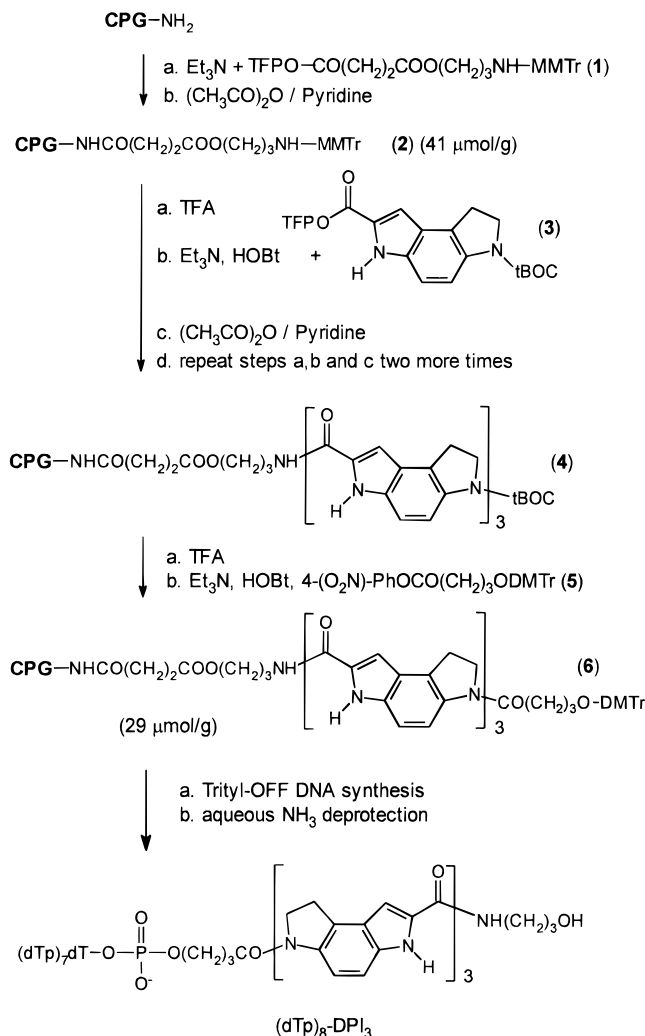


Figure 2. Synthetic scheme and structure of the (dTp)₈-DPI₃ conjugate.

modified with a specially designed reagent **1** to provide an ammonia-cleavable linker bearing an MMTr-protected amino group. The amino group was deprotected with trifluoroacetic acid and reacted with the activated DPI block (**3**), giving the solid support-bound DPI fragment with *N*-3-tBOC group as a new terminus. Capping with a mixture of pyridine and acetic anhydride, to eliminate possible DPI_{*n*-1} contamination, concluded each coupling cycle. The deprotection, coupling, and capping were repeated two more times to give a CPG (**4**) with the bound tBOC-DPI₃ residue. A DMTr-protected primary hydroxy group, the starting point for oligonucleotide synthesis, was introduced by removal of the last tBOC group, treatment with **5**, and capping with acetic anhydride in pyridine. The DMTr loading for this CPG (**6**) was found to be 29 μmol/g, reflecting an average 92% coupling yield during DPI₃ assembly.

The modified CPG (**6**) was then used for automated octathymidylate synthesis, using the standard cyanoethyl *N,N'*-diisopropylphosphoramidite chemistry. The trityl group was removed after the last coupling to simplify HPLC purification. The 3'-DPI₃-modified octathymidylate [(dTp)₈-DPI₃] was deblocked and cleaved from the CPG by aqueous ammonia treatment and isolated by reversed-phase HPLC. A chromatographic profile of the reaction mixture is shown in Figure 3A. The reaction mixture contained only one major peak, the product. As had been observed earlier (6) for CDPI₃ conjugates, the addition of the hydrophobic MGB residue to the octathymidylate significantly increased the retention time

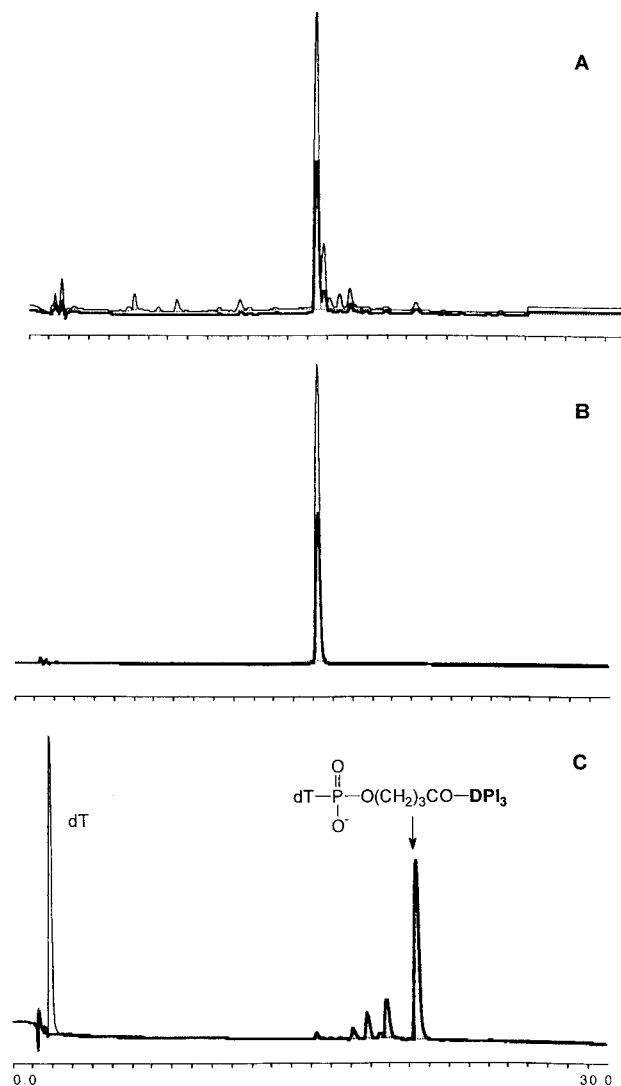


Figure 3. HPLC profiles of crude synthetic (**A**) and HPLC purified (**B**) (dTp)₈-DPI₃ conjugate. (**C**) HPLC analysis of nuclease digest of the conjugate with a mixture of RQ1 DNase, phosphodiesterase I, and alkaline phosphatase at 37 °C for 30 h in a buffer containing 50 mM Tris-HCl (pH 8.5) and 10 mM MgCl₂. Chromatography was performed on a Rainin C₁₈ column (4.5 × 150 mm) in a gradient of acetonitrile (0–60%) in 0.1 M triethylammonium acetate buffer (pH 7.5). Flow rate was 1 mL/min. Dual wavelength detection was used: fine line, 260 nm; bold line, 340 nm.

relative to that of the unmodified oligonucleotide. To further confirm the conjugation site, the purified conjugate (Figure 3B) was subjected to nuclease digestion using a mixture of RQ1 DNase, phosphodiesterase I and alkaline phosphatase. This gave free thymidine and dTp-DPI₃ as the final products. The hydrolysis of the last few phosphodiester bonds progressed slowly during this digestion (Figure 3C), explaining the presence of the faster eluting products. These were identified as products of incomplete digestion on the basis of their characteristic UV spectra; the latest eluting peak had absorbance at 350 and 260 nm corresponding to (DPI)₃ and one dT residue, respectively, while the next three small, faster eluting peaks (Figure 3C) had progressively more absorbance at 260 nm (data not shown). A second addition of digestion enzymes did not reduce these peaks.

The physical properties of the isolated (dTp)₈-DPI₃ conjugate were similar to those reported for the CDPI₃ analog (**6**). Besides the almost identical chromatographic mobilities, both types of conjugates had similar UV-visible spectra (Figure 4). A slight long wavelength shift

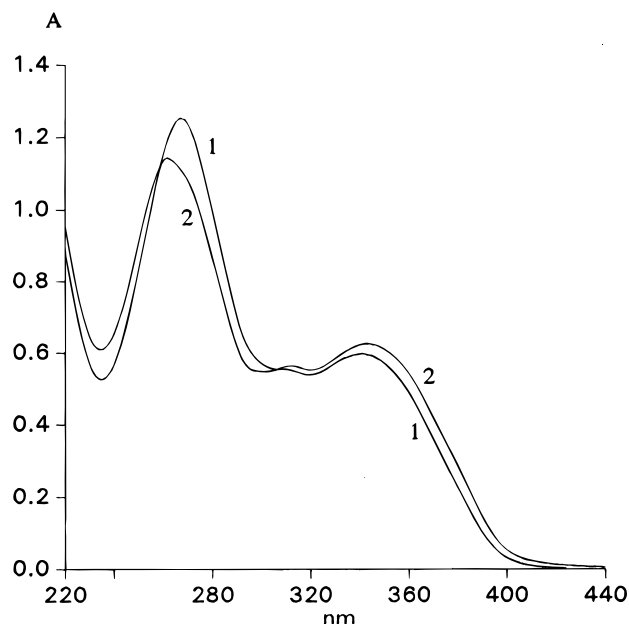


Figure 4. UV-visible spectra of 10 μ M (dTp)₈-CDPI₃ (1) and (dTp)₈-DPI₃ (2) in 10 mM Tris-HCl (pH 7.5).

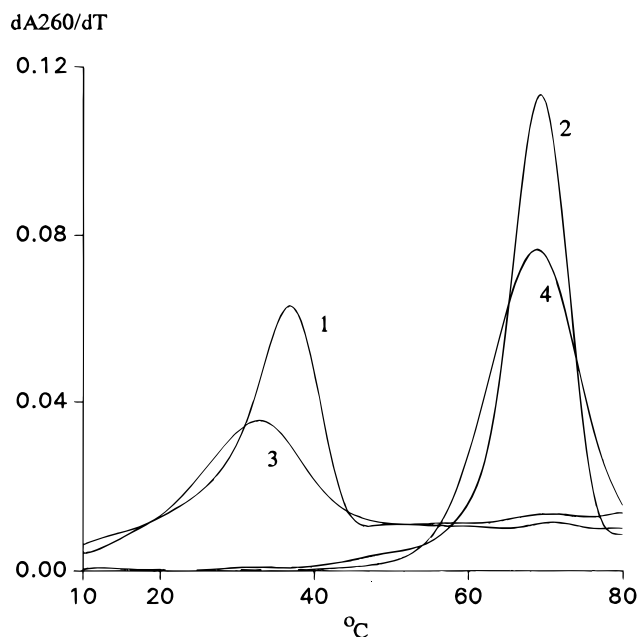


Figure 5. Differential melting curves for the complexes (dTp)₈-DPI₃ + poly(rA) (1), (dTp)₈-DPI₃ + poly(dA) (2), (dTp)₈-CDPI₃ + poly(rA) (3), and (dTp)₈-CDPI₃ + poly(dA) (4) in 140 mM KCl, 10 mM MgCl₂, and 20 mM HEPES-HCl (pH 7.2). The concentration of each ODN was 2 μ M, and the A:T ratio was 1:1.

for the DPI₃ residue was most likely due to the difference in structure of the linker and the end capping groups of the core MGB part (compare structures in Figures 1 and 2).

As had been already observed in case of the (dTp)₈-CDPI₃ conjugate, (dTp)₈-DPI₃ also formed hyperstabilized complexes with complementary polyribo- and polydeoxyriboadenylic acids (Figure 5). Both of these conjugates exhibited the same hybrid stability when bound to poly(dA) ($T_{\max}$ = 68–69 °C), although the (dTp)₈-DPI₃ derivative demonstrated a much higher cooperativity of melting transition than the CDPI₃ analog. The melting temperature of (dTp)₈-DPI₃ bound to poly(rA) ($T_{\max}$ = 35 °C) was 4 °C higher than the corresponding value of the CDPI₃ conjugate ($T_{\max}$ = 31

°C, see Figure 5), which gave an effect of MGB-assisted stabilization of 22 °C. These data show that the polarity of the conjugation (*N*–3 vs *C*–7) of the MGB in the minor groove does not dramatically affect the stability of the hybrid. The *N*–3 carbamoyl residue seems to be nonessential for the stabilization, as we found in the previous investigation (6).

This new solid phase approach for the preparation of oligonucleotides with conjugated DPI oligomers is equally applicable for the preparation of longer oligonucleotides and derivatives with MGBs of various length and structure. A major advantage over the solution phase methodology is elimination of the synthesis and purification of DPI oligomers (3-mer, 4-mer), which are complicated by the low solubility of the oligomers. Following the MGB assembly, an oligonucleotide sequence can be efficiently incorporated, allowing the direct preparation of the desired conjugate. Use of conventional 3'-phosphoramidite chemistry, which is described in this study, leads to 3'-type of conjugation. However, commercial availability of 5'-phosphoramidites (Glen Research) enables preparation of 5'-conjugates with the same solid support, making the method more versatile.

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LITERATURE CITED

- (1) Letsinger, R., and Schott, M. E. (1981) Selectivity in binding a phenanthridinium-dinucleotide derivative to homopolynucleotides. *J. Am. Chem. Soc.* 103, 7394.
- (2) Asseline, V., Delarue, M., Laucelot, G., Toulme, F., Thuong, N. T., Montenay-Garestier, T., and Helene, C. (1984) Nucleic acid binding with high affinity and base sequence specificity: intercalating agents covalently linked to oligodeoxynucleotides. *Proc. Natl. Acad. Sci. U.S.A.* 81, 3297.
- (3) Benimetskaya, L. Z., Bulychiev, N. V., Kozionov, A. L., Koshkin, A. A., Lebedev, A. V., Novozhilov, S. Yu., and Stockman, M. I. (1989) Site-specific laser modification (cleavage) of oligonucleotides. *Biopolymers* 28, 1129.
- (4) Lokhov, S. G., Podyminogin, M. A., Sergeev, D. S., Silnikov, V. N., Kutyavin, I. V., Shishkin, G. V., and Zarytova, V. P. (1992) Synthesis and high stability of complementary complexes of *N*-(2-hydroxyethyl)phenazinium derivatives of oligonucleotides. *Bioconjugate Chem.* 3, 414.
- (5) Sinyakov, A. N., Lokhov, S. G., Kutyavin, I. V., Gamper, H. B., and Meyer, R. B., Jr. (1995) Exceptional and selective stabilization of A-T rich DNA-DNA duplexes by *N*-methylpyrrole carboxamide peptides conjugated to oligonucleotides. *J. Am. Chem. Soc.* 117, 4995.
- (6) Lukhtanov, E. A., Kutyavin, I. V., Gamper, H. B., and Meyer, R. B., Jr. (1995) Oligodeoxyribonucleotides with conjugated dihydropyrroloindole oligopeptides: preparation and hybridization properties. *Bioconjugate Chem.* 6, 418.
- (7) Boger, D. L., Coleman, R. S., and Invergo, B. J. (1987) Studies on the total synthesis of CC-1065: preparation of a synthetic, simplified 3-carbamoyl-1,2-dihydro-3*H*-pyrrolo[3,2-*e*]indole dimer/trimer/tetramer (CDPI dimer/trimer/tetramer) and development of methodology for PDE-I dimer methyl ester formation. *J. Org. Chem.* 52, 1521.
- (8) Haralambidis, J., Dunkan, L., Angus, K., and Tregear, G. W. (1990) The synthesis of polyamide-oligonucleotide conjugate molecules. *Nucleic Acids Res.* 18, 493.
- (9) Riley, M., Maling, B., and Chamberlin, M. J. (1966) Physical and chemical characterization of two- and three-stranded adenine-thymidine and adenine-uracil homopolymer complexes. *J. Mol. Biol.* 20, 359.
- (10) Gamper, H. B., Reed, M. W., Cox, T., Virosco, J. S., Adams, A. D., Gall, A. A., Scholler, J. K., and Meyer, R. B., Jr. (1993) Facile preparation of nuclease resistant 3' modified oligodeoxynucleotides. *Nucleic Acids Res.* 21, 145.

Preparation of Conjugates between Oligonucleotides and *N*-Vinylpyrrolidone/*N*-Acryloxysuccinimide Copolymers and Applications in Nucleic Acid Assays To Improve Sensitivity

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A simple approach has been developed to prepare conjugates between oligodeoxyribonucleotides (ODNs) and linear copolymers of *N*-vinylpyrrolidone (NVP)/*N*-acryloxysuccinimide (NAS). These conjugates were of higher than expected molecular weight, as determined by light scattering. Side reactions between amino groups on heterocyclic bases, especially of cytosine, are proposed to explain the formation of aggregates. ODN–NVP/NAS conjugates used in both capture and detection steps in sandwich hybridization increased assay sensitivity. Sensitivity to 10⁷ DNA molecules/mL was reached for a 2.3 kb PCR fragment of hepatitis B virus (HBV) genomic DNA.

INTRODUCTION

Nucleic acid detection is an analytical tool showing great potential in the diagnostic detection of infectious and genetic diseases. Most detection strategies include amplification of the target sequence and detection of the amplified product (1). These techniques are very sensitive but are difficult to control for reproducibility and require extreme care to avoid cross-contamination. In addition, these strategies are not inherently quantitative, making it difficult to correlate the amplified product to the amount of target nucleic acid sequence present in the sample. Signal amplification presents an alternative to target amplification and offers a more direct method for quantitation.

One signal amplification strategy is to incorporate multiple reporter groups in oligonucleotide hybridization probes, such as biotin (2–9), fluorophores (7, 10–12), and 2,4-dinitrophenyl (13). The main problems with this strategy, however, are the control of background and an increase in sensitivity that is not very high.

An alternative strategy to traditional signal amplification methods employs the use of polymers with branched oligonucleotides in combination with short hybridization probes labeled with enzyme. These branched amplification multimers contain a primary sequence complementary to the target sequence and a set of secondary sequences that bind to the enzyme-labeled oligonucleotide. The theoretical amplification factor of this system depends on the ratio of linked labeled oligonucleotide sequences to target sequence. Amplification multimers used to link the enzyme-labeled oligonucleotide are large branched oligonucleotides, which have been synthesized first by a chemical procedure (14, 15) and more recently by using a combination of solid phase chemistry and enzymatic ligation methods (16). Though versatile in clinical applications (17), the preparation of these branched multimers is difficult and tedious.

We report a simple approach to the synthesis of a new class of oligonucleotide–copolymer amplification struc-

tures. These new structures were prepared by free radical polymerization of *N*-vinylpyrrolidone and *N*-acryloxysuccinimide (18) and then by covalently coupling oligonucleotides to these structures in an easy, one-step procedure (19). These new oligodeoxyribonucleotide (ODN)–copolymer conjugates were characterized, and their application in signal amplification was demonstrated for the detection of hepatitis B virus (HBV) DNA.

EXPERIMENTAL PROCEDURES

Materials. *N*-Vinylpyrrolidone (NVP) (from Aldrich) was purified by reduced-pressure distillation. *N*-Acryloxysuccinimide (NAS) was obtained commercially (Kodak) and recrystallized in ethyl acetate/pentane mixture or was synthesized from *N*-hydroxysuccinimide and acryloyl chloride according to the method of Pollack (20).

Copolymerization of NVP and NAS was performed at 60 °C in *N,N*-dimethylformamide with 4,4'-azobis(4-cyanopentanoic acid) as initiator (19). After the reaction, copolymers were precipitated at low conversion with ethyl ether, filtered, washed several times with the same solvent, and dried under vacuum.

One copolymer preparation, AZEO1, was of particular use in this study. It was prepared at the azeotropic composition of 60% NAS/40% NVP, which permitted the formation of homogeneous copolymer chains (see Figure 1 for the chemical structure). This copolymer was fully characterized in terms of composition, microstructure, and molecular weight as previously described (18). From light scattering studies, a weight-average molecular weight (M_w) of 70 000 was determined. One mole of this copolymer contained 288 equiv of NAS (M_w = 169) and 192 equiv of NVP (M_w = 111).

ODN Synthesis. The ODNs used in this study were synthesized by β -cyanoethyl phosphoramidite chemistry on an Applied Biosystems DNA synthesizer Model 394. The 5'-aminoethyl modification was introduced using the phosphoramidite derivative of trifluoroacetylaminohexanol, Aminolink 2, from Applied Biosystems. This substitution gave a primary amino group, which allowed specific reactions of conjugation. After deprotection with aqueous ammonia, ODNs were precipitated with 0.1 volume of 3 M sodium acetate and 2 volumes of cold ethanol at –20 °C. ODNs were analyzed by ion exchange

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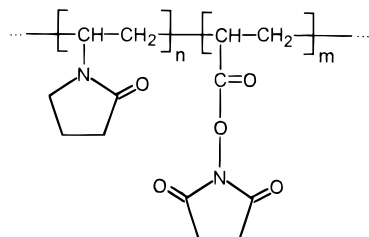


Figure 1. Linear structure of the NVP/NAS copolymer. For AZEO1, which is prepared at the azeotropic composition, $n = 1$ and $m = 1$ (alternate copolymer).

Table 1. Coupling of ODNs to NVP/NAS Copolymer

ODN ^a	sequence (5' to 3')	conjn proc	yield ^b (%)
ODN 1	TCATCCACCTGGCATTGGAC	A	35
		B	50
ODN 2	TTTTCTTTTCCCCCCT	B	61
ODN 3	GGTATGTTGCCCGTTTGTCTCTA	B	46
ODN 4	CTACTAATAGTAGTAGCGTTGCAC	B	70
	CTGTATTCCCATCCCATC		

^a ODN 1 and ODN 2 were used for chemical characterization. ODN 3 and ODN 4 were used for nucleic acid assays to detect HBV DNA. ^b Yield was measured by HPLC using eq 2 and represents the percent of coupling of ODN to the NVP/NAS copolymer AZEO1.

HPLC using a Gen Pak FAX column (Waters) and a gradient of 20–40% 1 M NaCl in 25 mM Tris-HCl, pH 8. The purity of ODNs was determined to be >80%. ODNs were quantified by measuring UV absorption at 260 nm, and concentration was determined by the equation

$$C = \frac{A_{260} \times 100}{1.54n_A + 0.75n_C + 1.17n_G + 0.92n_T} \quad (1)$$

where C is the concentration (nmol/mL) of the ODN at 260 nm and n_A , n_C , n_G , and n_T are the number of nucleotides A, C, G, and T, respectively, in the ODN sequence.

Coupling of ODN to the NVP/NAS Copolymer. Dried ODN (15×10^{-9} mol) was dissolved in X μ L of 0.1 M sodium borate buffer, pH 9.3, and then Y μ L of DMF was added (see below for volumes). Twenty-five microliters of copolymer AZEO1 solution at 1 mg/mL in DMF was mixed with the ODN preparation. The coupling reaction was carried out for 2 h at 37 °C. Solvents were removed by evaporation under vacuum, and 50 μ L of distilled water was added to redissolve the residue. Different coupling reaction conditions were tested. In procedure A, the more dilute condition, $X = 30$ μ L and $Y = 245$ μ L. In procedure B, $X = 10$ μ L and $Y = 68$ μ L. ODN–copolymer conjugates were purified by size exclusion chromatography (SEC). A Kontron device was equipped with an ultrahydrogel column (UH 500, Waters, 30 cm $\times$ 7.6 mm) having a porosity of 5×10^2 Å. The column buffer was 0.1 M sodium phosphate at pH 6.8, and a flow rate of 0.5 mL/min was used. Detection of products was performed by absorption at 260 nm. Purified conjugates were dialyzed against distilled water. Conjugates were stable and could be stored at –20 °C for months. The conjugates used in this study are described in Table 1.

Characterization of the ODN–Copolymer Conjugate. *Coupling Yield.* The yield of coupling between ODN and copolymer was analyzed by SEC (19) as previously described. In addition, the following method was used.

The NVP/NAS copolymer AZEO1 was labeled with the fluorescent dye fluorescein hydrazide, and the ODN was

coupled to the labeled copolymer. The amount of coupled ODN was determined by absorption at 260 nm and the amount of copolymer by fluorescence.

AZEO1 [120 mg; 1.71×10^{-6} mol of copolymer or 4.94×10^{-4} mol of NAS (420 equiv)] was dissolved in 10 mL of DMF (Aldrich). Then 100 μ L of triethylamine (Aldrich) and 100 μ L of an aqueous solution of fluorescein hydrazide (6 mg/mL; 1.20×10^{-6} mol or 1 equiv; Molecular Probes) were added. The reaction mixture was stirred at room temperature for 2 h. Labeled copolymer was precipitated with ethyl ether, filtered, washed with water to remove excess fluorescein, and dried under vacuum. Labeled copolymer (0.9 mg) was dissolved in 3 mL of DMF/water (85/15 v/v). The intensity of fluorescence was measured ($\lambda_{ex} = 506$ nm, $\lambda_{em} = 526$ nm) using a LS50 device (Perkin-Elmer) against a standard curve of dye in DMF/water.

The procedure for coupling ODN to the labeled copolymer and the purification of the conjugate were as described in procedure A above. The intensity of fluorescein was remeasured against a standard curve of dye in water, the ODN–copolymer solvent. Under these conditions, $\lambda_{ex} = 493$ nm and $\lambda_{em} = 513$ nm.

ODN concentration was determined as described above (eq 1).

Determination of Molecular Weights by SEC-LS. After filtration (0.22 μ m filter, Millipore), 5 μ L of the ODN–copolymer conjugate solution was analyzed by SEC using the UH500 ultrahydrogel column method. Detection was performed by refractometry (DR 410, Waters) and by light scattering using a multiangle detector (DawnF, Wyatt Technology).

Detection of HBV DNA. *Targets.* Two HBV DNA targets were used in the experiments described here.

The first target was a single-stranded oligonucleotide (synthetic target: 5' GAT GGG ATG GGA ATA CAG GTG CTA GAG GAC AAA CGG GCA ACA TAC C 3') with complementarity to the HBV capture and detection probes in the sandwich hybridization assay.

The second target was a PCR product of HBV DNA, subtype ayw (21), cloned into pBR322. This clone was a generous gift of Pr. Trépo (Inserm U271, Lyon, France). The sequences of the oligonucleotide primers used in PCR were (5' to 3') TGG AGA ACA TCA CAT CAG GA (158–177) and TAA AGC CCA GTA AAG TTC CC (2477–2496), giving a product of 2339 bp. These sequences are strongly conserved in all known HBV DNAs.

PCR was carried out on 20 ng of HBV plasmid in a 100 μ L reaction containing 50 mM KCl, 10 mM Tris, pH 8.3, 1.5 mM MgCl₂, 0.01% gelatin, 0.3 μ M of each primer, 200 μ M of each of the four deoxynucleotide triphosphates, and 1.5 units of Taq polymerase (Ampli Taq, Perkin-Elmer). The reaction was carried out in a Perkin-Elmer 9600 thermal cycler with cycling conditions at 94 °C for 1 min, 55 °C for 2 min, and 72 °C for 2 min for 40 cycles. PCR reaction products were analyzed by agarose gel electrophoresis, and the identity of the amplified band was confirmed by Southern blot analysis using a nonradioactive HBV DNA probe, 5' GCA CCT GTA TTC CCA TCC CAT C 3' (positions 595–616). The amplified PCR product was purified by agarose gel electrophoresis and electroelution.

Sample Treatment. HBV DNA targets amplified by PCR were diluted in sample buffer (0.1 M sodium phosphate, pH 7, 0.5 M NaCl, 0.65% Tween, 0.14 mg/mL salmon sperm DNA, 2% PEG 4000). Samples were denatured in 0.2 M NaOH (5 min at room temperature) and neutralized at 0.2 M acetic acid.

HBV DNA Detection on the Vidas. Three different systems for detection of HBV DNA were defined on

bioMérieux's Vidas immunoanalysis system. These tests were based on the sandwich hybridization format (22, 23) using specific probes, complementary to highly conserved regions of the HBV DNA sequence, for capture and detection. The capture probe ODN 3 consisted of the HBV specific sequence from position 460 to position 483 (Table 1). The detection probe ODN 4 contained the HBV specific sequence from position 595 to position 616 at its 3' end (Table 1) and a non-HBV sequence at its 5' end, which was complementary to a covalently linked alkaline phosphatase (AP) probe (24).

The capture probe ODN 3 was coated by passive adsorption on the inside of a pipet tip-like disposable, the Vidas solid phase receptacle (SPR), which served as the solid phase capture support and as the pipettor receptacle in the assay. SPRs were coated in 150 nM ODN 3, in 4× phosphate-buffered saline (PBS) buffer (10× PBS is 1.37 M NaCl, 27 mM KCl, 43 mM Na₂HPO₄, and 14 mM KH₂PO₄) for 1 h at 37 °C, on the Vidas instrument. In the sandwich hybridization assay, wells of a disposable polypropylene strip were appropriately filled with hybridization buffer (sample buffer containing 20% of deionized formamide), washing buffer (1× PBS made 0.5% in Tween 20), detection probe ODN 4 at 15 nM in hybridization buffer, AP-labeled probe at 15 nM in hybridization buffer, and the detection substrate 4-methylumbelliferyl phosphate (4-MUP).

Pretreated sample was introduced into the sample well of the prefilled Vidas strip (200 μL per test), and the strip was placed into the retaining tracks of the instrument. All reaction steps were performed automatically on the Vidas at 37 °C over a 3 h period during which the following occurred: capture of the target by ODN 3, washes, incubation with the detection probe ODN 4, washes, incubation with the AP-labeled probe, washes, and conversion of the AP substrate to a detectable fluorescent signal. The Vidas detector expresses the measured fluorescent signal in relative fluorescence values (RFV). This Vidas protocol was identical for the three HBV DNA assays, described in Figure 4: (A) classical sandwich hybridization without amplification (capture and detection of the target performed using the HBV capture ODN 3 and detection probe ODN 4 not conjugated to the NVP/NAS copolymer); (B) sandwich hybridization with signal amplification (detection probe ODN 4 used in conjugated form to the NVP/NAS copolymer); and (C) sandwich hybridization with capture and signal amplification (capture probe ODN 3 and detection probe ODN 4 used in forms conjugated to the NVP/NAS copolymer).

RESULTS AND DISCUSSION

Coupling of ODN to NVP/NAS Copolymer. Figure 2 shows an example of SEC analysis for the coupling of ODN 1 to the NVP/NAS copolymer AZEO1. In SEC, the ODN-copolymer conjugate and the free ODN are separable since they exhibit a large discrepancy in molecular weights and consequently various hydrodynamic volumes. The coupling yield *R* is determined from the peaks of free ODN and ODN-copolymer conjugate using the equation

$$R = \frac{\text{IP (ODN-copolymer)} \times 100}{\text{IP (ODN-copolymer)} + \text{IP (free ODN)}} \quad (2)$$

where IP is the integrated peak.

This calculation assumes that the absorbance at 260 nm of the ODN-copolymer conjugate is due to the ODN

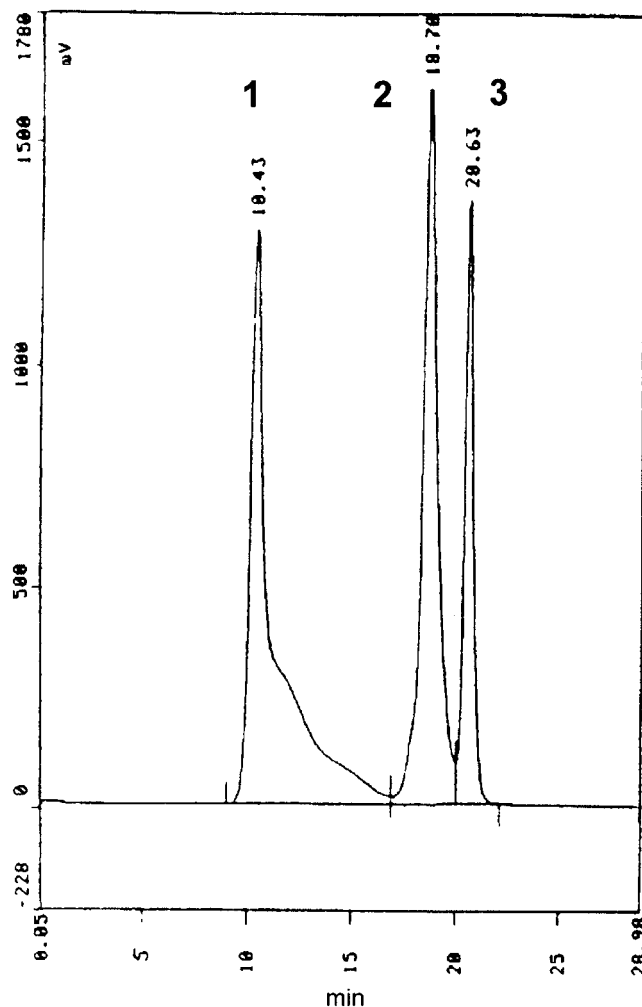


Figure 2. Analysis of the grafting reaction of ODN 1 to the NVP/NAS copolymer AZEO1 by size exclusion chromatography (coupling procedure B). 1, ODN-copolymer conjugate; 2, free ODN; 3, NHS.

and that the molar extinction coefficient of this ODN form is the same as that for the free ODN; that is, this calculation does not consider the copolymer concentration. In Figure 2, *R* is 50% according to this determination. Equation 2 can also be used to determine the yield from solution capillary electrophoresis by which the ODN-copolymer and free ODN are separated by charge. These two methods of determination are in good agreement (19). To discount the possibility of physical adsorption of the ODN-copolymer conjugate to the column, especially when considering the high molecular weight of the conjugate which will be discussed later, coupling yield was verified by fluorescence. This was done by covalently attaching the fluorophore fluorescein hydrazide to the NVP/NAS copolymer prior to the coupling reaction. We estimated that <5% of the reactive sites on the NVP/NAS copolymer reacted with the fluorophore, leaving available the majority of reactive site functions to react with the ODN in the coupling reaction. After purification of the conjugate ODN-fluoresceinated copolymer, the copolymer and the ODN were quantified using fluorescence and UV spectroscopy. Using this method, *R* was determined to be 46% for the example cited in Figure 2, whereas the determination by SEC analysis was 50%.

Characterization of the ODN-Copolymer Conjugate by SEC-LS. A multiangle light scattering detector was coupled to the SEC column. This technique permitted us to determine the *M_w* of the ODN-copolymer

Table 2. SEC-LS Characterization of ODN–NVP/NAS Copolymer Conjugates

analysis no.	ODN	copolymer	couplg proc	M_w^a	R_{gw}^b (nm)
1	ODN 1 ^c			10900	11
2		AZEO1 ^d		69200	14
3	ODN 1	AZEO1	A	1×10^6	40
4	ODN 1	AZEO1	B	12×10^6	46
5	ODN 1	AZEO1	B ^e	19×10^6	50
6	ODN 2	AZEO1	B	10×10^6	45

^a M_w , weight-average molecular weight. ^b R_g , gyration radii.

^c One milligram of ODN was used to determine molecular weight. Theoretically, $M_w = 7745$. In the low M_w range (<10 000), uncertainty on the measure significantly increases. ^d Hydrolyzed copolymer was used. Nonhydrolyzed copolymer is not soluble in phosphate buffer and therefore could not be analyzed under the conditions of this experiment. ^e This conjugate was the same as in analysis 4, but before analysis the conjugate was heated for 5 min at 95 °C and the SEC column was heated to 60 °C.

conjugate on the basis of the refractive index (dn/dc) and mass of the injected material. Since dn/dc could not be determined by classical differential refractometry, which requires much more conjugate than what was available, we used the value 0.20 mL/g for dn/dc . This value is based on the dn/dc of 0.18 mL/g for poly(vinylpyrrolidone) (PVP) (25) and that of 0.20 mL/g for DNA in water (25). Our estimate has been previously assumed (26).

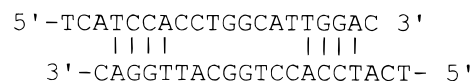
The results of the analysis of our conjugates by SEC-LS are summarized in Table 2. ODNs, hydrolyzed copolymer AZEO1, and ODN–AZEO1 conjugates were analyzed in phosphate buffer. The molecular weights of the ODN–copolymer conjugates were found to be much higher than expected, and this was independent of the coupling procedure, whether procedure A or B. Taking into consideration a grafting efficiency of 50% and based on calculations at 1 mol of ODN for 6 mol of NAS (reaction stoichiometry), assuming molecular weights of 7 745 [calculated as described by Brown et al. (27)] and 70 000 for ODN 1 and AZEO1, respectively, the molecular weight of the conjugate should not have exceeded 256 000. The following equation was used to determine the theoretical molecular weight:

$$M_{w,cj} = M_{w,cp} + M_{w,ODN}(n_{NAS} \times R \times 1/6) \quad (3)$$

$M_{w,cj}$ is the molecular weight of the conjugate ODN–copolymer, $M_{w,cp}$ is the weight-average molecular weight of the copolymer AZEO1, $M_{w,ODN}$ is the molecular weight of the ODN1, n_{NAS} is the number of NAS moieties per mole of copolymer, and R is the yield of coupling.

The SEC-LS method of analysis gives accurate molecular weights for ODN1 and hydrolyzed copolymer AZEO1 (Table 2). Thus, the high molecular weights obtained for the coupling reaction product might be attributable to light scattering properties. A more accurate estimate for dn/dc would not lead to a more accurate measurement of conjugate molecular weight, since only a highly skewed estimate of dn/dc would correct for the exceedingly high estimate of conjugate molecular weight.

Our observations suggest the formation of aggregates during the coupling reaction. Molecular weights are particularly high, $>10^7$, when the coupling reaction is performed with procedure B, where the reaction conditions are more concentrated and the conditions for hydrolyzing are less favorable. The reaction was carried out in an aqueous–organic mixture, since the copolymer sample is soluble only in polar solvents (DMF) and the ODN is water-soluble. Two competitive reactions should

**Figure 3.** Self-hybridization of ODN 1, most stable complex predicted by the program OLIGO 4.02, National Biosciences, Inc.

be considered under these conditions: hydrolysis of NAS functions and ODN coupling.

When the hydrolyzing conditions are more favorable, as in coupling procedure A, the conjugate molecular weight decreases; aggregate formation is reduced, but not totally eliminated, since the measured molecular weight of the conjugate (10^6) is also much higher than expected. In a previous study based on solution behavior, it was suggested that copolymer chains assume a random coil conformation (19). Considering this, and due to hydrolysis and coupling reactions, copolymer chains would tend to unfold and expand. However, as ionic charges increase due to the grafting of ODNs, solvency would decrease in the organic DMF coupling mixture, which in turn could cause the formation of more compact structures and, depending on their location, result in reduced accessibility of unreacted activated esters. This compaction of structure is suggested experimentally by the relatively small gyration radii (between 40 and 50 nm) of the high molecular weight conjugates.

One explanation for the formation of aggregates involves hydrogen bonding. Computer analysis of the ODN 1 sequence showed the potential for self-hybridization involving nonadjacent sequences of four bases (Figure 3). Thus, inter- and intra-ODN interactions are possible in the compacted aggregate structure even if the intramolecular interaction would be far more stable. It may be assumed that these aggregates can be broken, at least partially, by heat or by chaotropic agents. Therefore, to get a more accurate measurement of molecular weights, after the coupling reaction, the sample was heated to 95 °C and then injected into a thermostabilized SEC column at 60 °C. However, from the results in Table 2, analysis 5, no decrease in the measured molecular weight was observed. Therefore, we analyzed a 16 base ODN (ODN 2) composed of T and C nucleotides that was grafted onto the NVP/NAS copolymer (Table 2, analysis 6). No hydrogen bonding interactions are predicted in this ODN since T cannot hybridize with C. Nevertheless, a high molecular weight was estimated for the formed conjugate, suggesting the formation of aggregates and ruling out the hydrogen bonding explanation for the formation of high molecular weight conjugates. Finally, we had followed the kinetics of the grafting reaction using SEC-LS and found that aggregates are formed shortly after the grafting reaction begins (28).

Another explanation for the formation of aggregates involves covalent bonding. The aminoalkyl chain at the 5'-end of the ODN permits a specific grafting reaction, with the activated esters on the NVP/NAS copolymer. To explain the formation of high molecular weight conjugates, secondary reactions involving the NAS functions and the exocyclic amines on nucleotides A, C, and G could be considered. Coupling reactions occur in a mixture of DMF and alkaline buffer which, for the coupling of the aminealkyl at the 5'-end of ODNs, did not show side reactions with biotin-*p*-nitrophenol or dansyl chloride, even when these compounds were in excess (29). On the other hand, it can be assumed that in the case of cytosine, as demonstrated with the N-acylation strategy for protection of deoxyribonucleotides (30), the C₄-amino group would be sufficiently nucleophilic to induce secondary

reactions. To examine the effect of these reactions, grafting was done using 5'-end Aminolink 2 dT₁₃ and Aminolink 2 dT₂₀. Unfortunately, we were unable to draw any conclusions from this study since no coupling reaction was observed (28). Therefore, we examined the coupling of ODN 1 without the Aminolink 2 group at the 5'-end using coupling procedure B. A grafting yield of 15% was observed, suggesting that side reactions are possible with the NAS functions of the copolymer.

Hence, we can consider the possible effect of these side reactions to explain aggregate formation. In a first reaction, coupling could occur with 5'-end aminoalkyl functions, which are more reactive than heterocyclic amine functions. In a second reaction, once the ODN-copolymer is formed, side reactions could occur between the side chains of amino group on heterocyclic bases and the nonhydrolyzed NAS functions. If this reaction takes place between a function of the nucleic base and the NAS functions of other copolymer chains (intermolecular reaction), aggregate formation will occur. The same reaction is possible between two functions of the same chain (intramolecular reaction). In both cases, the structure of the ODN with two linkages, or even more, could reduce its hybridization ability. As it will be demonstrated later for HBV DNA detection, most of the oligonucleotides at the surface of the copolymer are available for hybridization.

Side reactions appear to be favored under the conditions of coupling using procedure B, which resulted in the formation of very compact structures. In the case of procedure A, NAS-activated esters might be more quickly hydrolyzed. The number of secondary reactions between exocyclic amino groups of ODN and unreacted activated esters is then reduced, resulting in less aggregate formation and a less compact structure.

The aim at the beginning of this study was to prepare amplification multimers, copolymers with multiple grafts of ODN, for signal amplification. The amplification factor was to depend on the number of oligonucleotides grafted to the copolymer. Formation of these amplification multimers, however, has two opposing effects: the higher the number of oligonucleotides per copolymer, the greater the potential amplification factor, but also the greater potential for aggregate formation because oligonucleotides reticulate and are less accessible. Nevertheless, we took advantage of the ODN-copolymer structure to try to improve capture and detection efficiencies.

The results are described next for HBV DNA detection.

Use of ODN-Copolymer Conjugates in the Detection of HBV DNA. The ODN-NVP/NAS copolymer conjugates were tested in sandwich hybridization for their ability to amplify the signal for nucleic acid detection. As our model, we chose the detection of HBV DNA.

The level of HBV DNA is the most informative marker for active viral replication and can be used to monitor patients with chronic hepatitis B who are undergoing antiviral therapy (31). HBV DNA can be detected by PCR, which is sensitive but laborious for routine laboratory detection, and by a variety of rapid hybridization methods including dot blot, solution hybridization, and sandwich hybridization, with either radiolabeled or non-radioactive probes (32, 33).

We developed our sandwich hybridization assay on the automated Vidas immunoanalyzer system (22, 23) making use of specific HBV DNA probes complementary to highly conserved regions of HBV DNA for capture and detection. Different detection systems were defined to compare no amplification (free capture and free detection oligonucleotides), capture amplification (capture oligonucleotide linked to NVP/NAS copolymer), and signal

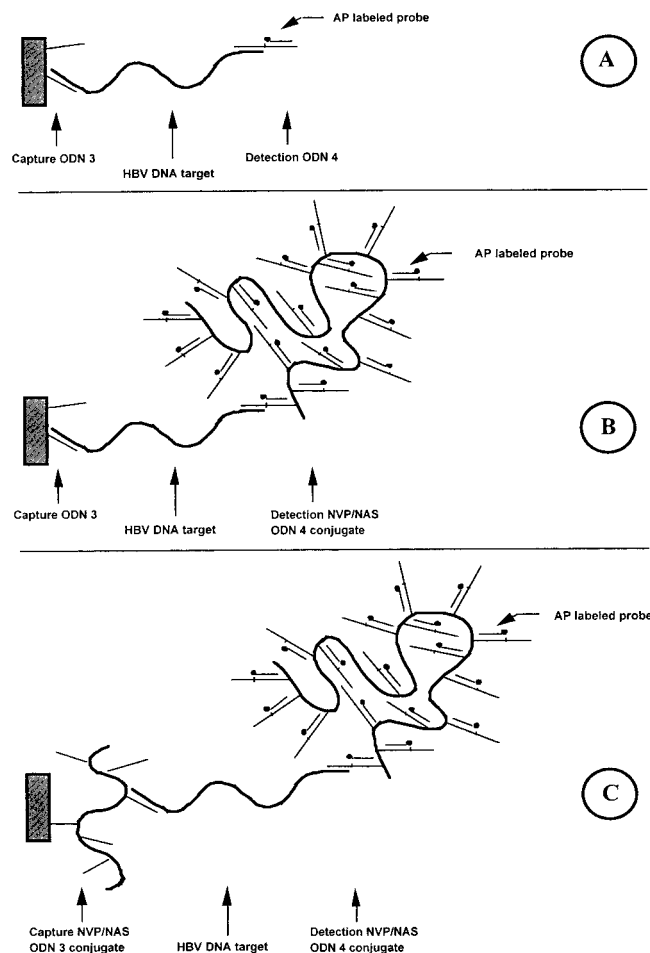


Figure 4. Three Vidas detection formats defined for HBV DNA detection: (A) classical sandwich hybridization assay without amplification; (B) sandwich hybridization assay with signal amplification (detection probe ODN 4 conjugated to the NVP/NAS copolymer); (C) sandwich hybridization assay with capture and signal amplification (capture probe ODN 3 and detection probe ODN 4 conjugated to the NVP/NAS copolymer).

amplification (detection oligonucleotide linked to NVP/NAS copolymer) as described under Experimental Procedures and outlined in Figure 4.

Direct comparison of these formats was possible because the same capture and detection sequences (ODN 3 and ODN 4), as well as the same Vidas program, could be used. This permitted us to evaluate the effect of ODN-copolymer conjugates in improving assay sensitivity. Serial dilutions were made of two kinds of target: a 46-mer synthetic single-stranded oligonucleotide target and a long 2339 bp double-stranded PCR amplified HBV DNA target. Sensitivity, the limit of detection, was defined as the minimum amount of target for which the intensity of the signal was greater than the blank plus 3 times its standard deviation.

The results (Table 3 and Figure 5) show that both capture and signal amplification increased the sensitivity of detection. Moreover, each permitted quantitation of HBV DNA with good precision and over a wide dynamic range. Importantly, no significant increase in background was observed. The best sensitivity was achieved using both capture and signal amplification; sensitivities were 1.5×10^6 and 3.5×10^7 DNA molecules/mL for the synthetic and double-stranded DNA targets, respectively (these detection limits were estimated in Figure 5, by considering for each system a sample/cut-off ratio of 1). The synthetic target was more easily detected probably because it is short and single-stranded; single-stranded-

Table 3. Detection of HBV DNA Using Capture and Signal Amplification

target (DNA molecules/mL)	no amplfn		signal amplfn		capture and signal amplfn	
	DS ^b (2339 bp)	SS ^b (46 b)	DS (2339 bp)	SS (46 b)	DS (2339 bp)	SS (46 b)
0	31	31	42	42	65	65
10 ⁶	31	31	42	42	65	65
10 ⁷	30	44	42	153	80	207
10 ⁸	30	64	45	972	105	2100
10 ⁹	30	350	51	7300	170	9700
10 ¹⁰	42	3279	131	8145	1320	10000
10 ¹¹	135	9449	997	9600	8189	ND ^c
10 ¹²	505	9431	5870	ND	ND	ND

^a Detection assays were done on the Vidas with no amplification, signal amplification, or both capture and signal amplification. The data expressed in RFV are mean values of duplicate measurements except for the blank, which represents the mean of six measurements. Standard deviations for the blank readings were 5.7, 7.1, and 10 for the no amplification, signal amplification, and capture and signal amplification systems, respectively. The ODN-copolymer conjugates used were those described in Table 2. ^b DS, double-stranded; SS, single-stranded. ^c ND, not determined.

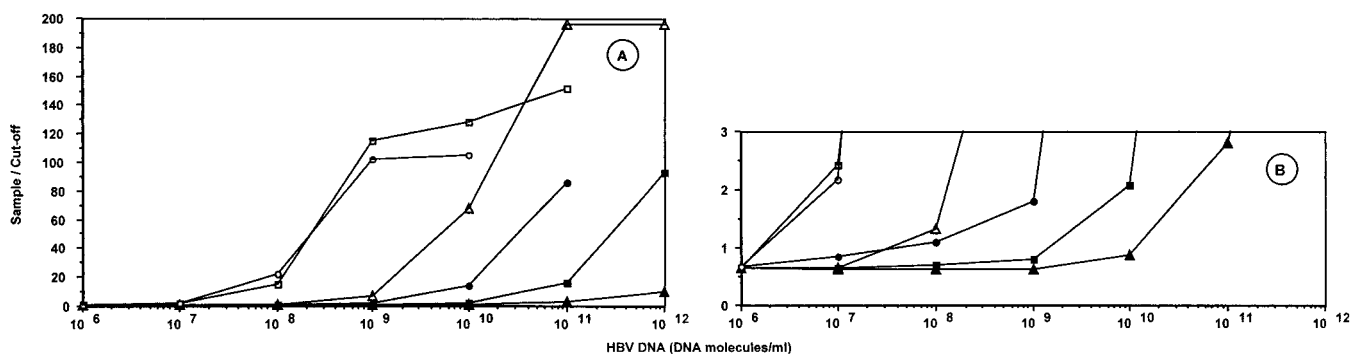


Figure 5. Detection of HBV DNA using capture and signal amplification. Assays were run on the Vidas with no amplification (Δ and ▲), signal amplification (□ and ■), or both capture and signal amplification (○ and ●). Open symbols, 46-mer synthetic single-stranded target; solid symbols, 2339 bp double-stranded target. The data represent ratios of sample RFV (detailed in Table 3) to cutoff RFV. Cutoff values, defined as the background RFV plus 3 times its standard deviation (see Table 3), were 48.1, 63.3, and 95 for the systems no amplification, signal amplification, and capture and signal amplification, respectively. A ratio >1 signifies a positive result. (A) Whole results; (B) enlargement of part A for the low values range, which was used to estimate the sensitivity of the different systems.

ness avoids the problem of reannealing after sample denaturation, as would occur for the double-stranded target.

To explain the increase in sensitivity, we believe that capture amplification may work by increasing the accessibility of the capture oligonucleotides which are removed, thereby reducing steric hindrance on the solid support and presenting more flexible capture probes. This explanation is supported by the observation that capture amplification resulted in increased sensitivity for the long double-stranded target but not for the short single-stranded target.

Increased sensitivity with signal amplification can be explained by an effective increase in the number of AP molecules hybridized to the detection ODN-copolymer. A similar principle based on the effective increase of AP molecules has been described by Urdea et al. (34), who developed the branched DNA (bDNA) signal amplification system. However, the structures of our and Urdea's signal amplification systems differ in three respects: (i) our capture and detection oligonucleotides are covalently linked to an NVP/NAS copolymer by a direct and simple conjugation procedure, whereas in the bDNA test, the branched DNA structure is synthesized by a combination of solid phase chemistry and enzymatic ligation methods (17); (ii) in our signal amplification system, the HBV specific detection oligonucleotide is directly conjugated to the signal amplification NVP/NAS copolymer, whereas in the bDNA system the detection oligonucleotide is used to link the bDNA amplifier by a complementary sequence; (iii) our system also incorporates a capture amplification step with NVP/NAS-conjugated oligonucle-

otides adsorbed to the solid phase, whereas the bDNA system uses adsorbed oligonucleotides.

The sensitivity of the bDNA test for HBV manufactured by Chiron Corp. (Quantiplex HBV assay) has been reported to range from 7×10^5 to 1×10^6 DNA molecules/mL using cloned HBV DNA (35, 36). The higher sensitivity of the Quantiplex HBV assay to our assay (we detect 3.5×10^7 DNA molecules/mL of the 2339 bp HBV double-stranded target) might be explained by the use of a pool of multiple-site HBV detection oligonucleotides to link the bDNA amplifier; in the latter, 12 sequences are used in capture and 36 sequences in detection (34), whereas our system makes use of only 1 sequence in capture and 1 sequence in detection. Furthermore, the bDNA system uses a chemiluminescent substrate (dioxetane) in detection, which is known to be more sensitive than the fluorescent substrate (4-MUP) used in our Vidas assay.

CONCLUSIONS

ODN-NVP/NAS copolymer conjugates have been prepared using a simple, one-step reaction procedure. These conjugates were characterized by SEC-LS and were shown to have a higher than expected molecular weight. Aggregate formation has been proposed to explain this, and the involvement of side reactions from heterocyclic base amino groups, especially of cytosine, has been implicated in this formation. The structure of these conjugates is currently under investigation using neutron scattering to confirm our hypothesis.

Our ODN-NVP/NAS copolymer conjugates were tested in nucleic acid detection and were shown to improve

assay sensitivity in both capture and signal efficiencies for a long double-stranded DNA.

The main advantages of our technology are ease of preparation and sensitivity. We have demonstrated sensitivity to 3.5×10^7 molecules/mL for HBV DNA and are now testing our system in other application, e.g., in ribosomal RNA detection. Because of the observed sensitivity ($\sim 10^7$ molecules/mL), the ODN-NVP/NAS copolymer technology should be suited to the direct detection of nucleic acid in assays requiring a sensitivity of 10^6 DNA molecules/mL, without the need for target sequence amplification.

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LITERATURE CITED

- (1) Abramson, R. D., and Myers, T. W. (1993) Nucleic acid amplification technologies. *Curr. Opin. Biotechnol.* 4, 41–47.
- (2) Nelson, P. S., Sherman-Gold, R., and Leon, R. (1989) A new and versatile reagent for incorporating multiple primary aliphatic amines into synthetic oligonucleotides. *Nucleic Acids Res.* 17 (18), 7179–7186.
- (3) Piele, U., Sproat, B. S., and Lamm, G. M. (1990) A protected biotin containing deoxycytidine building block for solid phase synthesis of biotinylated oligonucleotides. *Nucleic Acids Res.* 18 (15), 4355–4360.
- (4) Misiura, K., Durrant, I., Evans, M. E., and Gait, M. R. (1990) Biotinyl and phosphotriester phosphoramidite derivatives useful in the incorporation of multiple reporter groups on synthetic oligonucleotides. *Nucleic Acids Res.* 18 (15), 4345–4354.
- (5) Roget, A., Bazin, H., and Teoule, R. (1989) Synthesis and use of labelled nucleoside phosphoramidite building blocks bearing a reporter group: biotinyl, dinitrophenyl, pyrenyl and dansyl. *Nucleic Acids Res.* 17 (19), 7643–7651.
- (6) Pierlot, C., and Sergheraert, C. (1992) Solid phase synthesis of 5' nonradioactive multiple labelled oligodeoxyribonucleotides. *Bioorg. Med. Chem. Lett.* 2 (3), 267–270.
- (7) Haralambidis, J., Angus, K., Pownall, S., Duncan, L., Chai, M., and Tregear, G. W. (1990) The preparation of polyamide-oligonucleotide probes containing multiple non-radioactive labels. *Nucleic Acids Res.* 18 (3), 501–505.
- (8) De Vos, M. J., Van Elsen, A., and Bollen, A. (1994) New non nucleosidic phosphoramidites for the solid phase multi-labelling of oligonucleotides: comb- and multifork-like structures. *Nucleosides Nucleotides* 13 (10), 2245–2265.
- (9) Teigelkamp, S., Ebel, S., Will, D. W., Brown T., and Beggs, J. D. (1993) Branched poly-labelled oligonucleotides: enhanced specificity of fork-shaped biotinylated oligoribonucleotides for antisense affinity selection. *Nucleic Acids Res.* 21 (19), 4651–4652.
- (10) Conway, N. E., and McLaughlin, L. W. (1991) The covalent attachment of multiple fluorophores to DNA containing phosphorothioate diesters results in highly sensitive detection of single stranded DNA. *Bioconjugate Chem.* 2, 452–457.
- (11) Sund, C., Ylikoski, J., Hurskainen, P., and Kwiatkowski, M. (1988) Construction of europium (Eu^{3+})-labelled oligo DNA hybridization probes. *Nucleosides Nucleotides* 7 (5 and 6), 655–659.
- (12) Pachmann, K., Reinecke, K., Emmerich, B., and Thiel, E. (1991) Highly fluorochrome labeled gene probes for quantitative tracing of RNA in individual cells by in situ hybridization. *Bioconjugate Chem.* 2, 19–25.
- (13) Grzybowski, J., Will, D. W., Randall, R. E., Smith, C. A., and Brown, T. (1993) Synthesis and antibody-mediated detection of oligonucleotides containing multiple 2,4-dinitrophenyl reporter groups. *Nucleic Acids Res.* 21 (8), 1705–1712.
- (14) Horn, T., Warner, B. D., Running, J. A., Downing, K., Clyne, J., and Urdea, M. S. (1989) The synthesis of branched oligonucleotides as signal amplification multimers for use in nucleic acid assays. *Nucleosides Nucleotides* 8 (5 and 6), 875–877.
- (15) Horn, T., and Urdea, M. S. (1989) Forks and combs DNA: the synthesis of branched oligodeoxynucleotides. *Nucleic Acids Res.* 17 (17), 6959–6967.
- (16) Urdea, M. S., Horn, T., Fultz, T., Anderson, M., Running, J., and Hamren, S. (1991) Branched DNA amplification multimers for the sensitive direct detection of human hepatitis viruses. *Nucleic Acid Res. Symp. Ser. No. 24*, 197–200.
- (17) Urdea, M. S. (1993) Synthesis and characterization of branched DNA (bDNA) for the direct and quantitative detection of CMV, HBV, HCV, HIV. *Clin. Chem.* 39 (4), 725–726.
- (18) Erout, M. N., Elaissari, A., Pichot, C., and Llauro, M. F. (1996) Radical initiated copolymers of *N*-vinylpyrrolidone and *N*-acryloxysuccinimide. *Polymer* 37 (7), 1157–1165.
- (19) Erout, M. N., Elaissari, A., Pichot, C., Cros, P., and Kurfürst, K. (1996) Characterization of the coupling reaction between oligodeoxyribonucleotides and *N*-vinylpyrrolidone/*N*-acryloxysuccinimide copolymers by size exclusion chromatography and free solution capillary electrophoresis. *Int. J. Polym. Anal. Charact.* 2, 253–269.
- (20) Pollak, A., Blumenfeld, H., Wax, M., Baughn, R. L., and Whitesides, G. M. (1980) Enzyme immobilization by condensation copolymerization into crosslinked polyacrylamide gels. *J. Am. Chem. Soc.* 102, 6324–6336.
- (21) Galibert, F., Mandart, E., Fitoussi, F., Tiollais, P., and Charnay, P. (1979) Nucleotide sequence of the hepatitis B virus genome (subtype ayw) cloned in *E. coli*. *Nature* 281, 646–650.
- (22) Allibert, P., Cros, P., and Mandrand, B. (1992) Automated detection of nucleic acid sequences of HPV16, 18 and 6/11. *Eur. J. Biomed. Technol.* 14, 152–155.
- (23) Mabilat, C., Desvarenne, S., Panteix, G., Machabert, N., Bernillon, M. H., Guardiola, G., and Cros, P. (1994) Routine identification of *Mycobacterium tuberculosis* complex isolates by automated hybridization. *J. Clin. Microbiol.* 32 (11), 2702–2705.
- (24) Jablonsky, E., Moomaw, E. W., Tullis, R. H., and Ruth, J. L. (1986) Preparation of oligonucleotide-alkaline phosphatase conjugates and their use as hybridization probes. *Nucleic Acids Res.* 14, 6115–6128.
- (25) J. Brandrup and E. H. Immergut, Eds. (1989) *Polymer Handbook*, Wiley, New York.
- (26) Ladaviere, C. (1995) personal communication.
- (27) Brown, T., and Brown, D. J. S. (1991) Modern machine aided methods in oligodeoxy ribonucleotides synthesis, in *Oligonucleotides and Analogs, a Practical Approach* (F. Eckstein, Ed.) p 1–23, IRL Press, Oxford, U.K..
- (28) Erout, M. N. Doctorate thesis, Université Claude Bernard, Lyon, 1994.
- (29) Connolly, B. A. (1987) The synthesis of oligonucleotides containing a primary amino group at the 5'-terminus. *Nucleic Acids Res.* 15 (7), 3131–3139.
- (30) Jones, R. A. (1984) Preparation of protected deoxyribonucleosides, in *Oligonucleotide Synthesis, a Practical Approach* (M. J. Gait, Ed.) pp 23–34, IRL Press, Oxford, U.K.
- (31) Bonino, F., Hoyer, B., Nelson, J., Engle, R., Verme, G., and Gerin, J. (1981) Hepatitis B virus DNA in the sera of HBsAg carriers: a marker of active hepatitis B virus replication in the liver. *Hepatology* 1, 386–391.
- (32) Zaaijer, H. L., Terborg, F., Cuypers, H. T. M., Hermus, M. C. A. H., and Lelie, P. N. (1994) Comparison of methods for detection of hepatitis B virus DNA. *J. Clin. Microbiol.* 32, 2088–2091.
- (33) Valentine-Thon, E. (1995) Evaluation of SHARP signal system for enzymatic detection of amplified hepatitis B virus DNA. *J. Clin. Microbiol.* 33, 477–480.
- (34) Urdea, M. S., Running, J. A., Horn, T., Clyne, J., Ku, L., and Warner, B. D. (1987) A novel method for the rapid detection of specific nucleotide sequences in crude biological

- samples without blotting or radioactivity; its application to the analysis of hepatitis B virus in human serum. *Gene* 61, 253–264.
- (35) Chen, C. H., Wang, J. T., Lee, C. Z., Sheu, J. C., Wang, T. H., and Chen, D. S. (1995) Quantitative detection of hepatitis B virus DNA in human sera by branched-DNA signal amplification. *J. Virol. Methods* 53, 131–137.
- (36) Hendricks, D. A., Stowe, B. J., Hoo, B. S., Kolberg, J., Irvine, B. D., Neuwald, P. D., Urdea, M. S., and Perrillo, R. P. (1995) Quantitation of HBV DNA in human serum using a branched DNA (bDNA) signal amplification assay. *Am. J. Clin. Pathol.* 104 (5), 537–546.

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Maleimido Derivatives of Diethylenetriaminepentaacetic Acid and Triethylenetetraaminehexaacetic Acid: Their Synthesis and Potential for Specific Conjugation with Biomolecules

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Immunoconjugates sometimes suffer changes in immunoreactivity and tumor targeting because the chelators used interfere with antigen binding. To try to circumvent such problems, two new homobifunctional chelating agents, 2,6-bis[*p*-(β -maleimidopropionyl)amino]benzyl]diethylenetriamine-*N,N,N',N'',N'''*-pentaacetic acid (MPBz-DTPA) and 2,9-bis[*p*-(β -maleimidopropionyl)amino]benzyl]triethylenetetraamine-*N,N,N',N'',N'''*-hexaacetic acid (MPBz-TTHA), were synthesized for chelation of ¹¹¹In and ⁹⁰Y metal ions. Each of the new chelators contains two maleimido functional groups substituted at the carbon backbone. In this study, the overall chemical yields for MPBz-DTPA and MPBz-TTHA were approximately 5 and 4%, respectively. The intermediate and final compounds were purified by flash chromatography and fully characterized by ¹H-NMR, ¹³C-NMR, and mass spectroscopy. Chemical modification of the maleimido group on each of these derivatives allowed specific conjugation with a sulfhydryl group at the hinge region of an antibody molecule and conjugation with other peptides. For instance, the two Fab' fragments, through their hinge-region sulfhydryl groups, could covalently attach to a homobifunctional chelator via thioether linkage to generate a Fab'-S-chelator-S-Fab' immunoconjugate. By design, immunoconjugates containing these new chelators will contain only one chelator derivative at a known region of antibody away from the antigen binding site. Consequently, these chelators may minimize changes in the immunoreactivity and improve tumor targeting of immunoconjugates that contain them.

INTRODUCTION

The attachment of radiometal ions to monoclonal antibodies via bifunctional chelating agents has been beneficial to the development of radioimmunoconjugates for the diagnosis and therapy of cancer (1–4). In many of the studies reported to date, the diethylenetriaminepentaacetic acid (DTPA)¹ chelator and its modified substituted derivatives with various R groups attached to the carbon chain were used to improve the stability and the rigidity of the metal–ligand coordinate complexes (5–9). More recently, another series of bifunctional chelators has been introduced, consisting of macrocyclic frames of carbon and nitrogen (10–20). While macrocyclic structures provide higher complex stability for a particular radiometal, they produce a low yield of radiometal incorporation owing to the formation of competing coordination complexes with existing trace metal ions in conjugation buffers and in labeling solution (21). In all of these chelate structures, the ligand groups (–COOH, –N–) of the chelator hold the radiometal ion

while an activated functional (–N=C=S) group of the chelator covalently links the protein molecule with the chelator.

In general, chelate conjugation methodology takes advantage of the ϵ -amino group of the lysine residues of an antibody's peptide chain to join a chelator with an antibody molecule. This conjugation method is random, however, and can lead to problems. For instance, in the case of isothiocyanate–chelate conjugation, it is difficult to determine the exact position of conjugated chelators on an antibody; also, the number of chelators attached may vary from molecule to molecule. Moreover, because chelators are randomly attached to the available lysine residues on antibody molecules, they can interfere with antigen binding if any of the lysine residues happens to be in an antigen binding region. Consequently, loss of immunoreactivity after conjugation is a major concern (22–24). It follows that radioimmunoconjugates with only one or two chelates per antibody molecule will have high immunoreactivity because they are less likely to contain a chelate in the antigen binding region (25).

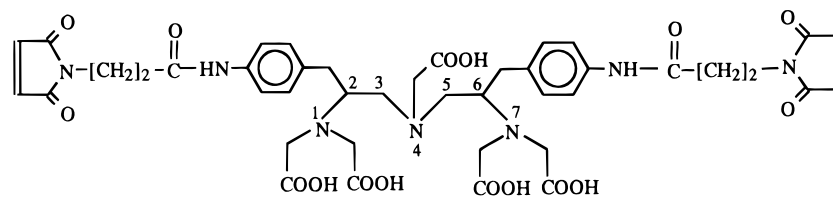
Here, we describe the synthesis and characterization of two novel maleimido chelators: 2,6-bis[*p*-(β -maleimidopropionyl)amino]benzyl]diethylenetriamine-*N,N,N',N'',N'''*-pentaacetic acid (MPBz-DTPA, **1**) and 2,9-bis[*p*-(β -maleimidopropionyl)amino]benzyl]triethylenetetraamine-*N,N,N',N'',N'''*-hexaacetic acid (MPBz-TTHA, **2**). The chemical structures of these chelators are illustrated in Figure 1.

A maleimido group reacts selectively with sulfhydryl groups (–SH) at pH 5–7, and for this reason the maleimido group is widely used to form stable protein conjugates (26, 27). This specific reactivity of maleimido groups for –SH groups has moved us to develop novel chelators containing maleimido derivatives (28, 29). These chelators may bind two Fab' fragments by site-

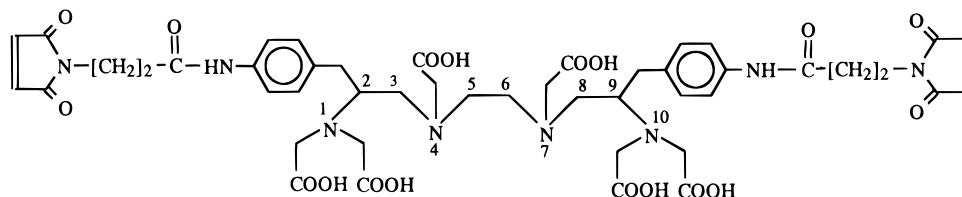
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¹ Abbreviations: DTPA, diethylenetriaminepentaacetic acid; TTHA, triethylenetetraaminehexaacetic acid; TFA, trifluoroacetic acid; NHS, *N*-hydroxysuccinimide; DCC, 1,3-dicyclohexylcarbodiimide; DMF, *N,N*-dimethylformamide; *t*-BOC, *tert*-butoxycarbonyl; MPBz-DTPA, 2,6-bis[*p*-(β -maleimidopropionyl)amino]benzyl]diethylenetriamine-*N,N,N',N'',N'''*-pentaacetic acid; MPBz-TTHA, 2,9-bis[*p*-(β -maleimidopropionyl)amino]benzyl]triethylenetetraamine-*N,N,N',N'',N'''*-hexaacetic acid; THF, tetrahydrofuran; MPA *N*-HSE, β -maleimidopropionic acid *N*-hydroxysuccinimide ester; CD, circular dichroism.



MPBz-DTPA (1)



MPBz-TTHA (2)

Figure 1. Structures of MPBz-DTPA (1) and MPBz-TTHA (2).

selective cross-linking through thioether linkages to generate the form Fab'-S-chelate-S-Fab'. Any resulting immunoconjugates will thus contain only one MPBz-DTPA or MPBz-TTHA chelator derivative at a known site of the antibody (the hinge region) away from the antigen binding site. Moreover, the consequent specific conjugation of antibodies should minimize changes in immunoreactivity and might even improve tumor targeting.

The versatility of these chelators lies in their providing two sites for the attachment of biomolecules, without significant loss of biological activity, and also of radiolabels with a diagnostic (^{111}In) or therapeutic (^{90}Y) radiometal. This new chelate conjugation method can be utilized for combining two Fab' fragments of different specificity to produce a site-radiolabeled bispecific antibody. One Fab' fragment reacts with a tumor-associated antigen; the other reacts with a hapten, which can be labeled with a therapeutic radioisotope. After administration of ^{111}In -labeled bispecific antibodies, tumor and normal tissue uptake of antibody can be visualized and quantified. In this way, the optimal timing for the subsequent administration of the ^{90}Y -labeled hapten can be determined more accurately.

EXPERIMENTAL PROCEDURES

Materials and Methods. 4-Nitro-L-phenylalanine monohydrate, borane–tetrahydrofuran complex (1 M solution in THF), ethylenediamine, trifluoroacetic acid (TFA), *N*-hydroxysuccinimide (NHS), 1,3-dicyclohexylcarbodiimide (DCC), anhydrous potassium carbonate, anhydrous *N,N*-dimethylformamide (DMF), anhydrous magnesium sulfate, *tert*-butyl bromoacetate, and TLC plates (250 nm, 60 Å) precoated with fluorescent indicators and silica gel (70–230 mesh, 60 Å) were purchased from Aldrich Chemical Co. (Milwaukee, WI). *N*-*t*-BOC-*p*-nitro-L-phenylalanine and β -maleimidopropionic acid *N*-hydroxysuccinimide ester were obtained from Sigma Chemical Co. (St. Louis, MO). All solvents of HPLC grade were obtained from Aldrich and used as received. ^1H -NMR and ^{13}C -NMR spectra were recorded on IBM BR 200/AF and General Electric GN 500 spectrometers. Mass spectra were recorded on a Kratos MS-50 RF double-focusing mass spectrometer fitted with an Ion-Tech fast atom bombardment gun using xenon as the bombardment gas. Circular dichroism (CD) was measured on a Jasco-170 spectropolarimeter using 1 cm path cells.

Methyl *p*-Nitrophenylalanine Hydrochloride (4).

Compound **4** was prepared by following a previously reported method (6). Thionyl chloride (2.0 mL, 27.41 mmol) was mixed with 40 mL of anhydrous methanol and stirred in an ice bath under nitrogen. To this solution was added 4-nitro-L-phenylalanine monohydrate (5.0 g, 23.78 mmol) as a solid. Stirring was then continued for 36 h. The solvent was removed under reduced pressure to get **4**: yield, 6.0 g (97%); TLC, R_f = 0.83 ($\text{CHCl}_3/\text{CH}_3\text{OH}$, 4:1 v/v).

***p*-Nitrophenylalanine Amide (5).** Amide was prepared as previously described (30). A slurry of compound **4** (5.50 g, 21.12 mmol) in 5 mL of anhydrous methanol was treated with 2.9 mL of triethylamine. Anhydrous ether (60 mL) was added and shaken well to get a clear solution. On cooling for 2 h at -10°C , a white solid was precipitated. This solid was filtered, and the filtrate was evaporated to dryness on a rotary evaporator. A limpid yellow oil was obtained. The oil was dissolved in 50 mL of anhydrous methanol, passed through $\text{NH}_3(\text{g})$ for 15 min, and then allowed to stand at room temperature for 4 days. Then, the solvent was evaporated to dryness. A light yellow compound, **5**, was obtained: yield, 4.25 g (96%); TLC, R_f = 0.6 ($\text{CHCl}_3/\text{CH}_3\text{OH}$, 4:1 v/v).

2-(*p*-Nitrobenzyl)ethylenediamine (6). Reduction was done according to a previously reported method (6). Compound **5** (3.90 g, 18.66 mmol) was dissolved in 40 mL of anhydrous THF. To this was added a solution of 1.0 M BH_3 –THF (158.6 mL, 158.6 mmol) via syringe with stirring under nitrogen at -10°C . After stirring continuously for 1.5 h, the solution was then refluxed for 18 h. The reaction mixture was cooled to -10°C , and 20 mL of anhydrous methanol was added. The solvent was removed under reduced pressure, another 25 mL of MeOH was added, and the mixture was concentrated to form a yellow oil. The yellow oil was purified by flash chromatography on silica gel and elution with $\text{CHCl}_3/\text{CH}_3\text{OH}/\text{NH}_4\text{OH}$ (12:4:1 v/v/v). A pure yellow oil of 2-(*p*-nitrobenzyl)ethylenediamine (**6**) was obtained: yield, 2.40 g (66%); TLC, R_f = 0.38 ($\text{CH}_3\text{Cl}/\text{CH}_3\text{OH}/\text{NH}_4\text{OH}$, 12:4:1 v/v/v).

***N*-(*tert*-Butoxycarbonyl)-*p*-nitrophenylalanine *N*-Hydroxysuccinimide Ester (8).** Procedure was adopted from a previously reported method (19). *N*-*t*-BOC-*p*-nitrophenylalanine (**7**) (10 g, 32.22 mmol) and *N*-hydroxysuccinimide (NHS) (4.35 g, 37.50 mmol) were dissolved in 55 mL of anhydrous THF, and to this solution was

added dropwise dicyclohexylcarbodiimide (DCC) (7.42 g, 36.0 mmol) in 20 mL of anhydrous THF. The reaction was kept stirring at 0 °C. After 2 h, 55 μ L of acetic acid was added to quench the reaction, and stirring was continued for another 1 h. Then the reaction mixture was filtered and the filtrate evaporated to dryness. The product was purified by chromatography on silica gel and elution with $\text{CHCl}_3/\text{CH}_3\text{CN}$ (8:2 v/v). A pure white solid **8** was obtained: yield, 11.95 g (91%); TLC, R_f = 0.83 ($\text{CHCl}_3/\text{CH}_3\text{CN}$, 8:2 v/v); FAB-MS, m/e 408 ($M + H^+$); ^1H NMR (CDCl_3) δ 8.19 (d, J = 8.6 Hz, 2H), 7.49 (d, J = 8.7 Hz, 2H), 4.95–5.10 (m, 1H), 3.25–3.50 (m, 2H), 2.85 (s, 4H), 1.40 (s, 9H); ^{13}C NMR (CDCl_3) δ 168.47, 167.18, 154.37, 147.34, 142.38, 130.71, 123.73, 80.98, 52.29, 38.17, 28.14, 25.54.

2,6-Bis(p-nitrobenzyl)-N,N,N',N'',N'''-pentakis(tert-butoxycarbonyl)-N-t-BOC-diethylenetriamin-5-one (9). A mixture of *p*-nitrophenylethylenediamine (**6**) (0.766 g, 3.93 mmol), triethylamine (2.90 mL, 20.82 mmol), and 5 mL of anhydrous acetonitrile was taken into a cleaned round-bottom flask and stirred for 30 min. Compound **8** (1.60 g, 3.93 mmol) was dissolved in 70 mL of anhydrous acetonitrile and added dropwise to the reaction mixture. Stirring was continued for 4 days. The reaction mixture was kept overnight at 0 °C and then filtered and dried. The crude product was dissolved in 5 mL of water, and the pH was adjusted to 11.5 with 1.0 M Na_2CO_3 solution. The contents were then mixed with 10 mL of saturated NaCl solution and extracted five times with 15 mL of chloroform. The extracts were dried over anhydrous sodium sulfate and filtered. The solvent was removed under reduced pressure to yield an off-white solid, which was purified by column chromatography on silica gel and elution with $\text{CHCl}_3/\text{MeOH}$ (8:1 v/v). After solvent evaporation, compound **9** was isolated as a white solid: yield, 1.77 g (92%); TLC, R_f = 0.4 ($\text{CHCl}_3/\text{CH}_3\text{OH}$, 8:1 v/v); FAB-MS, m/e 488 [$M + H^+$]; ^1H NMR ($\text{DMSO}-d_6$) δ 8.16 (t, J = 5.7 Hz, CONH), 8.03 (d, J = 5.4 Hz, CONH), 7.56 (d, J = 7.9 Hz, 4H), 7.48 (d, J = 8.5 Hz, 4H), 7.16 (t, J = 9.7 Hz, NH_2), 4.22 (br d, J = 13.4 Hz, NH), 3.09–3.18 (m, 1H), 2.82–3.10 (m, 4H), 2.70–2.80 (m, 3H) 1.21 (m, 9H); ^{13}C NMR (CDCl_3) δ 170.74, 155.58, 146.89, 144.62, 130.21, 123.76, 80.75, 55.66, 51.97, 44.92, 41.96, 38.06, 28.16.

2,6-Bis(p-nitrobenzyl)diethylenetriamine (10). Compound **9** (0.85 g, 1.74 mmol) was dissolved in 15 mL of TFA at 0 °C and allowed to stand for 2 h at room temperature. The solvent was then evaporated to dryness, dissolved in water, and lyophilized. The TFA salt of **9** was obtained as a white solid: yield, 1.04 g (97%); TLC, R_f = 0.86 ($\text{CHCl}_3/\text{MeOH}/\text{NH}_4\text{OH}$, 12:4:1 v/v/v); FAB+MS, m/e 388.2 [$M + H^+$]; ^1H NMR (D_2O) δ 8.24 (d, J = 10.0 Hz, 4H), 7.52 (d, J = 8.0 Hz, 4H), 4.35 (t, J = 10.0 Hz, 1H), 3.15–3.85 (m, 4H), 2.45–2.85 (m, 3H). This TFA salt (0.76 g, 1.23 mmol) was then dissolved in 10 mL of THF. To this solution was added 1.0 M BH_3 -THF (12.0 mL, 12.0 mmol) via syringe with stirring under N_2 at –10 °C for 1 h. Then, the mixture was refluxed for 18 h. Next, the reaction mixture was cooled to –10 °C, and 25 mL of anhydrous MeOH was added. The solvent was removed under reduced pressure, another 25 mL of MeOH was added, and the solution was concentrated to form **10** as a yellow oil. Compound **10** was purified by flash chromatography on silica gel and elution with chloroform/methanol/aqueous ammonium hydroxide (12:4:1 v/v/v): yield, 0.32 g (69%); TLC, R_f = 0.6 ($\text{CHCl}_3/\text{MeOH}/\text{NH}_4\text{OH}$, 12:4:1 v/v/v); FAB+MS, m/e 374 [$M + H^+$]; ^1H NMR (CD_3OD) δ 8.10 (d, J = 8.5 Hz, 4H), 7.35 (d, J = 8.6 Hz, 4H), 2.9–3.15 (m, 4H), 2.73–

2.85 (m, 2H), 2.35–2.68 (m, 4H); ^{13}C NMR (CDCl_3) δ 147.06, 146.52, 129.91, 123.57, 56.04, 52.52, 42.50.

2,6-Bis(p-nitrobenzyl)-N,N,N',N'',N'''-pentakis(tert-butoxycarbonyl)diethylenetriamine (11). To a suspension of **10** (0.32 g, 0.86 mmol) in 10 mL of acetonitrile were added *tert*-butyl bromoacetate (0.76 mL, 5.16 mmol) and anhydrous K_2CO_3 (1.18 g, 8.6 mmol) and the mixture was stirred for 2 days. The solvent was removed under reduced pressure to yield a residue, which was extracted with chloroform and dried with anhydrous Na_2SO_4 . The extract was filtered and evaporated to give an oil, which was purified by column chromatography on silica gel and elution with hexane/ethyl acetate (2:1 v/v). After evaporation of solvent, a yellow oil **11** was obtained: yield, 0.77 g (94%); TLC, R_f = 0.69 (hexane/ethyl acetate, 2:1 v/v); FAB+MS, m/e 944 [$M + H^+$]; ^1H NMR (CD_3OD) δ 8.09 (d, J = 10.0 Hz, 4H), 7.38 (d, J = 8.0 Hz, 4H), 3.29–3.39 (m, 10H), 2.95–3.20 (m, 4H), 2.75–2.90 (m, 2H), 2.35–2.64 (m, 4H), 1.36 (s, 36H) 1.30 (s, 9H); ^{13}C NMR (CDCl_3) δ 170.85, 168.98, 148.69, 146.16, 130.01, 123.53, 81.79, 80.85, 68.42, 63.18, 56.49, 53.04, 37.10, 28.35, 27.97.

2,6-Bis(p-aminobenzyl)-N,N,N',N'',N'''-pentakis(tert-butoxycarbonyl)diethylenetriamine (12). To a stirred solution of **11** (0.205 g, 0.217 mmol) in ethyl acetate was added 10% Pd (0.092 g, 0.086 mmol) on carbon with stirring under nitrogen. Hydrogen gas was then bubbled through the solution for 6 h. The reaction mixture was filtered through a prewashed (ethyl acetate) Celite pad. The filtrate was evaporated under reduced pressure to give **12** as a yellow oil. Compound **12** was then purified on silica gel by flash chromatography and elution with $\text{CHCl}_3/\text{CH}_3\text{OH}$ (85:15 v/v) to give a yellow oil: yield, 0.19 g (98%); TLC, R_f = 0.57 ($\text{CHCl}_3/\text{CH}_3\text{OH}$, 85:15 v/v); FAB+MS, m/e 884 [$M + H^+$]; ^1H NMR (CDCl_3) δ 6.89 (d, J = 7.3 Hz, 4H), 6.64 (d, J = 7.9 Hz, 4H), 3.42–3.37 (m, 10H), 2.85–3.10 (m, 4H), 2.60–2.75 (m, 2H), 2.30–2.40 (m, 4H), 1.18 (s, 36H), 1.15 (s, 9H).

2,6-Bis(p-aminobenzyl)diethylenetriamine-N,N,N',N'',N'''-pentaacetic Acid (13). A solution of **12** (0.191 g, 0.201 mmol) in 3 mL of concentrated hydrochloric acid was stirred at room temperature for 4 h and then evaporated to dryness under reduced pressure at 40 °C to form the pentahydrochloride salt of **13**. This compound was dried in a vacuum to yield **13**: yield, 0.15 g (95%); FAB MS, m/e 604 [$M + H^+$]. Anal. Calcd for $\text{C}_{28}\text{H}_{37}\text{N}_5\text{O}_{10} + 5\text{HCl}$: C, 42.77; H, 5.34; N, 8.91; Cl, 22.50. Found: C, 42.13; H, 5.41; N, 8.40; Cl, 22.40. CD (in CH_3OH , λ in nm, $\text{deg}/\text{M}^{-1}\text{cm}^{-1}$ in parentheses): 208.2 (–0.095), 212.5 (0.205), 214.4 (–0.329), 219.6 (0.761), 227.8 (–0.683), 234.4 (–0.826), 247.0 (–0.766).

2,6-Bis[p-(β -maleimido-N-propionyl)amino]benzyl]diethylenetriamine-N,N',N'',N'''-pentaacetic Acid (1). Compound **13** (0.068 g, 0.087 mmol) was dissolved in 2 mL of dimethylformamide (DMF) to give a pink solution. To this was added triethylamine (0.15 mL, 0.95 mmol), which changed the reaction mixture (pH 8) from pink to off-white. A white solid was precipitated. β -Maleimidopropionic acid *N*-hydroxysuccinimide ester (0.065 g, 0.24 mmol) was dissolved in 2 mL of DMF and added to the reaction mixture. A yellow solution was obtained, and a white precipitate settled to the bottom. The mixture was allowed to stand for 4 h at room temperature with occasional stirring. The precipitate formed was filtered, and the filtrate was evaporated to dryness. The impurities were removed by washing with chloroform and methanol: yield, 0.060 mg (76%); TLC, R_f = 0.2 ($\text{CH}_3\text{CN}/\text{H}_2\text{O}$, 30:8 v/v); FAB+MS, m/e 905 [$M + H^+$]; ^1H NMR ($\text{DMSO}-d_6$) δ 7.43 (d, J = 7.3 Hz, 2H), 7.04 (d, J = 11.7 Hz, 2H), 7.01 (d, J = 1.8 Hz, 4H), 6.78 (d, J = 7.32 Hz, 2H), 6.46 (d, J = 5.4 Hz, 2H), 3.75 (t, J

= 7.5 Hz, 4H), 2.35–3.15 (m, 10H), 3.18 (t, J = 6.8 Hz 4H); ^{13}C NMR (DMSO- d_6) δ 173.29, 173.21, 170.67, 168.20, 146.51, 136.99, 129.29, 124.53, 79.37, 63.40, 56.21, 53.13, 45.23, 34.68, 33.61. Anal. Calcd for $\text{C}_{42}\text{H}_{47}\text{N}_7\text{O}_{16} + 2\text{CH}_3\text{OH}$: C, 54.48; H, 5.67; N, 10.11. Found: C, 55.54; H, 5.78; N, 10.65; Cl, nil.

2,9-Bis(*p*-nitrobenzyl)-*N,N,N',N',N'',N''*-hexa(*tert*-butoxycarbonyl)-*N-t*-BOC-triethylenetetraamine-3,8-dione (14**).** To a solution of ethylenediamine (0.58 mL, 8.59 mmol) and triethylamine (6.35 mL, 45.57 mmol) in 5 mL of anhydrous DMF was added dropwise with stirring a solution of compound **8** (7.0 g, 17.19 mmol) in 40 mL DMF. The stirring was continued for 24 h. Next, the solution was filtered, evaporated to dryness under reduced pressure, and dissolved in 5 mL of water. To this was added 5 mL of saturated NaCl solution to dissolve the suspension and the mixture was extracted with chloroform. The extract was dried in anhydrous MgSO_4 , filtered, and then evaporated to dryness to obtain compound **14**. It was purified by chromatography on silica gel and elution with $\text{CHCl}_3/\text{MeOH}$ (8:1 v/v). After solvent evaporation, a white solid of compound **14** was isolated: yield, 5.88 g (53%); TLC, R_f = 0.7 ($\text{CHCl}_3/\text{MeOH}$, 8:1 v/v); ^1H NMR (CDCl_3) δ 8.11 (d, J = 20 Hz, 4H), 7.23 (d, J = 20 Hz, 4H), 4.20–4.35 (m, 2H), 3.30–3.60 (m, 4H), 2.90–3.25 (m, 4H), 1.35 (s, 18H); ^{13}C NMR (CDCl_3) δ 171.78, 155.55, 147.04, 144.54, 130.17, 123.75, 80.83, 55.59, 39.41, 37.84, 36.64; FAB-MS, m/e 645 [$\text{M} + \text{H}^+$].

2,9-Bis(*p*-nitrobenzyl)triethylenetetraamine (15**).** Compound **14** (1.19 g, 1.84 mmol) was dissolved in 10 mL of CF_3COOH at 0 °C, stirred continuously for 3 h, and then dried under reduced pressure. The residue was dissolved in water and lyophilized to obtain a white solid: yield, 1.18 g (95%); TLC, R_f = 0.45 ($\text{CHCl}_3/\text{CH}_3\text{OH}/\text{NH}_4\text{OH}$, 12:4:3 v/v/v); ^1H NMR (D_2O) δ 8.24 (d, J = 6.0 Hz, 4H), 7.46 (d, J = 6.0 Hz, 4H), 4.15 (t, J = 20 Hz, 2H), 3.17–3.34 (m, 4H), 2.91–3.03 (m, 4H); ^{13}C NMR (D_2O) δ 167.71, 146.84, 143.12, 130.95, 123.60, 53.26, 37.97, 36.64; FAB-MS, m/e 452.3 [$\text{M} + \text{H}^+$]. This TFA salt (0.46 g, 0.69 mmol) was then dissolved in 15 mL of THF, and to this was added a solution of 1.0 M $\text{BH}_3\text{--THF}$ (12.11 mL, 12.11 mmol) via a syringe with stirring under nitrogen at –10 °C. After continuous stirring for 1.5 h, the mixture was refluxed for 18 h. Then, the reaction mixture was cooled to –10 °C, and 25 mL of anhydrous MeOH was added. The solvent was removed under reduced pressure, another 25 mL of MeOH was added, and the solution was concentrated to form **15** as a yellow oil. Compound **15** was purified by flash chromatography on silica gel and elution with chloroform/methanol/aqueous ammonium hydroxide (12:4:1 v/v/v). Finally, solvent was evaporated to provide **15** as a yellow oil: yield, 0.19 g (66.6%); TLC, R_f = 0.4 ($\text{CHCl}_3/\text{MeOH}/\text{NH}_4\text{OH}$, 12:4:1 v/v/v); ^1H NMR (CD_3OD) δ 8.09 (d, J = 8.0 Hz, 4H), 7.44 (d, J = 8.6 Hz, 4H), 3.24–3.61 (m, 12H), 3.03–3.18 (m, 4H), 2.77–2.95 (m, 2H), 2.57–2.71 (m, 4H), 2.30–2.58 (m, 4H), 1.37 (s, 36H), 1.26 (s, 18H); ^{13}C NMR (CD_3OD) δ 149.15, 131.45, 124.61, 108.32, 55.35, 53.06, 48.99, 42.27; FAB+MS, m/e 417 [$\text{M} + \text{H}^+$].

2,9-Bis(*p*-nitrobenzyl)-*N,N,N',N',N'',N''*-hexa(*tert*-butoxycarbonyl)triethylenetetraamine (16**).** To a suspension of **15** (0.15 g, 0.36 mmol) in 10 mL of acetonitrile and 3 mL of DMF solvent mixture were added *tert*-butyl bromoacetate (0.32 mL, 2.15 mmol) and anhydrous K_2CO_3 (0.49 g, 3.59 mmol) and the mixture was stirred for 2 days. The solvent was removed under reduced pressure to yield a residue. The residue was extracted with chloroform and dried with anhydrous Na_2SO_4 . The extract was filtered and evaporated to give a

yellow oil, which was purified by column chromatography on silica gel and elution with hexane/ethyl acetate (2:1 v/v). After evaporation of solvent, a yellow oil (**11**) was obtained: yield, 0.36 g (91%); TLC, R_f = 0.61 (hexane/ethyl acetate, 2:1 v/v); FAB+MS, m/e 1101 [$\text{M} + \text{H}^+$]; ^1H NMR (CDCl_3) δ 8.09 (d, J = 8.8 Hz, 4H), 7.44 (d, J = 8.6 Hz, 4H), 3.24–3.61 (m, 12H), 3.0–3.18 (m, 4H), 2.71–2.98 (m, 2H), 2.55–2.70 (m, 4H), 2.30–2.58 (m, 4H), 1.48 (s, 36H), 1.37 (s, 18H); ^{13}C NMR (D_2O) δ 170.92, 166.97, 148.92, 146.50, 130.67, 123.60, 82.13, 80.79, 62.79, 60.33, 56.39, 56.08, 53.35, 37.36, 28.14, 28.07.

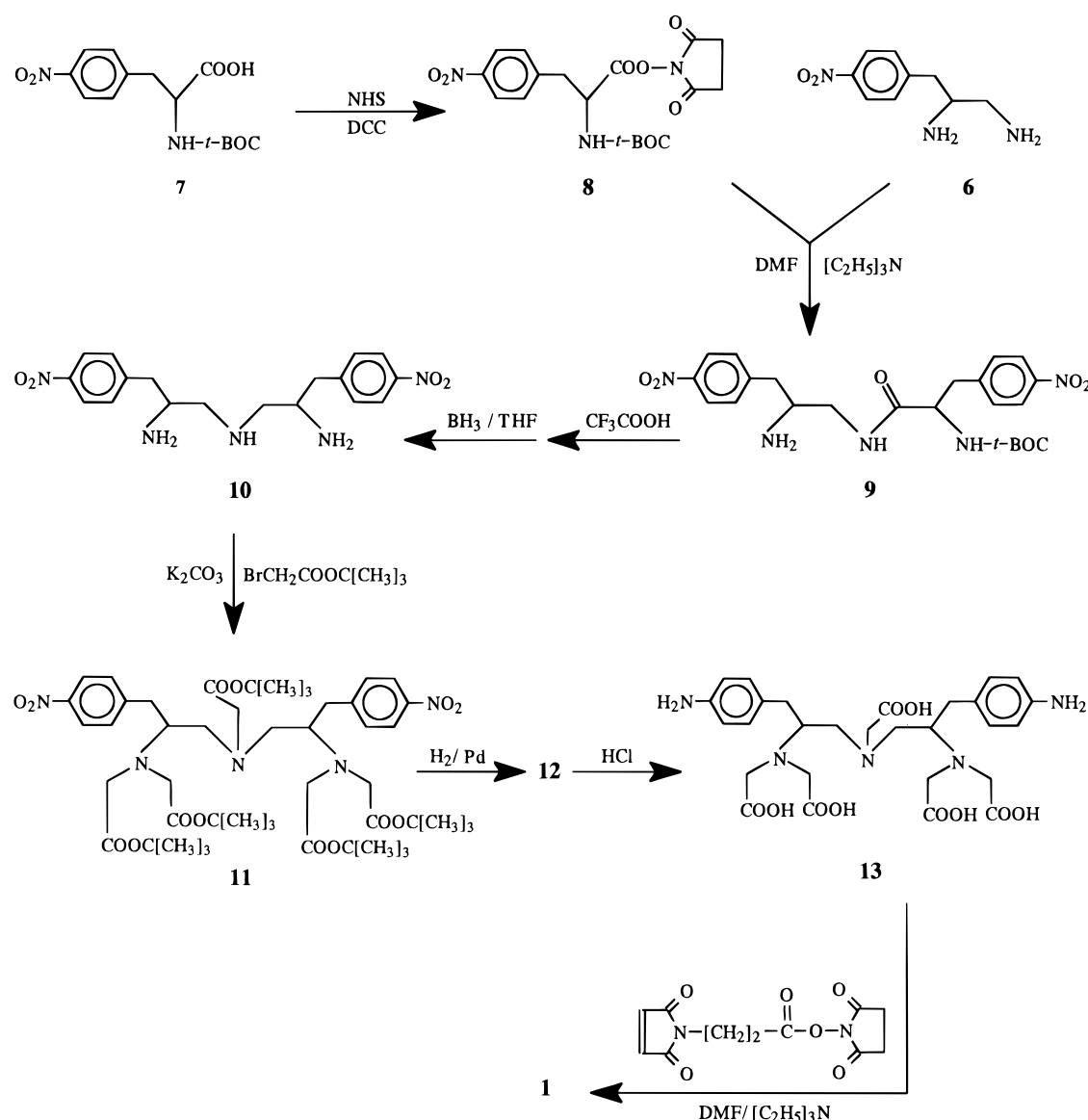
2,9-Bis(*p*-aminobenzyl)-*N,N,N',N',N'',N''*-hexa(*tert*-butoxycarbonyl)triethylenetetraamine (17**).** To a stirred solution of **16** (0.20 g, 0.18 mmol) in 20 mL of anhydrous ethyl acetate was added with stirring 10% Pd on carbon (0.072 g, 0.077 mmol) under nitrogen. Hydrogen gas was bubbled through the solution for 8 h. The reaction mixture was filtered through a prewashed (ethyl acetate) Celite pad. The filtrate was evaporated under reduced pressure to give **16** as a yellow oil. Compound **16** was then purified on silica gel by flash chromatography and elution with $\text{CHCl}_3/\text{MeOH}$ (2:1 v/v) to give the pure compound: yield, 0.17 g (90%); TLC, R_f = 0.58 ($\text{CHCl}_3/\text{MeOH}$, 85:15 v/v); FAB-MS, m/e 1041 [$\text{M} + \text{H}^+$]; ^1H NMR (CDCl_3) δ 6.94 (d, J = 7.3 Hz, 4H), 6.65 (d, J = 7.9 Hz, 4H), 3.20–3.30 (m, 12H), 2.98–3.10 (m, 4H), 2.62–2.80 (m, 2H), 2.50–2.65 (m, 4H), 2.32–2.42 (m, 4H), 1.24 (s, 36H), 1.21 (m, 18H).

2,9-Bis(*p*-aminobenzyl)triethylenetetraamine-*N,N,N',N',N'',N''*-hexaacetic Acid (18**).** A solution of **17** (0.175 g, 0.168 mmol) in 5 mL of 12 M hydrochloric acid was stirred at room temperature for 3 h and then evaporated to dryness under reduced pressure at 40 °C to form the hexahydrochloride salt of **18**: yield, 0.145 g (93%); FAB-MS, m/e 704 ($\text{M} + \text{H}^+$). Anal. Calcd for $\text{C}_{32}\text{H}_{44}\text{N}_6\text{O}_{12} + 6\text{HCl}$: C, 41.60; H, 4.26; N, 9.10; Cl, 23.07. Found: C, 41.37; H, 5.45; N, 8.51; Cl, 23.19. CD (in CH_3OH , λ in nm, $\delta\epsilon/\text{M}^{-1}\text{cm}^{-1}$ in parentheses): 211.5 (–0.312), 224.0 (0.156), 235.0 (–1.767), 258.0 (–0.385).

2,9-Bis[*p*-(β -maleimido-*N*-propionyl)amino]benzyl]triethylenetetraamine-*N,N,N',N',N'',N''*-hexaacetic Acid (2**).** Compound **18** (0.078 g, 0.084 mmol) was dissolved in 2 mL of anhydrous DMF to give a pink solution. To this was added triethylamine (0.34 mL, 2.45 mmol), which changed the reaction mixture (pH 8) from pink to off-white. A white solid was precipitated. β -Maleimidopropionic acid *N*-hydroxysuccinimide ester (0.062 g, 0.240 mmol) was dissolved in 2 mL of DMF and added to the reaction mixture. A yellow solution was obtained, and a white precipitate settled to the bottom. The mixture was allowed to stand for 4 h at room temperature with occasional stirring. The precipitate formed was filtered, and the filtrate was evaporated to dryness. The impurities were removed by washing with chloroform and methanol: yield, 0.065 g (76%); TLC, R_f = 0.25 ($\text{CH}_3\text{CN}/\text{H}_2\text{O}$, 30:8 v/v); FAB+MS, m/e 1007 [$\text{M} + \text{H}^+$]; ^1H NMR (DMSO- d_6) δ 7.43 (d, J = 9.7 Hz, 2H), 7.12 (d, J = 9.2 Hz, 2H), 7.02 (d, J = 35 Hz, 4H), 6.84 (d, J = 8.2 Hz, 2H), 6.46 (d, J = 8.6 Hz, 2H), 3.71 (t, J = 7.0 Hz, 4H), 2.58–3.68 (m, 12H), 3.28 (t, J = 8.2 Hz, 4H); ^{13}C NMR (DMSO- d_6) δ 173.26, 173.05, 170.62, 168.15, 145.21, 138.97, 134.45, 129.24, 120.47, 77.91, 62.17, 56.02, 49.13, 45.55, 42.95, 33.77, 31.79. Anal. Calcd for $\text{C}_{46}\text{H}_{54}\text{N}_8\text{O}_{18} + \text{CH}_3\text{OH}$: C, 54.33; H, 5.58; N, 10.78. Found: C, 53.24; H, 5.68; N, 10.82; Cl, nil.

RESULTS AND DISCUSSION

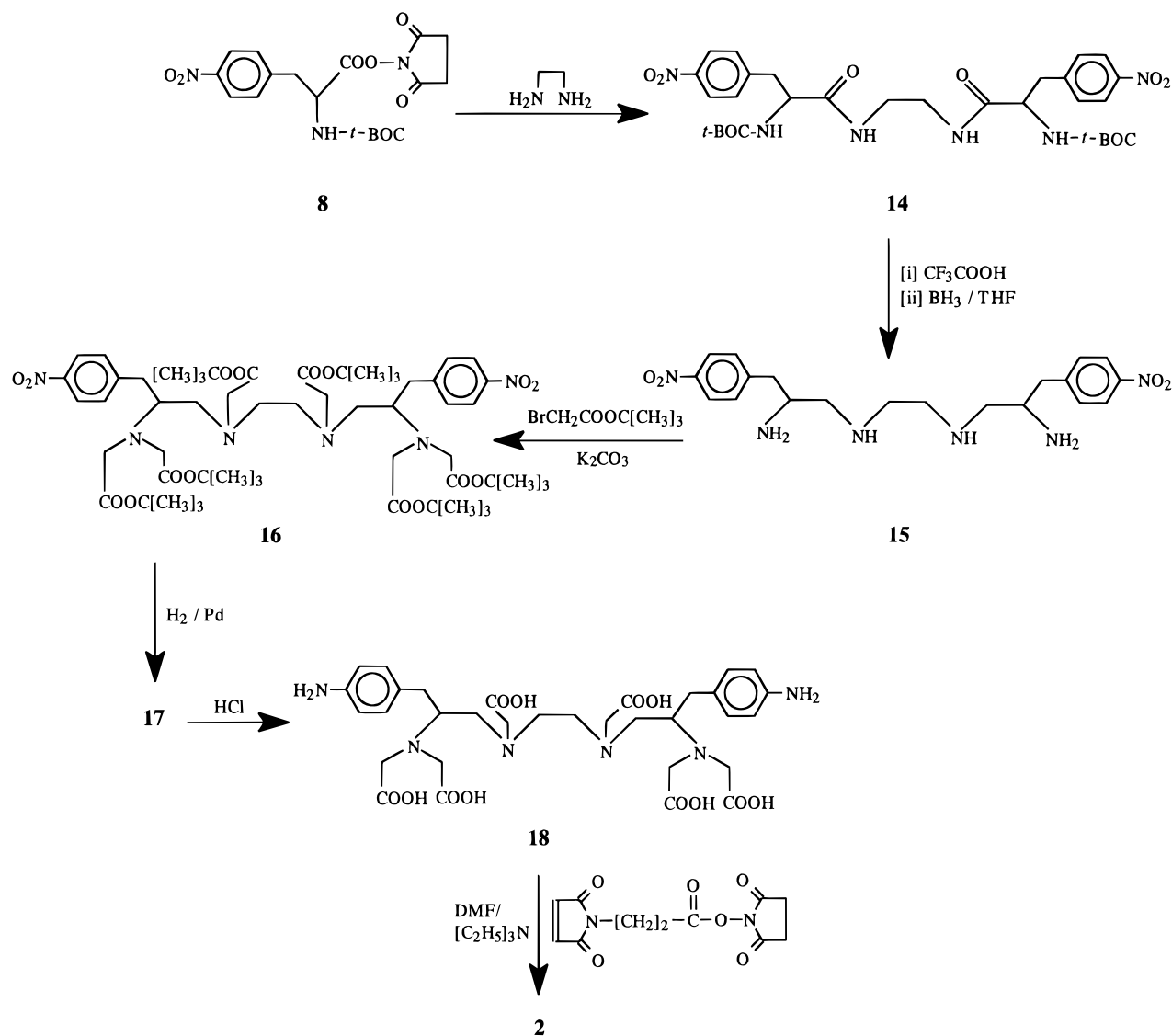
Strategies were devised for synthesizing maleimido derivatives of both MPBz-DTPA and MPBz-TTHA. The reaction sequences in the synthesis are shown in Schemes

Scheme 1. Synthesis of 2,6-Bis[*p*-(β -maleimidopropionyl)amino]benzyl]diethylenetriamine-*N,N,N',N'',N'''*-penta-acetic Acid

1 and 2, respectively. Commercially available 4-nitro-L-phenylalanine (**3**) was converted to methyl ester hydrochloride **4** according to Brenner and Huber's procedure (31). When compound **4** was neutralized with triethylamine base and saturated with ammonia gas for a short time, the product *p*-nitro-L-phenylalanine amide (**5**) was obtained. It was isolated and purified on silica gel by column chromatography (30, 32). Compound **5** was reduced in turn with 1 M BH₃-THF to form 2-(*p*-nitrobenzyl)ethylenediamine (**6**). Reduction was accomplished by using an amide/diborane ratio of 1:8.5. The reaction temperature was controlled to minimize the reduction of the nitro group. The resultant crude 2-(*p*-nitrobenzyl)ethylenediamine was isolated after the usual workup procedure, and product was purified by column chromatography on silica gel (33).

The basic approach we used to construct the MPBz-DTPA (**1**) and MPBz-TTHA (**2**) derivatives was based on two key intermediate steps. One of these steps was the reaction (34, 35) of commercially available *N*-*t*-BOC-*p*-nitro-L-phenylalanine (**7**) with NHS in the presence of DCC, which provided succinimide ester **8** as a starting material in both schemes. The second intermediate step was displacement of the NHS group of **8** with the amino group of **6**, which gave the monoamide derivative **9** for

the DTPA backbone (Scheme 1), and the reaction of **8** with ethylenediamine, which gave the BOC-protected dipeptide amide **14** backbone of TTHA (Scheme 2). Selection of solvent is critical for high yield of the compounds. In Schemes 1 and 2 for the preparations of compounds **9** and **14**, reactions were optimized by using a mixture of aprotic solvents CH₃CN and DMF, which resulted a significant yield. The resulting amide and diamide derivatives were deprotected with TFA (36), and then the resulting salts were reduced with diborane to yield the required diethylenetriamine and triethylenetetraamine backbones of DTPA **10** and TTHA **15**, respectively. A serious complication was encountered during this sequence in that ~5% of the nitrobenzyl was reduced to an aniline. This problem was obviated by use of a stoichiometric amount of hydride equivalent required for the reduction of amides, neutralization of the TFA, and reduction of the TFA itself. The reduction of **9** with diborane under amide/BH₃ (1:13 molar ratio) yielded 69% **10**, whereas the reduction of **14** under similar conditions using diamide/BH₃ (1:17 molar ratio) yielded 66% **15**. After the usual acidic workup to cleave boron adduct, crude amines were purified on silica gel by flash chromatography using a mixture of CHCl₃/CH₃OH/NH₄OH as eluent. Pure compounds **10** and **15** were used for

Scheme 2. Synthesis of 2,9-Bis[*p*-(β -maleimidopropionyl)amino]benzyl]triethylenetetraamine-*N,N,N',N'',N''',N''''*-hexaacetic Acid

subsequent procedures. Conversion of these free amines into their corresponding HCl salts was avoided as the alkylation of these amines with *tert*-butyl bromoacetate was done immediately after isolation of free amines. The compounds **11–13** (Scheme 1) and **16–18** (Scheme 2) were prepared by following previously described methodologies (29, 37). Alkylation of **10** and **15** with *tert*-butyl bromoacetate provided good yields of **11** and **16**. Catalytic hydrogenation of **11** and **16** with Pd/C in ethyl acetate gave **12** and **17** in quantitative yields. Cleavage of the *tert*-butyl ester groups in **12** and **17** with concentrated hydrochloric acid (12 N) provided pentahydrochloride and hexahydrochloride salts **13** and **18**, respectively. The hydrochloride salts of these compounds were confirmed by elemental analysis.

The two primary amine groups of **13** or **18** are the target sites for introducing the maleimido function by means of an activated NHS ester of β -maleimidopropionic acid. Consequently, one maleimido function was linked to each primary amino group of the chelator via a stable amide bond. The reaction was completed using an aprotic polar solvent (DMF) and triethylamine as a base to trap the proton generated from the reaction mixture. The final compounds **1** and **2** were obtained in good yields by reacting the amino derivatives **13** and **18** with activated cross-linkers at a 1:2.8 molar ratio. The pH of

the reaction was maintained at 7.5 (38). The overall yields of compounds **1** and **2** were approximately 5 and 4%, respectively.

The structures of final products and intermediates were assigned on the basis of ^1H -NMR and ^{13}C -NMR data. Mass spectra provided molecular ions of the compounds and interpretable fragmentation ion patterns for further confirmation of the structures of the compounds. In the ^1H -NMR spectra of compound **8**, the appearance of a peak for a new singlet for the $-\text{CH}_2$ group of succinimide indicated the formation of compound **8**. This was further supported by the ^{13}C -NMR spectra, which exhibited three characteristic carbonyl ($\text{C}=\text{O}$) peaks. In the ^1H -NMR spectra for amide and diamide (compounds **9** and **16**), a singlet at 2.5 ppm for the $-\text{CH}_2$ group of succinimide completely disappeared with the displacement of an NHS group in each compound. The structures of amide **9** and diamide **14** were confirmed by ^{13}C -NMR and mass spectra.

In the preparation of compound **9**, the two amino groups of diamine **6** can possibly react with ester, resulting in two possible isomers. The product mixture depends upon the rate of reaction of the two amino groups of diamine with ester. Theoretically, the less hindered group has higher reactivity than the more sterically hindered group. Therefore, compound **9** contained two

positional isomers, whose isomeric ratio was determined by a spectroscopic method. A high-resolution ^1H -NMR spectrum at 500 MHz in $\text{DMSO}-d_6$ was obtained to identify and confirm the isomer structures in compound **9** by means of their amide NH signals. A large triplet of amide NH connected to a methylene carbon (8.16 ppm) and a small doublet of amide NH connected to a methine carbon (8.03 ppm) occurred in an 85:15 ratio. This ratio established that compound **9** is a mixture of one major and one minor positional isomer. The major isomer product has *p*-nitrobenzyl groups at positions 2 and 6, whereas the minor isomer product constitutes *p*-nitrobenzyl groups at positions 2 and 5 (6). The established isomeric composition was used for the remainder of the synthesis.

Deprotection of the BOC (*tert*-butoxy) group of amide and diamide by TFA was indicated in the ^1H -NMR spectra by the disappearance of the strong *tert*-butyl group peak. Successful reduction of **9** and **14** with diborane was indicated by the ^{13}C -NMR spectra, in which the two C=O peaks disappeared and the new peaks in the alkyl region appeared as evidence of the formation of compounds **10** and **15**. Alkylation of **10** and **15** led to compounds **11** and **16**, which was corroborated by ^1H -NMR spectra showing new peaks of the *tert*-butoxy group and $-\text{CH}_2$ groups and ^{13}C -NMR spectra showing C=O peaks. Central acetate esters were unique, and their distinct signals were observed in ^{13}C -NMR spectra. Reduction of NO_2 groups in **11** and **16** to NH_2 groups in **12** and **17** was corroborated by the ^1H -NMR spectra, which showed phenyl peaks at 6.9 and 6.6 ppm as well as upfield shifts of 0.5 and 1.1 ppm, respectively, when compared with the analogous peaks of compounds **11** and **16**.

Optical activity was confirmed by measuring circular dichroism (CD) of starting compound **8** and intermediate compounds **13** and **18** in CH_3OH . Reaction Schemes 1 and 2 were started with the optically active *N*-*t*-BOC-*p*-nitro-L-phenylalanine (**8**). The CD data of compounds **13** and **18** have shown similar patterns when compared to compound **8**, indicating the retention of L-configurations at both chiral carbons. Hence, in the course of reactions the two asymmetric centers were not disturbed and the optical activity was retained throughout the schemes.

In the ^1H -NMR spectra of compounds **1** and **2**, four doublets were observed in the phenyl region, indicating the magnetic inequivalence of the four phenyl protons. Additionally, one singlet and two triplets were due to one $-\text{CH}$ and two $-\text{CH}_2$ groups, respectively, for the maleimido moiety. Removal of NHS was proved by the disappearance of the $-\text{CH}_2$ peak of the succinimide group. Both compounds **1** and **2** were soluble in DMSO. Starting materials MPA *N*-HSE and HCl salts of **13** and **18** were fairly soluble in CHCl_3 and CH_3OH , respectively. Therefore, compounds **1** and **2** were purified by washing repeatedly with CHCl_3 and CH_3OH solvents until pure compounds were obtained. Finally, both compounds **1** and **2** were confirmed by their respective mass ion peaks. ^1H -NMR and ^{13}C -NMR spectra and elemental analysis further established the purity of the synthesized maleimido derivatives of chelators.

CONCLUSION

The chelators MPBz-DTPA (**1**) and MPBz-TTHA (**2**) were successfully prepared in multistep syntheses and confirmed by spectroscopic analysis. The maleimido substituents in these chelate structures should allow their cross-linked conjugation with sulfhydryl groups at the hinge region of antibody fragments or with other peptides containing free sulfhydryl groups.

In light of our findings, we are working to optimize the conjugation of these ligands with antibody fragments to generate hinge-specific chelate-immunoconjugates of the form Fab'-S-chelate-S-Fab'. Moreover, we are currently evaluating these immunoconjugates for their *in vitro* and *in vivo* stability, binding affinity, and tumor targeting and hope to report on these in the near future. Interestingly, the ligands we are studying contain two conjugation sites and can covalently cross-link with two biomolecules or peptides for enhanced specific receptor or antigen binding *in vivo*. Additionally, biomolecules with $-\text{NH}_2$ or $-\text{COOH}$ functions available for conjugation can also be coupled at the two activated sites using isothiocyanato or amino derivatives of these chelators.

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LITERATURE CITED

- (1) Kozak, R. W., Raubitschek, A., Mirzadeh, S., Brechbiel, M. W., Junghaus, R., Gansow, O. A., and Waldmann, T. A. (1989) Nature of the bifunctional chelating agents used for radioimmunotherapy with yttrium-90 monoclonal antibodies: critical factors in determining *in vivo* survival and organ toxicity. *Cancer Res.* 49, 2639–2644.
- (2) Meares, C. F., McCall, M. J., Reardan, D. T., Goodwin, D. A., Diamanti, C. J., and McTigue, M. (1984) Conjugation of antibodies with bifunctional chelating agents: isothiocyanate and bromoacetamide reagents, methods of analysis, and subsequent additional metal ions. *Anal. Biochem.* 142, 68–78.
- (3) Stimmel, J. B., Stockstill, M. E., and Kull, F. C., Jr. (1995) Yttrium-90 chelation properties of tetraazatetraacetic acid macrocycles, diethylenetriamine pentaacetic acid analogues, and a novel terpyridine acyclic chelator. *Bioconjugate Chem.* 6, 219–225.
- (4) Gansow, O. A., Brechbiel, M. W., Mirzadeh, S., Colcher, D., and Roselli, M. (1990) Chelates and antibodies: current methods and new directions. In *Cancer Imaging and Radio-labeled Antibodies* (D. M. Goldenberg, Ed.), pp 153–171, Kluwer Academic Publishers, Boston.
- (5) Brechbiel, M. W., and Gansow, O. A. (1991) Backbone-substituted DTPA ligands for ^{90}Y radioimmunotherapy. *Bioconjugate Chem.* 2, 187–194.
- (6) Cummins, C. H., Rutter, E. W., Jr., and Fordyce, W. A. (1991) A convenient synthesis of bifunctional chelating agents based on diethylenetriaminepentaacetic acid and their coordination chemistry with yttrium (III). *Bioconjugate Chem.* 2, 180–186.
- (7) Gansow, O. A. (1991) Newer approach to the radiolabeling of monoclonal antibodies by use of metal chelates. *Nucl. Med. Biol.* 18, 369–381.
- (8) Gansow, O. A., and Brechbiel, M. W. (1989) Backbone polysubstituted chelates for forming a metal chelate protein conjugate. U.S. Pat. 4,831,175.
- (9) Quadri, S. M., and Mohammadapur, H. (1992) Synthesis of 2-*p*-aminobenzyl-3-methyl and 2-*p*-aminobenzyl-3-benzyl derivatives of diethylenetriamine-pentaacetic acid (DTPA): carbon backbone modified bifunctional chelating agents. *Biorg. Med. Chem. Lett.* 2, 1661–1664.
- (10) Li, M., and Meares, C. F. (1993) Synthesis, metal chelate stability studies, and enzyme digestion of a peptide-linked DOTA derivative and its corresponding radiolabeled immunoconjugates. *Bioconjugate Chem.* 4, 275–283.
- (11) Moran, J. K., Greiner, D. P., and Meares, C. F. (1995) Improved synthesis of 6-[*p*-(bromoacetamido)benzyl]-1,4,8,11-tetraazacyclotetradecane-*N,N',N'',N'''*-tetraacetic acid and development of a thin-layer assay for thio-reactive bifunctional chelating agents. *Bioconjugate Chem.* 6, 296–301.

- (12) Kukis, D. L., Diril, H., Greiner, D. P., DeNardo, S. J., DeNardo, G. L., Salako, Q. A., and Meares, C. F. (1994) A comparative study of copper-67 radiolabeling and kinetic stabilities of antibody-macrocyclic chelate conjugates. *Cancer (Suppl.)* 73, 779–786.
- (13) Brechbiel, M. W., McMurray, T. J., and Gansow, O. A. (1993) A direct synthesis of a bifunctional chelating agent for radiolabeling proteins. *Tetrahedron Lett.* 34, 3691–3694.
- (14) Moi, M. K., and Meares, C. F. (1988) The peptide way to macrocyclic bifunctional chelating agents: synthesis of 2-(*p*-nitrobenzyl)-1,4,7,10-tetraazacyclododecane-*N,N',N'',N'''*-tetraacetic acid and study of its yttrium (III) complex. *J. Am. Chem. Soc.* 110, 6266–6267.
- (15) Gansow, O. A., and Kumar, K. (1990) Process for synthesizing macrocyclic chelates. U.S. Pat. 4,923,985.
- (16) McMurray, T. J., Brechbiel, M., Kumar, K., and Gansow, O. A. (1992) Convenient synthesis of bifunctional tetraaza macrocycles. *Bioconjugate Chem.* 3, 108–117.
- (17) Cox, J. P. L., Craig, A. S., Helps, I. M., Jankowski, K. J., Parker, D., Eaton, M. A. W., Millican, A. T., Miller, K., Beeley, N. R. A., and Boyce, B. A. (1990) Synthesis of C- and N-functionalized derivatives of 1,4,7-triazacyclononane-1,4,7-triyltriacetic acid (NOTA), 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetrayltetraacetic acid (DOTA), and diethylenetriaminepentaacetic acid (DTPA): bifunctional complexing agents for the derivatization of antibodies. *J. Chem. Soc., Perkin. Trans. 1*, 2567–2576.
- (18) Meares, C. F., and Studer, M. (1992) Synthesis of novel 1,4,7-triazacyclononane-*N,N',N''*-triacetic acid derivatives suitable for protein labeling. *Bioconjugate Chem.* 3, 337–341.
- (19) Renn, O., and Meares, C. F. (1992) Large-scale synthesis of bifunctional chelating agents 2-(*p*-nitrobenzyl)-1,4,7,10-tetraazacyclododecane-*N,N',N'',N'''*-tetraacetic acid, and the determination of its enantiomeric purity by chiral chromatography. *Bioconjugate Chem.* 3, 563–569.
- (20) McMurry, T. J., Brechbiel, M., Wu, C., and Gansow, O. A. (1993) Synthesis of 2-(*p*-thiocyanatobenzyl)-1,4,7-triazacyclononane-1,4,7-triacetic acid: application of the 4-methoxy-2,3,6-trimethylbenzenesulfonamide protecting group in the synthesis of macrocyclic polyamines. *Bioconjugate Chem.* 4, 236–245.
- (21) Kukis, D. L., Li, M., and Meares, C. F. (1993) Selectivity of antibody-chelate conjugate for binding copper in the presence of competing metals. *Inorg. Chem.* 32, 3981–3982.
- (22) Sumerdon, G. A., Rogers, P. E., Lombardo, C. M., Schnobrich, K. E., Melvin, S. L., Hobart, E. D., Tribby, I. I. E., Stroupe, S. D., and Johnson, D. K. (1990) An optimized antibody-chelator conjugate for imaging of carcinoembryonic antigen with indium-111. *Nucl. Med. Biol.* 17, 247–254.
- (23) McCall, M. J., Diril, H., and Meares, C. F. (1990) Simplified method for conjugating macrocyclic bifunctional chelating agents to antibodies via 2-iminothiolane. *Bioconjugate Chem.* 1, 222–226.
- (24) Kosmas, C., Snook, D., Gooden, C. S., Courtenay-Luck, N. S., McCall, M. J., Meares, C. F., and Epenetos, A. A. (1992) Development of humoral immune responses against a macrocyclic chelating agent (DOTA) in cancer patients receiving radioimmunoconjugates for imaging and therapy. *Cancer Res.* 52, 904–911.
- (25) Paik, C. H., Yokoyama, J. C., Reynold, J. C., Quadri, S. M., Min, C. Y., Shin, S. Y., Maloney, P. J., Larson, S. M., and Reba, R. C. (1989) Reduction of background activities by introduction of a diester linkage between antibody and a chelate in iodine immunodetection of tumor. *J. Nucl. Med.* 30, 1693–1701.
- (26) Ahlem, C. N., Huang, A. E., and Anderson, L. D. (1991) Tris-maleimido compounds as intermediates in trifunctional antibody synthesis. Eur. Pat. Appl. 91301962.6 (Publication 0 446 071 A2); *Chem. Abstr.* 116 (Book 11), 102257z.
- (27) Ji, T. H. (1983) Bifunctional reagents. *Methods Enzymol.* 91, 580–609.
- (28) Wilbur, D. S., Stray, J. E., Hamlin, D. K., Curtis, D. K., and Vessella, R. L. (1994) Monoclonal antibody Fab' fragment cross-linking using equilibrium transfer alkylate reagents: a strategy for site-specific conjugation of diagnostic and therapeutic agents with F(ab')₂ fragments. *Bioconjugate Chem.* 5, 220–235.
- (29) Koyama, K. (1993) Preparation of maleimido chelating agents for radiodiagnosis. Jpn. Pat. 05 178826; *Chem. Abstr.* 120, 217262t.
- (30) Brechbiel, M. W., Gansow, O. A., Atcher, R. W., Schlom, J., Esteban, J., Simpson, D. E., and Colcher, D. (1986) Synthesis of 1-(*p*-isothiocyanatobenzyl) derivatives of DTPA and EDTA: antibody labeling and tumor-imaging studies. *Inorg. Chem.* 25, 2772–2781.
- (31) Brenner, M., and Huber, W. (1953) Herstellung von α -Aminosäureestern durch Alkoholyse der Methylester. *Helv. Chim. Acta* 36, 1109–1115.
- (32) Chambers, R. W., and Carpenter, F. H. (1955) On the preparation and properties of some amino acid amides. *J. Am. Chem. Soc.* 77, 1522–1526.
- (33) Brown, H. C., and Heim, P. (1973) Selective reduction. XVIII. The fast-reaction of primary, secondary, and tertiary amides with diborane: a simple, convenient procedure for the conversion of amides to the corresponding amines. *J. Org. Chem.* 38, 912–916.
- (34) Arano, Y., Matsushima, H., Tagawa, M., Koizumi, M., Endo, K., Konishi, J., and Yokoyama, A. (1991) A novel bifunctional metabolizable linker for the conjugation of antibodies with radionuclides. *Bioconjugate Chem.* 2, 71–76.
- (35) Nerstrom, V. M., Henriksen, U., Nielsen, P. E., Buchardt, O., Schmiegelow, K., and Koch, C. (1994) Monoclonal antibodies to thioguanine: influence of coupling position on fine specificity. *Bioconjugate Chem.* 5, 357–363.
- (36) Lundt, B. F., Johansen, N. L., Volund, A., and Markussen, J. (1978) Removal of *t*-butyl and *t*-butoxycarbonyl protecting groups with trifluoroacetic acid. *Int. J. Pept. Protein Res.* 12, 258–268.
- (37) Brechbiel, M. W. (1988) New bifunctional chelates for radioimmunotherapy. Ph.D. Thesis, The American University, Washington, DC.
- (38) Smyth, D. G., Blumenfeld, O. O., and Konigsberg, W. (1964) Reactions of *N*-ethylmaleimide with peptides and amino acids. *Biochem. J.* 91, 589–595.

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Development and in Vitro Studies of Epidermal Growth Factor–Dextran Conjugates for Boron Neutron Capture Therapy

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A delivery molecule for directed boron neutron capture therapy against epidermal growth factor (EGF) receptor-rich tumors, such as gliomas, squamous carcinomas, and breast cancers, is presented. EGF and sulfhydryl boron hydride (BSH) were covalently coupled to an allylated 70 kDa dextran chain to form a conjugate. Conjugates with low and high substitution rates of BSH, as well as without BSH, were investigated. The conjugate with a low amount of boron had approximately 6 BSH (72 boron atoms) per dextran, while the conjugates with higher amounts had an average substitution of 55 BSH (660 boron atoms) per dextran. The maximum substitution of boron to dextran in a single experiment was over 800 boron atoms. Binding, retention, and internalization of ^{125}I -labeled conjugates were investigated on cultured human glioma cells. Binding of the conjugates was EGF receptor specific, but the amount of BSH coupled to dextran affected specificity, more than the presence of dextran. The nonspecific binding of the conjugates increased with the amount of attached boron. This was partly due to nonspecific adhesion to the plastic in the culture dishes. ^{125}I -EGF–allyldextran with 6 BSH had a binding maximum after 4 h of continuous incubation and thereafter decreased in binding, while ^{125}I -EGF–allyldextran with the higher substitution rate had a slow increase of binding during 24 h. Over 93% of the radioactivity bound to the cells was internalized, but the retention was quite poor. Only one-third of the cell-bound activity was still associated to the cells 4 h after incubation had ended. In conclusion, it is possible to load the conjugates produced with high amounts of boron, and they retained specificity for the EGF receptor and internalized into cultured cells. Theoretical calculations show that about 10^3 boron atoms per EGF-based conjugate are needed to give a satisfactory therapeutic response. These conjugates are within reach of that level.

INTRODUCTION

A major unresolved problem in cancer treatment is how to kill those tumor cells that remain after local therapy. The present work is an attempt to develop a therapeutically interesting substance planned to bind dispersed tumor cells that overexpress the epidermal growth factor receptor (EGFR). The basic principle was to synthesize a conjugate of EGF, dextran, and boron. The EGF ligand would deliver sufficient amounts of boron to EGFR-rich malignant cells. After boron capture of externally applied neutrons, locally emitted high energy particles would then specifically damage the target cells.

The overexpression of EGFR in malignancies, such as gliomas, squamous carcinomas, and breast cancers, is due to gene amplification and/or increased transcription rates (Bigner et al., 1988; Collins and James, 1993). Such tumors are candidates for directed tumor cell specific therapy in which the receptors are the targets. This type of targeting therapy could hopefully become a supplement to conventional cancer therapy.

Targeting agents suggested are antibodies (Mendelsohn, 1988; Ozawa et al., 1989; Vollmar et al., 1987; Wikstrand et al., 1995), fragments of antibodies (Reiter et al., 1994), or receptor–ligand conjugates (Andersson et al., 1992; Olsson et al., 1994). Growth factors carrying toxic agents could be superior to antibodies, due to their

smaller size giving better penetration in tumor tissues and the fact that conjugated antibodies often have poor tumor specificity (Vaughan et al., 1987; Wheldon et al., 1988). The noxious agents in growth factor conjugates could be toxins (Cawley et al., 1980; Siegall et al., 1990), radioactive nuclides (Andersson et al., 1992; Capala et al., 1990), or stable nuclides for neutron activation (Barth et al., 1990; Carlsson et al., 1994).

Neutron capture therapy was proposed 60 years ago (Locher, 1936) as a form of treatment for brain tumors and other malignancies. The principle is to deliver stable nuclides into the tumor that will react with a beam of low-energy neutrons to produce two high-energy fission particles. The nuclide most often used for boron neutron capture therapy (BNCT) is ^{10}B , which captures a neutron to produce ^7Li and ^4He . The alpha particle has a range of $9\text{ }\mu\text{m}$ and the lithium particle $5\text{ }\mu\text{m}$ in tissue (Fairchild et al., 1990; Gabel et al., 1987). Since a mammalian cell diameter is about $10\text{ }\mu\text{m}$, the particle may deposit virtually all of its lethal energy in a single cell that has bound or internalized ^{10}B (Charlton, 1991). The probability for ^{10}B to capture a neutron vastly exceeds that of other atoms in biological tissues, thus minimizing risk of unwanted side effects. Boron can rather easily be incorporated into a multitude of different chemical structures (Barth et al., 1990; Hawthorne, 1993), and a number of compounds have already been tested, such as sulfhydryl boron hydride (BSH), which consists of 12 boron atoms per molecule (Soloway et al., 1967).

Gliomas have been a favorite target for experimental BNCT mainly because these malignancies are unusually difficult to cure in spite of the fact that they do not metastasize outside the skull. It is generally hypothesized that their intractability depends on early wide-

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spread dissemination of cancer cells in the brain, rendering local therapy inefficient. Only if specific targeting by therapeutic molecules is accomplished is there a chance of substantial improvement of cure rate.

We have, in the present work, used the strategy of conjugating BSH to EGF via dextran. The use of EGF-based conjugates for BNCT has been described by Carlsson et al. (1994). EGF, consisting only of 53 amino acids (6 kDa), has a compact stable structure. Conjugation via its amino terminus to dextran and toxins without loss of biological activity of the ligand has been described by Andersson et al. (1991), Cawley et al. (1980), and Olsson et al. (1994). Dextran can easily be biochemically modified with a variety of methods to allow coupling to proteins and other substances (Holmberg and Meurling, 1993; Kohn and Wilchek, 1984). Furthermore, dextran stabilizes proteins against degradation (Andersson et al., 1991; Foster, 1975; Melton et al., 1987).

In this study, the amino terminus of EGF was modified, using *N*-succinimidyl-3-(2-pyridyldithio)propionate (SPDP) to introduce a thiol group. The thiol group was then directly coupled to allyl groups present on 70 kDa dextran. Allyl groups were also used to attach various amounts of BSH to the dextran. Radical additions to carbon–carbon unsaturates were used to form sulfur–carbon bonds (Stacey and Harris, 1963). These EGF-based conjugates were tested for their receptor binding specificity and kinetics of internalization and retention in a cultured human malignant glioma cell line, which overexpresses the EGF receptor.

EXPERIMENTAL PROCEDURES

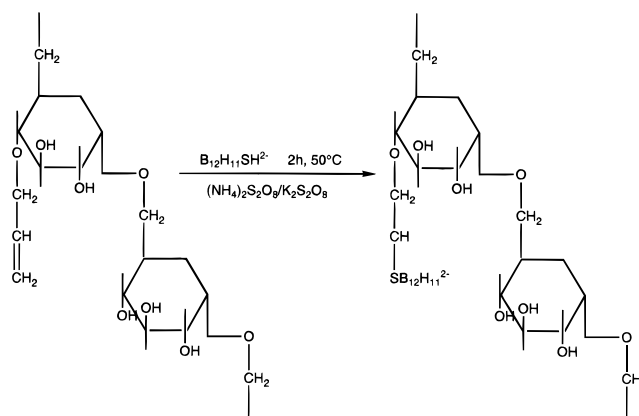
Materials. EGF, tissue culture grade, of murine origin (Janssen, Geel, Belgium) was labeled by ^{125}I (Amersham Int plc, Little Chalfont, Buckinghamshire, U.K.). *N*-Succinimidyl-3-(2-pyridyldithio)propionate (SPDP; Pharmacia, Uppsala, Sweden) was dissolved in ethanol to a concentration of 4 mM and used for thiolation of the growth factor. Dextran (Sigma, St. Louis, MO; industrial grade) with an average molecular mass of 70 kDa was used. The compound disodium undecahydromercapto-*closo*-dodecarborate (BSH; $\text{Na}_2\text{B}_{12}\text{H}_{11}\text{SH}$ or sulfhydryl boron hydride) was obtained from Callery Chemical Co., Pittsburgh, PA.

Inductively coupled plasma-atomic emission spectrometry (ICP-AES) was used for boron analysis (Lindström et al., 1994). Elemental analysis of dextrans was performed by Micro Kemi AB, Uppsala, Sweden. NAP-5 columns (Sephadex G-25 gel, Pharmacia, Sweden) were used for separation of conjugates from low molecular weight compounds and Sephadex G-50 gel (Pharmacia, Sweden) for separations of conjugates from unconjugated growth factor. Radioactivity measurements were performed on an automatic gamma counter and liquid scintillation counter (LKB Wallac Oy, Turku, Finland).

The cell line U-343 MGaCl2:6 is a subclone of the U-343 MG family of human malignant glioma lines. It has approximately 2×10^5 EGFRs per cell (Westermarck et al., 1982). Cells were grown in 35 mm Petri dishes with a cell density of 10^4 – 10^5 cells/cm². Binding of EGF to U-343 MGaCl2:6 is not cell density dependent in this range (Capala et al., 1990). Ham's F10 medium supplemented with 10% FCS, L-glutamine (2 mM), and PEST (penicillin 100 IU/mL and streptomycin 100 µg/mL) was used, all from Kebo, Stockholm, Sweden. The cells were incubated at 37 °C in humidified air containing 5% CO₂ if not stated otherwise.

Methods. *Modification of Dextran.* Allyl groups were introduced into dextran as described by Holmberg and Meurling (1993). Briefly, 2 g of 70 kDa dextran, 0.5 g of

Scheme 1

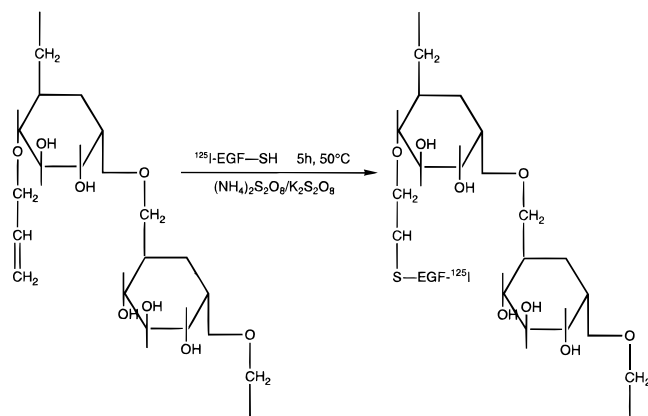


NaOH, and 20 mg of NaBH_4 were mixed in 15 mL of distilled water at 40 °C. Allyl bromide (3.5 g; 2.5 mL) was added, and the reaction was allowed to proceed at 60 °C for about 3 h. After neutralization, dextran was purified by precipitation with ethanol three times and thereafter lyophilized and stored at –70 °C. The degree of allyl substitution was calculated by elemental analysis of carbon and hydrogen in the allylated and native dextran.

Introduction of BSH to Allyldextran (Scheme 1). Two slightly different methods were used. Dextran–BSH(A): 20 mg of BSH and 20 mg of $(\text{NH}_4)_2\text{S}_2\text{O}_8$ were mixed with 20 mg of allyldextran dissolved in 200 µL of distilled H₂O. Dextran–BSH(B): 20 mg of BSH and 1 mg of $\text{K}_2\text{S}_2\text{O}_8$ were mixed with 20 mg of allyldextran dissolved in 200 µL of distilled H₂O. Both reactions continued for 2 h at 50 °C with stirring. Thereafter, dextran–BSH was purified from uncoupled BSH and other low molecular weight compounds on a NAP-5 column (equilibrated with distilled H₂O). Nine hundred microliters of the high molecular weight fraction was eluted instead of the recommended 1.0 mL to avoid any contaminants of low molecular weight compounds. The amount of boron added to the dextran was determined with ICP-AES, and substitution rates were calculated.

Thiol- and ^{125}I -Labeling of EGF. EGF (5 µg in 25 µL of 0.25 M phosphate buffer, pH 7.5) was mixed with a 10-fold molar excess of SPDP for 30 min. Thereafter, ^{125}I (37 MBq) and chloramine T (20 µg in 10 µL of 0.5 M phosphate buffer, pH 7.5) were added and mixed for 1 min. The reaction was interrupted with sodium metabisulfite (50 µg in 25 µL of 0.5 M phosphate buffer, pH 7.5). Thiol groups on [^{125}I]EGF were generated by reducing the introduced SPDP with 10 mM dithiothreitol at pH 3.5 (0.1 M NaAc buffer). The low pH was used to prevent reduction of native disulfide bonds present in the growth factor. Thiolated [^{125}I]EGF is hereafter referred to as [^{125}I]EGF-SH. Excess reagents and low molecular weight substances were separated from macromolecules on a NAP-5 column (equilibrated with distilled water). Nine hundred microliters of the high molecular weight fraction was eluted.

Optimization of the [^{125}I]EGF-SH Coupling to Allyldextran. Coupling of [^{125}I]EGF to allyldextran or dextran–BSH (Scheme 2) was done according to the same principle as coupling of BSH to allyldextran. To obtain acceptable yield of conjugate and preserve good binding to cellular EGF receptors, different coupling conditions were applied. [^{125}I]EGF-SH (150 µL; 0.8 µg) and 20 mg of allyldextran were mixed in 650 µL of distilled water with either (a) 1 mg of $\text{K}_2\text{S}_2\text{O}_8$ at 30 °C, (b) 1 mg of $\text{K}_2\text{S}_2\text{O}_8$ at 40 °C, (c) 1 mg of $\text{K}_2\text{S}_2\text{O}_8$ at 50 °C, (d) 1 mg of $(\text{NH}_4)_2\text{S}_2\text{O}_8$ at 50 °C, or (e) 20 mg of $\text{K}_2\text{S}_2\text{O}_8$ at 50 °C.

Scheme 2

Furthermore (f), 900 μL (5 μg) of $[^{125}\text{I}]\text{EGF-SH}$ and 20 mg of allyldextran were mixed with 20 mg of $(\text{NH}_4)_2\text{S}_2\text{O}_8$ at 50°C . All reactions were continued for 5 h, and thereafter conjugates were separated from $[^{125}\text{I}]\text{EGF-SH}$ and other reaction compounds with Sephadex G-50. Fractions of 1 mL were collected and analyzed regarding radioactivity. The fractions containing conjugate were pooled and diluted to a radioactivity concentration of about 1 kBq/mL. These conjugates were tested in receptor binding assays.

Preparation of Complete Conjugates. Conjugate I: 900 μL (5 μg) of $[^{125}\text{I}]\text{EGF-SH}$ was stirred with 900 μL (20 mg) of allyldextran and 1 mg of $\text{K}_2\text{S}_2\text{O}_8$ at 50°C for 5 h. Conjugate II: 900 μL (5 μg) of $[^{125}\text{I}]\text{EGF-SH}$ was stirred with 900 μL (20 mg) of dextran-BSH(B) and 1 mg of $\text{K}_2\text{S}_2\text{O}_8$ at 50°C for 5 h. Conjugate III: 900 μL (5 μg) of $[^{125}\text{I}]\text{EGF-SH}$ was stirred with 900 μL (20 mg) of dextran-BSH(A) and 1 mg of $\text{K}_2\text{S}_2\text{O}_8$ at 50°C for 5 h. All of these conjugates were separated from unconjugated growth factor and other low molecular weight reaction compounds with Sephadex G-50. Fractions of 1 mL were collected and analyzed regarding radioactivity. The fractions containing conjugate were pooled, diluted to a radioactivity concentration of 37 kBq/mL, and tested in receptor binding assays and retention studies.

Receptor Binding Assays. Cells were presaturated with 0 or 1 $\mu\text{g/mL}$ of nonradioactive EGF and thereafter incubated with culture medium containing either $[^{125}\text{I}]\text{-EGF-SH}$ or any of the conjugates. The incubation time used for $[^{125}\text{I}]\text{EGF-SH}$ was 25 min, which is the binding maximum for native EGF. The conjugates were incubated for 3 h to enable them to reach near their binding maximum, since EGF-dextran conjugates have shown slower kinetics than EGF (Andersson et al., 1991). After incubation, the cells were washed six times with cold serum-free medium and detached with 0.5 mL of trypsin-EDTA for 10 min at 37°C . One milliliter of medium was added, and the cells were resuspended; 0.5 mL of the cell suspension was used for cell counting, and 1.0 mL was used for radioactivity measurement in a liquid scintillation counter.

Displacement. Cells were washed with serum-free medium and incubated in triplicates with 0.5 mL of nonradioactive EGF (concentrations: 0, 10^{-3} – 10^3 ng/mL) and 0.5 mL of conjugate III or $[^{125}\text{I}]\text{EGF}$. The cells were incubated during 25 min with $[^{125}\text{I}]\text{EGF}$ and for 3 h with conjugate III. The amounts added, with respect to EGF, were the same for both conjugate III and $[^{125}\text{I}]\text{EGF}$. Culture Petri dishes without any cells were also incubated with conjugate III to examine the nonspecific binding to the dishes. After incubation, cells were thoroughly washed, trypsinized, and counted and the radioactivity was measured, as described above.

Binding Patterns. Cells were continuously incubated for 1, 2, 4, 8, and 24 h with conjugates II and III. The radioactivity concentrations for both conjugates were 18.5 kBq/mL. After incubation, cells were thoroughly washed, trypsinized, and counted and the radioactivity was measured, as described above.

Retention and Internalization. The method described by Haigler et al. (1980) was used. Cells were incubated for 4 h with conjugates II and III. The radioactivity concentrations for both conjugates were 37 kBq/mL. After incubation, cells were washed six times with cold serum-free medium and then incubated in ordinary culture medium for an additional 0, 4, and 8 h. The cells were washed, and retained cell-associated radioactivity was measured. Thereafter, cells were treated with 0.5 mL of 0.1 M glycine-HCl, pH 2.5, for 6 min at 4°C , to peel membrane-bound activity off the cells. An additional 0.5 mL of glycine-HCl buffer was used to quickly wash the cells and to collect any remaining activity on the cell surface. The radioactivity, considered to be membrane bound, was measured in a well counter. The cells were then treated with 0.5 mL of 1 M NaOH for 60 min at 37°C and an additional 0.5 mL of NaOH was used to wash remaining cells off the dishes. The cell-associated radioactivity, considered to be internalized, was measured in a well counter.

RESULTS

Introduction of allyl groups into dextran increased its contents of carbon from 43 to 46%, corresponding to a substitution of 6.6 kDa and giving an average molecular mass of about 76.6 kDa. About 160 allyl groups were thus added to each dextran chain, which means that 37% of the glucose units became allylated.

BSH coupling to dextran mediated by ammonium persulfate/dextran-BSH(A), resulted in a 10-fold higher boron content compared to mediation by potassium persulfate/dextran-BSH(B). On average, the substitutions corresponded to 660 and 72 boron atoms or 55 and 6 BSH, respectively. With dextran-BSH(A) a maximum of 827 boron atoms per dextran was recorded; that is, 69 BSH or 43% of the allyl groups were substituted with BSH.

Variations in coupling conditions for EGF, when coupled to allyldextran, were performed to optimize the yield of conjugate as well as binding to the EGFR on cultured cells. Both of these parameters were taken into consideration when the coupling conditions for further generation of conjugates were chosen. Figure 1 shows how the yield of $[^{125}\text{I}]\text{EGF-allyldextran}$ increased with elevated temperature and increasing amount of radical initiators. However, as shown in Figure 2, the increase in yield accomplished by stronger or higher concentration of radical initiator was offset by decreased EGFR binding. Conjugation using 20 mg of ammonium persulfate is not shown since the resulting $[^{125}\text{I}]\text{EGF-allyldextran}$ did not bind at all. Temperature during coupling was only of minor importance for cellular EGFR binding. The best compromise was to use 1 mg of potassium persulfate at 50°C for coupling $[^{125}\text{I}]\text{EGF}$ to allyldextran or boronated allyldextran, since this resulted in acceptable yield of conjugate and still good binding to the cells.

Separations of complete conjugates on Sephadex G-50 are shown in Figure 3. The peaks from left to right correspond to conjugate, $[^{125}\text{I}]\text{EGF}$, and low molecular weight substances. The gel-bed volume was not the same for the different chromatograms; hence, the conjugates were not eluted at the same fractions. The yield of conjugate seemed to be higher for production of conjugate

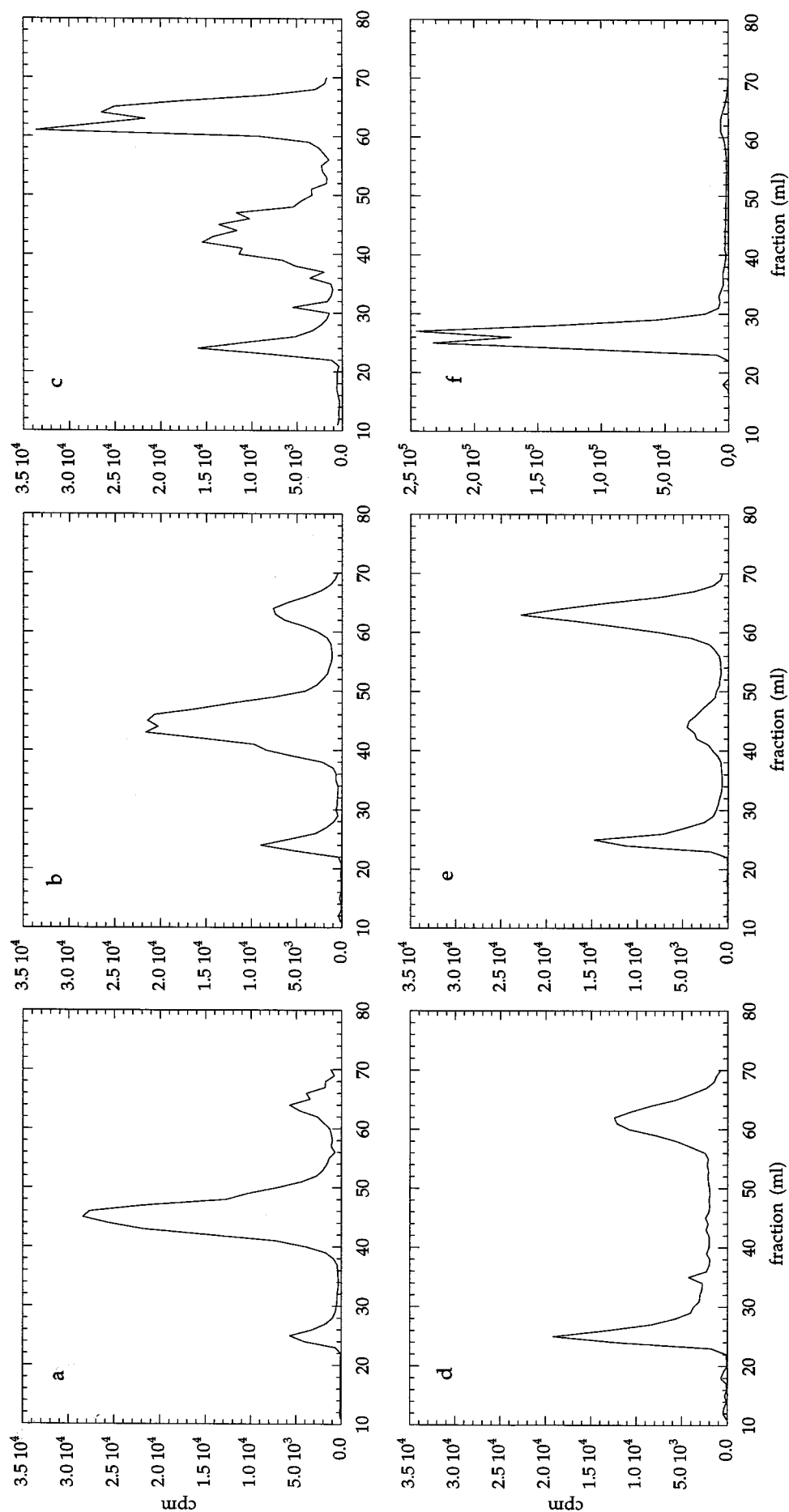


Figure 1. Chromatograms for separation of reaction mixtures with Sephadex G-50 after coupling of $[^{125}I]EGF$ to allyldextran under different reaction conditions. No boron was coupled in these reactions. (a) 1 mg of $K_2S_2O_8$ at 30 °C; (b) 1 mg of $K_2S_2O_8$ at 40 °C; (c) 1 mg of $(NH_4)_2S_2O_8$ at 50 °C; (d) 20 mg of $(NH_4)_2S_2O_8$ at 50 °C; (e) 20 mg of $[^{125}I]EGF-SH$, and low molecular weight compounds, respectively. Fractions 22–28 were pooled for further tests (Figure 2).

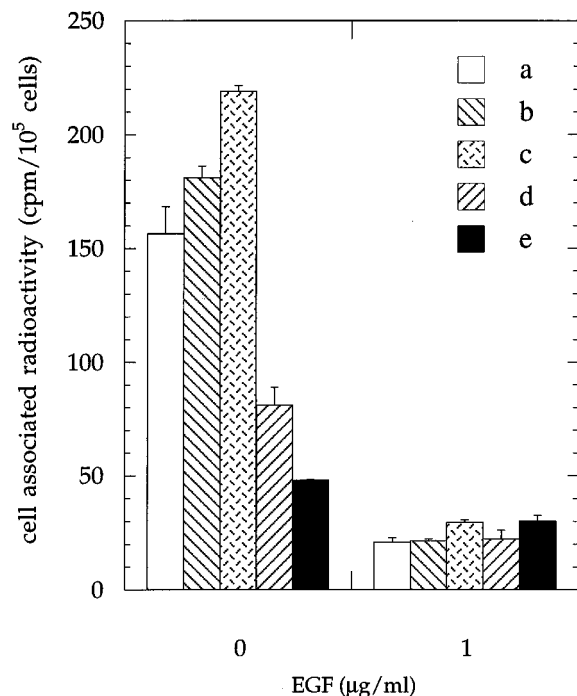


Figure 2. Binding of [^{125} I]EGF–allyldextran (pooled from fractions 22–28, in Figure 1), produced under different reaction conditions [(a) 1 mg of $K_2S_2O_8$ at 30 °C; (b) 1 mg of $K_2S_2O_8$ at 40 °C; (c) 1 mg of $K_2S_2O_8$ at 50 °C; (d) 1 mg of $(NH_4)_2S_2O_8$ at 50 °C; (e) 20 mg of $K_2S_2O_8$ at 50 °C] to cultured glioma cells with and without blocking of receptors, using 1 or 0 μ g/mL EGF. Incubation time was 3 h, and added radioactivities for a, b, c, d, and e were 0.7, 1.0, 1.4, 1.7, and 1.6 kBq/mL, respectively. Each bar corresponds to the analysis of three dishes. Mean values and maximum variations are given. (f from Figure 1 is not shown since that conjugate had no specific binding at all to cells.)

III compared with production of conjugates I and II. Table 1 shows an overview of the conjugates.

Binding of conjugates I, II, and III and [^{125} I]EGF–SH to cultured cells is shown in Figure 4. Specific cell binding decreased when EGF was modified with allyldextran. When boron was bound to allyldextran, binding decreased even more, especially if large amounts of boron were added. Nonspecific binding seemed to increase when boron was bound to the dextran and was about 30% for conjugate II and 40% for conjugate III. These results showed that the presence of BSH had a negative effect on both receptor specific and nonspecific binding to cells by the conjugates.

Figure 5 shows the displacement of conjugate III and [^{125} I]EGF with increasing concentrations of native EGF. The radioactivity in culture dishes without cells corresponded to 40% of the radioactivity in culture dishes with cells incubated with both the conjugate and the highest concentration of native EGF. This background activity was subtracted from all dishes with conjugate III in the displacement test. As in Figure 4 the conjugate showed specific binding to the EGFR but it could not fully be displaced by EGF due to nonspecific binding to the cells of about 20%. The figure also shows that the amount of EGF needed to displace the conjugate is less than what was needed to displace [^{125} I]EGF.

The binding patterns for continuous incubation with conjugates II and III are shown in Figure 6. Conjugate II bound rapidly to the cells with a maximal binding at 4 h and thereafter slowly decreased. Conjugate III showed quite a different pattern with a peak already after 1 h and a slight decrease followed by a slow increase of binding from 4 h until, at least, 24 h. This indicated

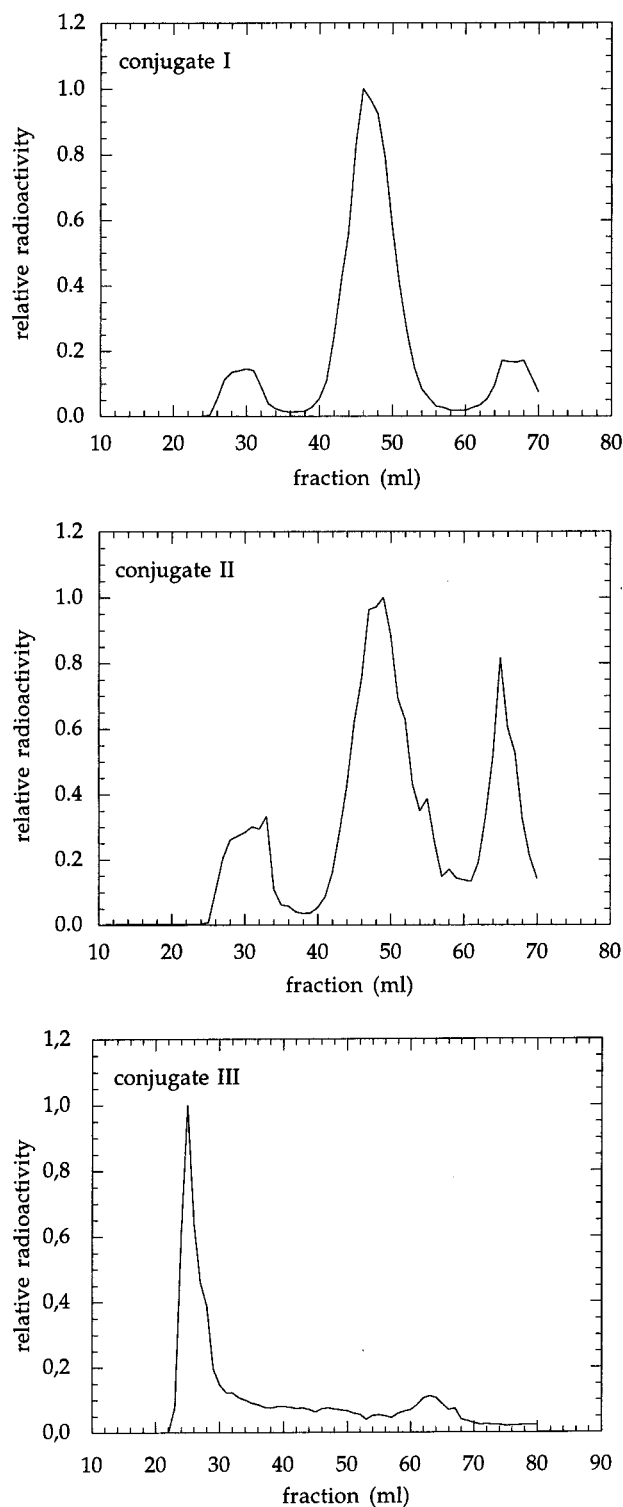


Figure 3. Chromatograms for separation of boron-containing conjugates with Sephadex G-50 gel. One milliliter fractions were collected, and pools were used in further tests (Figures 4–6). Conjugate I (top), fractions 26–33 were pooled. This conjugate did not contain any boron. Conjugate II (middle), fractions 26–33 were pooled. This conjugate contained 72 boron atoms on average. Conjugate III (bottom), fractions 24–28 were pooled. This conjugate contained 660 boron atoms on average.

that after 24 h there was more cell-associated activity with conjugate III than with conjugate II. The nonspecific binding for both conjugates was less or the same as shown in Figure 4.

The retention curves of conjugates II and III in cultured cells are shown in Figure 7. Both conjugates showed similar patterns, though the binding was higher

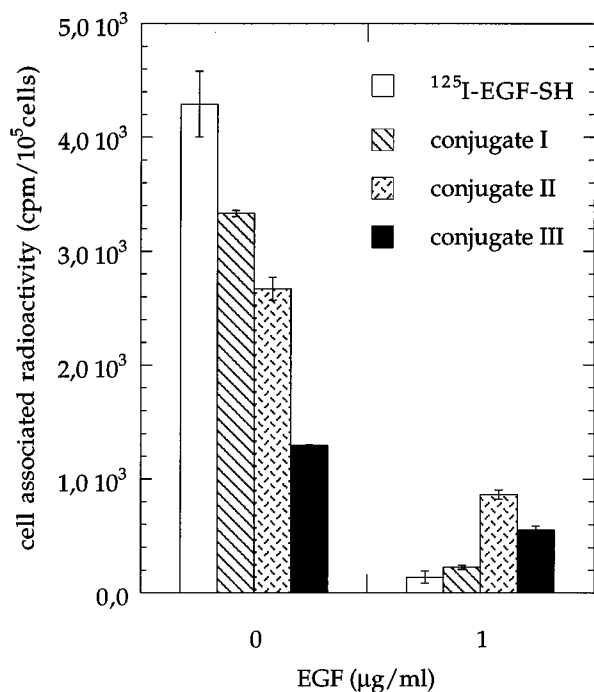


Figure 4. Binding of conjugates I–III (pools from Figure 3) and [^{125} I]EGF-SH to cultured glioma cells with and without blocking of receptors, using 1 or 0 μ g/mL EGF. Incubation time was 3 h (25 min for [^{125} I]EGF-SH), and added radioactivity was 37 kBq/mL in all cases. Each bar corresponds to the analysis of three dishes. Mean values and maximum variations are given.

Table 1. Schedule over Complete Conjugates Constructed and the Average Amount of Boron Bound to the Dextran

conjugate	EGF	dextran	BSH (av)	boron atoms (av)
EGF-SH	[^{125} I]EGF			
I	[^{125} I]EGF	allyldextran		
II	[^{125} I]EGF	dextran-BSH(B)	6	72
III	[^{125} I]EGF	dextran-BSH(A)	55	660

for conjugate II. After 4 h, one-third of the original activity was still bound to the cells for both conjugates. After 8 h, only 15 and 25% of the radioactivity from conjugates II and III, respectively, was still associated to the cells. As shown in Table 2, the major part of this association was in the form of internalized radioactivity and <10% was bound to receptors on the cell surface.

DISCUSSION

Dextran was easily modified by allylation of the hydroxyl groups in the glucose units, since more than one-third of them were substituted. This implies that the desired therapeutic range is within reach. If all 160 available substitution sites would be occupied by BSH, nearly 2×10^3 boron atoms could theoretically be attached in a single dextran chain. It has been calculated that roughly 10^3 boron atoms per EGF-based conjugate would suffice if a tumor target cell expressed about 10^6 EGFRs (Hartman and Carlsson, 1994). Such overexpression has been established in some gliomas at least in vitro (Bigner et al., 1981; Werner et al., 1988).

In this study the maximum substitution was about 70 BSH per dextran, corresponding to over 800 boron atoms. This was achieved when using ammonium persulfate as initiator for the coupling of BSH to the dextran chain. When the same initiator was used for coupling of [^{125} I]-EGF to allyldextran, the yield was high (Figure 1) but the growth factor lost its binding properties to the EGFR

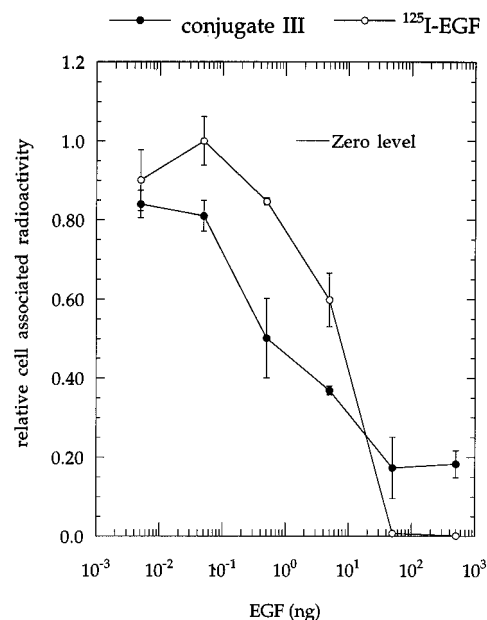


Figure 5. Displacement of conjugate III and [^{125} I]EGF with increasing concentrations of EGF. Added amounts of conjugate and [^{125} I]EGF were the same, with respect to the growth factor. Incubation time was 25 min for [^{125} I]EGF and 3 h for conjugate III. Each bar corresponds to the analysis of three dishes. Mean values and maximum variations are given.

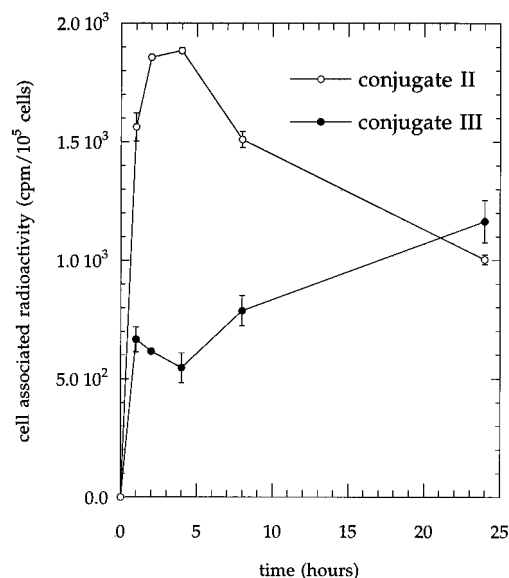


Figure 6. Continuous incubation with conjugates II and III on cultured glioma cells. The radioactivity concentrations for both conjugates were 18.5 kBq/mL. The nonspecific binding was less than or the same as shown in Figure 4. Each point corresponds to the analysis of three dishes. Mean values and maximum variations are given.

(Figure 2). The biological inactivation was probably due to the drop of pH connected with this procedure. To circumvent this problem, potassium persulfate was used instead, and this gave a good binding of the conjugate to the EGFR (Figure 2), although the coupling efficiency of BSH (Table 1) and EGF (Figure 1) to the dextran chain was lower than with ammonium persulfate. To create a conjugate with maximum load of boron as well as retained affinity to the EGFR, ammonium persulfate was used for the coupling of BSH to allyldextran, while potassium persulfate was used for coupling EGF to dextran-BSH.

The differences in yield of conjugate III and of the others (Figure 3) could be caused by small contamination

Table 2. Internalized and Membrane-Bound Radioactivity, after 4 h of Incubation and Thorough Washes, with Conjugates II and III on Cultured Glioma Cells^a

conjugate	time after wash (h)	internalized (%)	membrane-bound (%)
II	0	93.0	7.0
	4	31.4	3.2
	8	15.7	0.7
III	0	93.5	6.5
	4	31.7	1.3
	8	24.5	1.2

^a Figures are given in percent of the originally cell-associated radioactivity.

of ammonium persulfate from the production of dextran-BSH(A). This contamination could then affect the coupling of dextran-BSH(A) to EGF, mediated by potassium persulfate. As shown before, ammonium persulfate gave much higher yield of conjugate compared with potassium persulfate (Figure 1), thus the high yield of conjugate III. This was not the case for conjugates I and II since their production did not involve ammonium persulfate at all.

When allyldextran was coupled to [¹²⁵I]EGF-SH, the specific binding to cells decreased by about 20%, as shown in Figure 4. This was surprisingly little since the molecular weight increased more than 10-fold by the dextran. On the other hand, addition of BSH affected the specific receptor binding much more. Six BSH bound to the [¹²⁵I]EGF-allyldextran decreased binding by another 20%, although the molecular weight was only increased by about 1%. Furthermore, if 55 BSH were attached, specific binding decreased by an additional 30%, ending up with only one-third of the binding of [¹²⁵I]EGF-SH. The nonspecific binding was also much more affected by the boron than by the dextran chain. Adding an allylated dextran chain to [¹²⁵I]EGF-SH did not considerably alter the nonspecific binding to cells. However, the increased amount of boron bound to the conjugate gave more nonspecific binding. It is possible that BSH strongly altered the charge of the conjugate and thus changed its binding properties.

Adding BSH to dextran also increased the nonspecific binding to the plastic in cell culture dishes, and this partly explains the increasing nonspecific binding of conjugates when tests are made in culture dishes with cells. As much as half of the background radioactive signal could be from conjugates bound to the plastic. This nonspecific binding to the plastic was subtracted in the displacement test (Figure 5). The figure shows that the background of nonspecific cellbound conjugate was thereafter <20%, instead of the earlier 40% (Figure 4). The figure also shows, besides the receptor specific binding of the conjugate, that displacement of the conjugate is possible with only about 1/10 of the amount of EGF compared to [¹²⁵I]EGF at these incubation times.

[¹²⁵I]EGF-allyldextran-BSH conjugates showed different binding patterns depending on the amount of boron attached to the conjugate (Figure 6). Conjugate II, with 6 BSH, bound rapidly to the cells and had a maximum binding within 2–4 h and thereafter a slight decrease until 24 h, while for conjugate III with 10 times more boron, maximum binding had not been reached within 24 h. It is possible that differences in charge are the cause of this.

The difference in binding between conjugates II and III was not due to delayed degradation and excretion of the EGF part of conjugate III. Since the radioactivity was associated to EGF, it was the [¹²⁵I]EGF part of the conjugates that was presented in Figure 7. It indicates that, at least the [¹²⁵I]EGF part of conjugate II as well

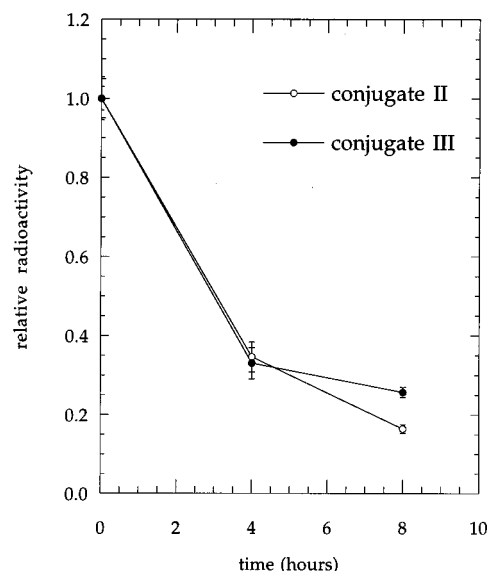


Figure 7. Retention of conjugates II and III in cultured glioma cells. The cultures were preincubated with the conjugates for 4 h before washing and the retention study. The radioactivity concentrations for both conjugates were, during preincubation, 18.5 kBq/mL. Each point corresponds to the analysis of three dishes. Mean values and maximum variations are given.

as conjugate III were excreted to about one-third of the original cell-associated amount within 4 h. Likewise, there were no differences in internalization between the conjugates as over 90% of the cell-associated radioactivity was present as internalized activity during all of the investigated times (Table 2). This suggests that the conjugates are rapidly taken up by the cells once they have bound to the receptors and the radioactive part of the conjugates is subsequently excreted.

The uptake and retention of the dextran and BSH parts of the conjugates have not been evaluated in this study. However, earlier studies with U-343 cells showed that the dextran part in EGF conjugates has a longer retention time than the EGF part (Andersson et al., 1991). The reason for not evaluating uptake and retention of dextran and BSH in this study is due to the molar ratios between EGF and dextran-BSH at conjugation. There is a 300-fold excess of dextran-BSH compared with EGF, which leads to approximately one conjugate for each 300 dextran-BSH. Separation between conjugate and dextran-BSH was shown not to be trivial due to their similarities in size and charge, and therefore no separation was performed. No separation implies that the boron signal from cell specific bound conjugates would drown in the nonspecific binding of dextran-BSH when administered to cells. A lot of effort is now being focused on solving this problem.

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LITERATURE CITED

- Andersson, A., Holmberg, A., Carlsson, J., Carlsson, J., Pontén, J., and Westermark, B. (1991) Binding of epidermal growth factor-dextran conjugates to cultured glioma cells. *Int. J. Cancer* 47, 439–444.

- Andersson, A., Capala, J., and Carlsson, J. (1992) Effects of EGF-dextran-tyrosine- ^{131}I conjugates on the clonogenic survival of cultured glioma cells. *J. Neuro-Oncol.* 14, 213–223.
- Barth, R. F., Soloway, A. H., and Fairchild, R. G. (1990) Boron neutron capture therapy of cancer. *Cancer Res.* 50, 1061–1070.
- Bigner, D. D., Bigner, S. H., Pontén, J., Westermarck, B., Mahaley, M. S., Jr., Ruoslahti, E., Herschman, H., Eng, L. F., and Wikstrand, C. J. (1981) Heterogeneity of genotypic and phenotypic characteristics of fifteen permanent cell lines derived from human gliomas. *J. Neuropathol. Exp. Neurol.* 40, 201–229.
- Bigner, S. H., Burger, P. C., Wong, A. J., Werner, M. H., Hamilton, S. R., Muilbaier, L. H., Vogelstein, B., and Bigner, D. D. (1988) Gene amplification in malignant human gliomas: clinical and histopathologic aspects. *J. Neuropath. Exp. Neurol.* 47, 191–205.
- Capala, J., Práhl, M., Scott-Robson, S., Pontén, J., Westermarck, B., and Carlsson, J. (1990) Effects of ^{131}I -EGF on cultured human glioma cells. *J. Neuro-Oncol.* 9, 201–210.
- Carlsson, J., Gedda, L., Grönvik, C., Hartman, T., Lindström, A., Lindström, P., Lundqvist, H., Löfqvist, A., Malmqvist, J., Olsson, P., Pontén, J., Sjöberg, S., and Westermarck, B. (1994) Strategy for boron neutron capture therapy against tumor cells with over-expression of the epidermal growth factor-receptor. *Int. J. Radiat. Oncol., Biol. Phys.* 30, 105–115.
- Cawley, D. B., Herschman, H. R., Gilliland, D. G., and Collier, R. J. (1980) Epidermal growth factor-toxin A chain conjugates: EGF-ricin A is a potent toxin while EGF-Diphtheria fragment A is nontoxic. *Cell* 22, 563–570.
- Charlton, D. E. (1991) Energy deposition in small ellipsoidal volumes by high-LET particles: application to thermal neutron dosimetry. *Int. J. Radiat. Biol.* 59 (3), 827–842.
- Collins, V. P., and James, C. D. (1993) Gene and chromosomal alterations associated with the development of human gliomas. *FASEB J.* 7, 926–930.
- Fairchild, R. G., Kahl, S. B., Laster, B. H., Kalef-Ezra, J., and Popenoe, E. A. (1990) In vitro determination of uptake, retention, distribution, biological efficacy and toxicity of boronated compounds for neutron capture therapy; a comparison of porphyrines and sulfhydryl boron hydrides. *Cancer Res.* 50, 4860–4865.
- Foster, R. L. (1975) Preparation and properties of a soluble trypsin-dextran conjugate. *Experientia* 31, 773–774.
- Gabel, D., Foster, S., and Fairchild, R. G. (1987) The Monte Carlo simulation of the biological effect of the $^{10}\text{B}(\text{n},\alpha)^7\text{Li}$ reaction in cells and tissue and its implication for boron neutron capture therapy. *Radiat. Res.* 111, 14–25.
- Haigler, H. T., Maxfield, F. R., Willingham, M. C., and Pastan, I. (1980) Dansylcadaverine inhibits internalization of ^{125}I -epidermal growth factor in Balb 3T3 cells. *J. Biol. Chem.* 255 (4), 1239–1241.
- Hartman, T., and Carlsson, J. (1994) Radiation dose heterogeneous in receptor and antigen mediated boron neutron capture therapy. *Radiother. Oncol.* 31, 61–75.
- Hawthorne, M. F. (1993) The role of chemistry in the development of boron neutron capture therapy of cancer. *Angew. Chem., Int. Ed. Engl.* 32, 950–984.
- Holmberg, A., and Meurling, L. (1993) Preparation of sulfhydrylborane-dextran conjugates for boron neutron capture therapy. *Bioconjugate Chem.* 4 (6), 570–573.
- Kohn, K., and Wilchek, M. (1984) The use of cyanogen bromide and other novel cyanylating agents for the activation of polysaccharide resins. *Appl. Biochem. Biotechnol.* 9, 285–305.
- Lindström, P., Olsson, P., Malmqvist, J., Pettersson, J., Lemmen, P., Werner, B., Sjöberg, S., Olin, Å., and Carlsson, J. (1994) New carborane-based compounds for boron neutron capture therapy: binding and toxicity of ANC-1, DAC-1 and B-Et-11-OMe in cultured human glioma and mouse melanoma cells. *Anti-Cancer Drugs* 5, 43–52.
- Locher, G. L. (1936) Biological effects and therapeutic possibilities of neutrons. *Am. J. Roentgenol. Radium Ther.* 36, 1–13.
- Melton, R. G., Wiblin, C. N., Foster, R. L., and Sherwood, R. F. (1987) Covalent linkage of carboxypeptidase G_2 to soluble dextrans—I. Properties of conjugates and effects on plasma resistance in mice. *Biochem. Pharmacol.* 36 (1), 105–112.
- Mendelsohn, J. (1988) Growth factor receptors as targets for antitumor therapy with monoclonal antibodies. *Monoclonal Antibody Ther.* 45, 147–160.
- Olsson, P., Lindström, A., and Carlsson, J. (1994) Internalization and excretion of epidermal growth factor-dextran-associated radioactivity in cultured human squamous-carcinoma cells. *Int. J. Cancer* 56, 529–537.
- Ozawa, S., Masakazu, U., Nobutoshi, A., Osahiko, A., Minoshima, S., and Shimizu, N. (1989) Selective killing of squamous carcinoma cells by an immunotoxin that recognizes the EGF receptor. *Int. J. Cancer* 43, 152–157.
- Reiter, Y., Brinkmann, U., Jung, S.-H., Lee, B., Kasprzyk, P. G., King, C. R., and Pastan, I. (1994) Improved binding and antitumor activity of a recombinant anti-erbB2 immunotoxin by disulfide stabilization of the Fv fragment. *J. Biol. Chem.* 269 (28), 18327–18331.
- Siegall, C. B., FitzGerald, D. J., and Pastan, I. (1990) Selective killing of tumor cells using EGF and TGF α -Pseudomonas exotoxin chimeric molecules. *Semin. Cancer Biol.* 1, 345–350.
- Soloway, A. H., Hatanaka, H., and Davis, M. A. (1967) Penetration of brain and brain tumor VII. Tumor-binding sulfhydryl boron compounds. *J. Med. Chem.* 10, 714–717.
- Stacey, F. W., and Harris, J. F., Jr. (1963) Formation of carbon-hetero atom bonds by free radical chain additions to carbon-carbon multiple bonds. In *Org. React. (N.Y.)* 13.
- Vaughan, A. T. M., Anderson, P., Dykes, P. W., Chapman, C. E., and Bradwell, A. R. (1987) Limitations to the killings of tumors using radiolabelled antibodies. *Br. J. Radiol.* 60, 567–578.
- Vollmar, A. M., Banker, D. E., Mendelsohn, J., and Herschman, H. R. (1987) Toxicity of ligand antibody-directed ricin A-chain conjugates recognizing the epidermal growth factor receptor. *J. Cell. Physiol.* 131, 418–425.
- Werner, M. H., Humphrey, P. A., Bigner, D. D., and Bigner, S. H. (1988) Growth effects of epidermal growth factor (EGF) and a monoclonal antibody against the EGF receptor on four glioma cell lines. *Acta Neuropathol.* 77, 196–201.
- Westermarck, B., Magnusson, A., and Heldin, C.-H. (1982) Effect of Epidermal growth factor on membrane motility and cell locomotion in cultures or human clonal glioma cells. *J. Neurosci. Res.* 8, 491–507.
- Wheldon, T. E., Donoghue, J. A., Hilditch, T. E., and Barrett, A. (1988) Strategies for systemic radiotherapy of micrometastases using antibody-targeted ^{131}I . *Radiother. Oncol.* 11, 133–142.
- Wikstrand, C. J., Hale, L. P., Batra, S. K., Hill, M. L., Humphrey, P. A., Kurpad, S. N., McLendon, R. E., Moscatello, D., Pegram, C. N., Reist, C. J., Traweck, S. T., Wong, A. J., Zalutsky, M. R., and Bigner, D. D. (1995) Monoclonal antibodies against EGFRvIII are tumor specific and react with breast and lung carcinomas and malignant gliomas. *Cancer Res.* 55, 3140–3148.

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Chemical Modification of Surface Active Poly(ethylene oxide)–Poly(propylene oxide) Triblock Copolymers

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A general route has been developed to chemically modify a series of poly(ethylene oxide)–poly(propylene oxide) triblock copolymers with molecular weights from 6500 to 14 600. It is initiated by the introduction of *p*-nitrophenyl groups; such nitrophenyl conjugated copolymers are stable in an organic milieu and in a dry state but are seen to react easily with amino-containing molecules including small peptides. Among them, introduction of 2-pyridyl disulfide groups after coupling with 2-(2-pyridyldithio)ethylamine enables the selective attachment of thiol-containing molecules. The released thiopyridone in such thiol–disulfide reactions can be used to quantify the content of 2-pyridyl disulfide groups. In addition, a new type of modified copolymers was developed for the radioisotope (¹²⁵I) labeling purpose that consists of a reaction of nitrophenyl conjugated copolymers with hydrazine and a subsequent coupling with *N*-succinimidyl 3-(4-hydroxyphenyl)propionate (Bolton–Hunter reagent). Adsorption studies of ¹²⁵I-labeled and 2-pyridyl disulfide conjugated copolymers on polystyrene particles are consistent with previous determinations of surface coverage using other technologies, in turn indicating that this new chemical modification does not alter their surfactant properties on hydrophobic solid phase. The coating of common hydrophobic surfaces with 2-pyridyl disulfide conjugated copolymers has been demonstrated as a general and robust immobilization method to generate a high-sensitivity bioactive surface with low nonspecific binding. The optimal space between immobilized ligands can also be controlled by incubating the solid phase with solutions containing mixtures with different ratios of unmodified and modified copolymers.

INTRODUCTION

Pluronic (BASF Corp.) surfactants, a group of poly(ethylene oxide)/poly(propylene oxide)/poly(ethylene oxide) (PEO/PPO/PEO), triblock copolymers also known as poloxamers, have been shown to possess many unique properties leading to numerous valuable applications (1). Generally, the poloxamers are utilized as delivered, and a broad range of relative block compositions is available from the manufacturer. Thus, low molecular weight, liquid poloxamers are used as washing detergents, while high molecular weight surfactants with high ethylene oxide contents may be selected for the purpose of dispersion stabilization. Several members of the Pluronic family are known to adsorb to hydrophobic plastic surfaces in a pseudo-irreversible way, forming complexes that are stable for long periods of time following suspension in a surfactant-free medium.

Pluronic compounds are of considerable interest in the biotechnological and pharmaceutical industry because of their unique surfactant abilities and their extremely low toxicity and immunogenic response. In fact, Pluronic compounds are among a small number of surfactants that have been approved by the U.S. Food and Drug Administration for direct use in medical applications and as food additives (1). Recently, surface treatments with Pluronic have been found to efficiently reduce platelet adhesion (2), protein adsorption (3–6), bacterial adhesion (7), and phagocytosis (8–10.) Furthermore, several Pluronic compounds have been found to enhance the therapeutic

effect of drugs, both *in vitro* and *in vivo* (11, 12). In addition, surfactants of this type have seen a variety of uses including serving as vehicles for the introduction of antisense oligonucleotides to inhibit the proliferation of vascular smooth muscle cells (13) and as a drug administered to reduce myocardial injury (14).

Despite the numerous sophisticated applications of this class of surfactants, relatively few attempts have been made to subject them to chemical derivatization. Here, to broaden their versatility, we will introduce a simple and reliable means for their modification, which is based on the introduction of reactive intermediates for conjugating other functional groups, e.g., for radioisotope labeling or solid phase immobilization. Through these reactions, quantification of Pluronic can be simplified by a direct probing of labeled surfactants, and biological molecules can be tethered to surfaces of interest after physical adsorption of the conjugated surfactants.

Nonspecific immobilization of biological molecules, such as enzymes or antibodies, can be achieved by simple adsorption onto a solid phase. For example, polystyrene (PS) in the form of microtiter plates is a common solid phase in many heterogeneous enzyme immunoassay systems. Since such assays are based on the specific interactions between reagents and analytes, the preparation of a bioactive surface is a critical factor in developing a testing assay with high sensitivity and selectivity. The effectiveness of a passive coating is based on the retention of a specific biological activity upon adsorption, be it an antibody–antigen interaction or an enzymatic reaction. The fact that antibodies spontaneously bind to hydrophobic surfaces and in most cases preserve their immunochemical activity is the basis for the common use of PS as the solid phase in heterogeneous immunoassays (15, 16).

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Yet, sensitive monoclonal antibodies sometimes lose their activities due to conformational changes caused by their sharing hydrophobic interactions with the surface, and such a physical adsorption method appears to be unsuitable for the immobilization of many enzymes on PS surfaces. Indeed, Sandwich and Schray (17) demonstrated that enzymes such as alkaline phosphatase, β -galactosidase, lysozyme, horseradish peroxidase, catalase, and glucose-6-phosphate dehydrogenase lose their catalytic activity when they adsorb onto small PS latex particles from low concentration ($<1 \times 10^{-3}$ mg/mL) protein solutions. Because of the tendency of proteins, particularly in low concentration, to denature on hydrophobic surfaces, adsorption is of limited applicability as an immobilization method. Here, we will introduce a new technique for immobilizing proteins and their ligands to hydrophobic surfaces. This technique, although still based on adsorption, involves precoating of surfaces with a 2-pyridyl disulfide derivatized Pluronic surfactant and a subsequent attachment of thiol-containing ligands.

As discussed previously (4, 5), it is believed that a Pluronic surfactant adsorbs to hydrophobic PS surfaces via its hydrophobic PPO center block, while leaving its terminal hydrophilic PEO chains to extend from the surface into the hydration surrounding, thus rendering it hydrophilic with a much reduced tendency to nonspecifically adsorb proteins from the environment. A derivatized Pluronic coating layer may then ideally serve the dual purpose of providing reactive groups for specific coupling of a desired ligand in a preferred orientation as well as creating a protein-compatible environment for the immobilized molecules. Since most molecules can be easily thiolated by the modification of suitable functional groups, this new technique can be applicable to a large array of molecules of interest. In addition, the coating layer, which is rich in dynamic PEO chains, offers very little opportunity for nonspecific adsorption of biomolecules.

The most attractive chemical modification of Pluronic surfactants involves the hydroxyl ends of the PEO blocks, and the PEO chemistry developed by other groups (18–20) may thus be applied also to the PEO/PPO/PEO triblock copolymers. We have found that an introduction of reactive carbonate structures into the Pluronic with the aid of 4-nitrophenyl chloroformate, previously used to activate polyols for biomolecule binding (21, 22), is a straight-forward and reliable starting step for derivatizing the functional groups of interest. Here, we will first discuss a general synthetic procedure for radioisotope labeling of all types of Pluronic and will then specifically focus on Pluronic F108, which has the highest molecular weight and highest PEO content in a series of PEO–PPO triblock copolymers examined in this study for further introduction of functional groups.

EXPERIMENTAL PROCEDURES

Materials. The Pluronic surfactants, P105, F68, F88, and F108, with molecular weights of 6500, 8400, 11 400, and 14 600 corresponding to numbers of monomer units in each PEO block of 37, 76, 104, and 129, respectively, were kindly donated by BASF Co. PS latex particles with a diameter of 0.272 μm were from Seradyn, and porous monodisperse PS beads (10 μm) in dry form were kindly supplied by Dr. P.-Å. Pernemalm at Pharmacia-BTG-LKB, Uppsala, Sweden. The 4-nitrophenyl chloroformate and 1,3-aminopropane were obtained from Aldrich, hydrazine was from EM Science, *N*-succinimidyl 3-(4-hydroxyphenyl)propionate (Bolton–Hunter reagent) was from Pierce, chloramine T was from Eastman Kodak, Na^{125}I (100 mCi/mL) was from Amersham, sodium met-

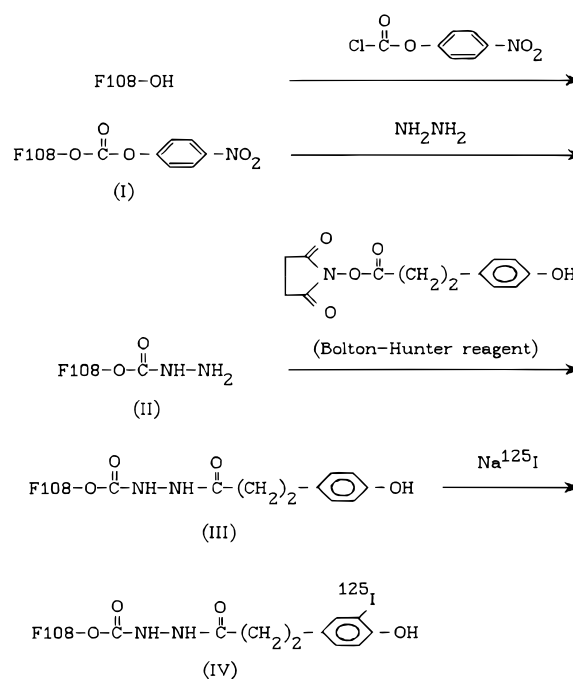


Figure 1. Modification of Pluronic F108 for radioisotope ^{125}I labeling.

abisulfite was from Fisher, triethylamine (TEA) was from Baker, and dithiothreitol (DTT) was from Bio-Rad. Mercaptoethylamine hydrochloride, 2,2'-dithiopyridine, 2-aminoethanesulfonic acid (taurine), glycyltryptophan (Gly-Trp), β -galactosidase (grade 8 from *Escherichia coli*) were all purchased from Sigma. Disposable prepacked PD-10 columns (Sephadex G-25 M with an approximate bed volume of 8.5 mL) were from Pharmacia, and the dialysis membranes (Spectra/Por) were purchased from Spectrum Medical.

General Methods for Activation of Pluronic with 4-Nitrophenyl Chloroformate. Two grams of Pluronic of either type was first dissolved in 6 mL of benzene, and this solution was slowly added to a stirred solution of 4-nitrophenyl chloroformate in 6 mL of benzene. The content of 4-nitrophenyl chloroformate varied with the molecular weight of Pluronic as the ratio of $-\text{OH}/\text{nitrophenyl}$ was kept at 1:3. After 24 h of shaking, the reaction product was precipitated with ether, recovered by filtration, and redissolved in benzene. This procedure was repeated at least three times and the product (I) (Figure 1, where F108 represents the Pluronic F108 molecule) was finally recovered by evaporation of the remaining solvent under vacuum overnight. For Pluronic P105, the precipitation was done by using an 80 times excess petroleum ether (bp 35–60 $^{\circ}\text{C}$). The degree of substitution was determined spectrophotometrically by measuring the amount of *p*-nitrophenolate ions released in an alkaline solution. For this purpose, an accurately weighed sample of the 4-nitrophenyl chloroformate-activated Pluronic (product I in Figure 1) was dissolved in 0.1 M NaOH. After 1 h of mixing under rotation, its absorbance was measured at 402 nm and converted to concentration, using a molar extinction coefficient of $18400 \text{ cm}^{-1} \text{ M}^{-1}$ (19).

Radioisotope ^{125}I Labeling of Pluronic. The modification of Pluronic for radioisotope labeling is schematically shown in Figure 1. A 1.5 g portion of activated Pluronic, product I in Figure 1, was dissolved in 3 mL of methanol and mixed with 97% hydrazine; the molar ratio of hydrazine to Pluronic was kept as 100:1. After an 8 h reaction time, the product II was precipitated with ethyl

ether. Redissolution in methanol followed by precipitation was repeated at least three times, and the final product was dried under vacuum overnight. For Pluronic P105, the recovery of product **II** was not so straightforward, and after considerable trial and error the following dialysis process was found to efficiently remove all possible traces of the strongly colored *p*-nitrophenolate ions. The mixture after reaction was concentrated in a rotating evaporator for 1–1.5 h and was then dissolved in 6 mL of methanol. This solution was transferred to a dialysis bag with a molecular weight cutoff of 1000 and was dialyzed against a 30% methanol solution. The methanol/water mixture (500 mL) was changed three times in 24 h and was then replaced by 0.1 M NaHCO₃ solution (500 mL). This weak alkaline solution was changed every 8 h, and the procedure was repeated three or four times. Then, deionized water was used as incubation solution and was changed three times in 24 h, whereupon product **II** was recovered by lyophilization.

The Bolton–Hunter reagent was found to be an efficient chemical for coupling to the introduced –NH₂ groups and for subsequent labeling with radioisotope ¹²⁵I (23, 24). One volume of a 17 mM solution of product **II** in dimethyl sulfoxide was vigorously mixed with 4 volumes of a 43 mM Bolton–Hunter reagent also in dimethyl sulfoxide. After 4 h, product **III** was precipitated from the solution using ether and then dried under vacuum overnight. For Pluronic P105, tetrahydrofuran replaced dimethyl sulfoxide as the solvent. After 4 h of reaction, this mixture was concentrated in a rotating evaporator and was then dissolved in 3 mL of methanol. This solution was transferred to a dialysis bag with a molecular weight cutoff of 1000 and dialyzed against methanol for 8 h and subsequently against deionized water for 8 h. The incubation solution was then replaced by fresh deionized water, and after 8 h, the product was sent to lyophilization. The substitution rate of this reagent can be calculated by measuring the ratio of conjugated phenol groups to the moles of Pluronic molecules present. A series of samples with Bolton–Hunter reagent in various concentrations were prepared in methanol, and their UV absorbances were determined at a wavelength of 270 nm using a Perkin-Elmer Lambda 6/PECSS spectrophotometer. From these measurements a molar extinction coefficient of the Bolton–Hunter phenol group was calibrated as 1270 cm⁻¹ M⁻¹. Finally, 0.5 mL of a solution containing 0.6 mg/mL of product **III** in PBS buffer (0.01 M phosphate, 0.14 M NaCl, pH 7.4) was treated with 3 μL of Na¹²⁵I, and 50 μL of 4 mg/mL chloramine T was added as an oxidizing reagent. This iodination reaction was terminated after 4 h by using 50 μL of a solution containing 4.8 mg/mL sodium metabisulfite. The ¹²⁵I-labeled Pluronic and free Na¹²⁵I were then separated by passing through a PD-10 column using PBS as the mobile phase, and the eluate was fractionated into 12 culture tubes. Twenty microliters of each 1 mL fraction of the eluate was transferred into a counting vial, and the radioactivity was determined by a radioisotope detector (Beckman 170M).

Coupling of Amines to Pluronic. (A) *1,3-Diaminopropane*. 1,3-Diaminopropane (3.3 g) was mixed with 5 mL of deionized water. After the pH was adjusted to 8.2 with concentrated HCl, the solution was mixed with a solution of 0.5 g of 4-nitrophenyl chloroformate activated Pluronic F108 (prepared as described above) in 5.0 mL of deionized water. The reaction mixture, which immediately turned yellow, was kept at 25 °C for 15 h. This solution was transferred to a dialysis tubing (with a molecular weight cutoff of 3500) and was dialyzed against 4 L of deionized water. During the 48 h dialysis process,

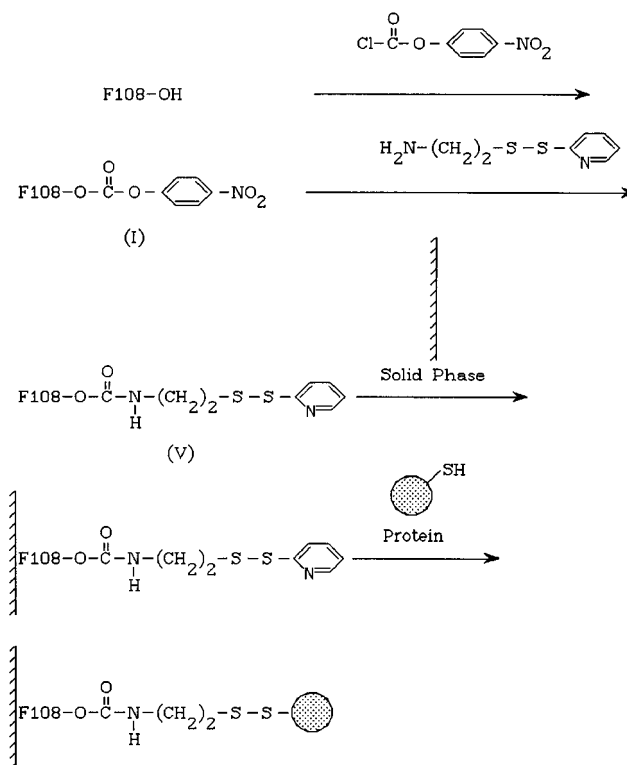


Figure 2. Preparation of a F108–PDS surface for protein specific binding.

water was changed five times until the low molecular weight material was assumed to be completely removed. The product was then recovered by lyophilization. The degree of substitution was determined by elemental nitrogen analysis. In this calculation the nitrogen content determined per a given mass of product was taken to exclusively derive from the attached diamine.

(B) *2-Aminoethanesulfonic Acid (Taurine)*. Taurine (3.4 g) was dissolved in 7 mL of deionized water, and the pH of the solution was adjusted to 9.4 with 2 M HCl. The solution was mixed with a 5 mL water solution of 0.5 g of 4-nitrophenyl chloroformate activated Pluronic F108. The resulting reaction mixture was kept at 25 °C overnight, and the product was obtained after dialysis and lyophilization as described previously. The degree of substitution was determined through sulfur and nitrogen analysis of a known amount of product; its molar taurine content was then readily calculated.

(C) *Glycyltryptophan (Gly-Trp)*. Two 11 mg portions of Gly-Trp were each dissolved in a vial with 2 mL of methanol. One of the vials contained 0.05 mL of 1.2 M TEA. To both vials was added 11 mg of 4-nitrophenyl chloroformate activated Pluronic F108, and the final solutions were kept at 25 °C overnight. The reaction mixtures were then passed through PD-10 Sephadex G-25 columns, and the void fractions were pooled. The amount of bound Gly-Trp was determined by dry weight determination and photometric analysis using a molar extinction coefficient of 6170 cm⁻¹ M⁻¹ for the tryptophan residue (25).

Preparation of F108–2-Pyridyl Disulfide Derivative. 2-(2-Pyridyl)dithioethylamine was prepared first for conjugating the pyridyl disulfide group to Pluronic F108 (Figure 2). In this step, 1.13 g of mercaptoethylamine hydrochloride was dissolved in a mixture of 1.2 mL of methanol and 0.8 mL of acetic acid and was then added dropwise to a stirred solution of 6.74 g of 2,2'-dithiopyridine in 30 mL of methanol. The yellow reaction mixture was stirred for 30 min at room temperature and

was then slowly poured into a beaker with 200 mL of stirred ether, where it precipitated. The product that was separated from the ether phase was dissolved in a small volume of methanol and was again precipitated by ether. This procedure was repeated until the crystals appeared white, and the product was then dried under vacuum. A 0.6 g portion of this 2-(2-pyridyldithio)ethylamine hydrochloride was dissolved in 3 mL of methanol, and 300 mg of TEA was added (mole ratio is 1:1). After the addition of TEA, 2-(2-pyridyldithio)ethylamine hydrochloride becomes 2-(2-pyridyldithio)ethylamine and is ready for the next reaction. Subsequently, 1 g of 4-nitrophenyl chloroformate activated F108 (product **I**) was dissolved in 3 mL of methanol and was added to the above stirred solution. The reaction mixture, which immediately turned yellow, was left at room temperature for 15–20 h. The TEA was then neutralized by adding 0.2–0.25 mL of 10 M HCl. Four milliliters of deionized water was subsequently added to this mixture, and the whole was transferred to a dialysis tubing (3500 cutoff) and was dialyzed against 4 L of deionized water. Water was changed five times during the 48 h dialysis process, and the F108–2-pyridyl disulfide (F108–PDS) product (**V**) in Figure 2 was finally recovered by lyophilization. The derivative was stored in a desiccator over P_2O_5 at room temperature. The determination of its content of 2-pyridyl disulfide groups was essentially as described by Carlsson et al. (26). Exactly weighed F108–PDS was dissolved in phosphate–NaCl buffer (0.05 M phosphate, 0.15 M NaCl, pH 7.3). The UV absorbance at a wavelength of 343 nm was measured before as well as 10 min after the addition of 0.1 mL of 25 mM DTT to both the reference cuvette, containing pure phosphate–NaCl buffer, and the sample cuvette containing F108–PDS. The concentration of released 2-thiopyridone, which is identical to the original concentration of F108–PDS, was calculated using a molar extinction coefficient of $8060 \text{ cm}^{-1} \text{ M}^{-1}$.

Pluronic Adsorption to Polystyrene Latex Particles. A suspension containing 2.5% (w/v) of PS particles ($0.272 \mu\text{m}$ in diameter) was incubated with 4% Pluronic (^{125}I -labeled and plain surfactants were mixed with a ratio of 1:50) in PBS for 24 h with end-over-end shaking at room temperature. The free surfactants were then removed after coated PS spheres were pelleted in a Fisher microcentrifuge, Model 235A. Particles were resuspended in PBS and were again centrifuged. These washing cycles were repeated until the supernatant was free from measurable radioactivity. The radioactivity of the particles was then measured, and the surface density was therefore calculated on the basis of the known amount of particles present in the examination. For the sake of comparison, the surface density was also determined by a nonlabeling method, namely sedimentation field-flow fractionation (sedFFF). The instrumentation as well as the operation and use of sedFFF in characterizing colloidal adsorption complexes have been described in detail elsewhere (27, 28). Briefly, sedFFF is a colloid separation technique that causes selective retention of colloidal particles based on difference in buoyant mass. In the case of Pluronic adsorption, the increases in particle retention associated with uptake of surfactants can be directly related to the mass adsorbed per particle. From this information one can easily obtain the surface density provided the size of the spherical particles had been well characterized in a previous run.

F108–2-Pyridyl Disulfide Coating of Porous Polystyrene Particles. Twenty milligram of dry porous PS particles with a size of $10 \mu\text{m}$ were incubated with 1.0 mL of ethanol. The suspension was mixed by end-over-

end rotation for 2 h at 25°C . After being settled by centrifugation, the porous PS particles were separated from the ethanol and incubated in 1 mL of 0.2 M phosphate–NaCl buffer. The PS particles were again centrifuged, the buffer was removed, and the particles were resuspended in 1 mL of phosphate–NaCl buffer. After five consecutive washings, the PS particles were incubated in 2.0 mL of 4% F108–PDS solution, which was prepared in 0.2 M phosphate–NaCl buffer, and the mixture was rotated end-over-end for 20 h at 25°C . The coated beads were then washed eight times using phosphate–NaCl buffer as described above, and the amount of coated modified Pluronic F108 was determined by measuring the disulfide reactive groups. The coated PS particles were first incubated in 25 mM DTT for thiolation. After 10 min of incubation, the coated particles were pelleted by centrifugation and the supernatant containing the released 2-thiopyridone was carefully transferred to a new centrifuge tube and was subjected to one more centrifugation to remove possible remaining particles. The 2-thiopyridone content was then determined photometrically as described above. After the weight of the PS particles that were trapped on a preweighed Millipore filter and dried extensively over P_2O_5 was determined, the content of reactive disulfide groups per dry weight of PS porous particles was finally calculated.

Immobilization of β -Galactosidase on Porous Polystyrene Coated with F108–2-Pyridyl Disulfide.

Eleven milligrams of β -galactosidase was dissolved in 1.0 mL 0.2 M phosphate–NaCl solution containing 30 mM reduced glutathione. After 30 min of reduction, the solution was passed through a PD-10 column equilibrated with phosphate–NaCl buffer, and 2.0 mL of the reduced β -galactosidase containing solution was collected, of which 1.0 mL was mixed with a 0.5 mL suspension containing 10 mg of F108–PDS-coated porous particles and the other 1.0 mL was reserved for determination of its thiol content. To measure the amount of thiol groups present in this sample, 0.2 mL of a saturated solution of 2,2'-dithiopyridine in water (1.5 mM) was added to both the reference cuvette with 1 mL of 0.2 M phosphate–NaCl buffer and the sample cuvette with the above reduced β -galactosidase. After 10 min of incubation, the thiol concentration was calculated by measuring the concentration of released 2-thiopyridone as described above. The mixture of coated particles and β -galactosidase was rotated end-over-end for 48 h at 4°C , and the particles were then washed with the 0.2 M phosphate–NaCl buffer as described previously. The amount of immobilized β -galactosidase was determined by amino acid analysis. The amino acid analysis based on the phenyl isothiocyanate derivatization method has been detailed elsewhere (5).

RESULTS AND DISCUSSION

To develop a general procedure for activation of a Pluronic surfactant to enable its conjugation with other functional groups of interest, we selected Pluronic P105, F68, F88, and F108 in this investigation. The conjugation of *p*-nitrophenyl groups to the PEO ends of these surfactants was found to provide an efficient coupling chemistry and was independent of molecular weight. The coupling yield was readily determined from the release of yellow *p*-nitrophenolate ions in alkaline solution. In this calculation, the degree of substitution was based on the assumption of two available $-\text{OH}$ groups in each Pluronic molecule and was generally found to be around 80% (Table 1). The degree of substitution of each Pluronic type reported in this table is the average value

Table 1. Chemical Modification of Pluronic Surfactants

	degree of substitution (%)	
	4-nitrophenyl chloroformate	Bolton–Hunter reagent
Pluronic P105	82	64
Pluronic F68	78	84
Pluronic F88	85	93
Pluronic F108	83	93

of at least three repeated experiments performed under the same conditions ($-\text{OH}:\text{nitrophenyl}$ was kept at 1:3) and was not found to increase significantly when a larger excess of reagents was used. Such activated Pluronic surfactants can be stably stored in a desiccator over P_2O_5 at room temperature and have been found not to lose their activity after months of storage.

Radioisotope ^{125}I Labeling of Pluronic. It is difficult to quantify a Pluronic surfactant in solution or in its adsorption complexes precisely because it does not have any chromophore or other functional groups that can be specifically determined by a common instrument. Although the turbidity measurement of Pluronic is the most common technique for its quantification, this method usually has many limitations and is only applicable to polymers in a dust-free solution (29). Our approach is to modify the *p*-nitrophenyl-activated Pluronic molecules by means of the introduction of phenol groups. Through the iodination of such groups, biomolecules or polymers have been found to be easily labeled with radioactive iodine (30). The Bolton–Hunter reagent, with its succinimidyl and phenol groups, has been found to be a good reagent for coupling to $-\text{NH}_2$ groups in proteins, and such conjugated biological molecules can subsequently be radioiodinated with high specific activity. By this technique, proteins in nanogram quantities can be detected and quantified (21). To provide available $-\text{NH}_2$ groups for attachment of the Bolton–Hunter reagent to Pluronic surfactants, hydrazino groups were introduced by reacting hydrazine with the *p*-nitrophenyl-activated Pluronic (product **I**, Figure 1). This reaction usually went to completion, and measurements of the amount of released *p*-nitrophenol were in close agreement with the amounts of conjugate **I** used for the reaction. In Table 1, a high degree of substitution is also indicated for the reaction of the Bolton–Hunter reagent's hydroxysuccinimide ester with $-\text{NH}_2$ groups of the modified Pluronic (product **II**, Figure 1). The purified phenol-conjugated Pluronic was then ready for labeling with radioactive iodine. Following iodination by means of the chloramine T method, ^{125}I -labeled Pluronic and free Na^{125}I were separated using a disposable PD-10, Sephadex G-25 column. The radioisotope-labeled polymer was collected and passed through the PD-10 column a second time to check for the presence of free Na^{125}I in the solution. By this procedure all free Na^{125}I was proven to be completely removed from the labeled polymer. Although the labeling efficiency cannot be calculated by a general precipitation method such as the trichloroacetic acid method used in protein labeling (3), the efficiency estimated from the second GPC profile was around 95–96%. In the above three-step reaction, Pluronic F68, F88, and F108 were all modified chemically in organic solvents and were successfully recovered by precipitation with ether. The product yield for each step was from 75% to 80% as shown in Table 1. By contrast, the Pluronic P105 analog was not easily separated from unreacted reagents and often had to be recovered by other elaborate schemes. This solubility difference in ether may be the result of differences in chemical composition between P105 and the other selected Pluronic surfactants; P105 contains

Table 2. Coupling of Amino-Containing Molecules to Pluronic F108

reactive groups	reaction milieu	degree of substitution (mol/mol F108)
hydrazine	CH_3OH	1.7
1,3-diaminopropane	aqueous pH 8.2	1.4
2-aminoethanesulfonic acid	aqueous pH 9.4	1.5
Gly-Trp	CH_3OH	1.0
Gly-Trp	$\text{CH}_3\text{OH}/\text{TEA}$	1.1
2-(2-pyridyldithio)ethylamine	$\text{CH}_3\text{OH}/\text{TEA}$	1.0

50% PEO, whereas the others contain up to 80% of PEO. The ^{125}I -labeled surfactants have been used to quantify the amounts adsorbed on PS colloids (see below) and to trace the stability of such coated colloids in various environments (4).

Coupling of Amines. In addition to the isotope labeling described above, the *p*-nitrophenyl conjugated derivative is a useful intermediate for the introduction of various other substituents into Pluronic surfactants. By this route it has for instance been possible to introduce groups that carry a negative or a positive charge at physiological pH, by reacting the surfactant derivative with bifunctional molecules containing a primary amine. Activated Pluronic F108 (product **I** in Figure 1) was thus chosen to show this useful route by reacting with 2-aminoethanesulfonic acid (taurine), 1,3-diaminopropane, and the dipeptide Gly-Trp, respectively (Table 2). The coupling can be performed in both organic and aqueous environments. In water the coupling should be performed at an alkaline pH (between 8 and 10) in which milieu most primary amino functions are at least partially deprotonated. In organic solvents deprotonated amines seem to react well even without a base catalyst. Thus, the same degree of substitution was obtained when the dipeptide Gly-Trp was reacted with *p*-nitrophenyl-activated F108 in methanol with or without the base TEA (Table 2).

A particularly interesting derivative is the reactive disulfide derivative of Pluronic obtained by reacting 4-nitrophenyl chloroformate activated surfactant with 2-(2-pyridyldithio)ethylamine (Figure 2). The 2-pyridyl disulfide group reacts specifically with thiols in a so-called thiol–disulfide exchange reaction leading to the release of 2-thiopyridone (Figure 2) and the formation of a more stable disulfide. This reaction occurs at both acidic and alkaline pH and usually goes to completion even under equimolar concentrations of the reactants. Thiol–disulfide exchange is a specific and reversible reaction in which the hydrolysis rate of the reactive disulfide is negligible at pH <8.5. This reaction has been the base for several useful applications, such as covalent chromatography, reversible protein immobilization, and protein–protein conjugation (31–33). When the disulfide functional groups have been immobilized on a solid phase designed to bind thiol-containing molecules, the bound molecules can subsequently be easily released and removed from the solid surface by the addition of low molecular weight thiol-containing reagents (Figure 2). Here, the 2-pyridyl disulfide was substituted into Pluronic F108 by the reaction of *p*-nitrophenyl-activated F108 and 2-(2-pyridyldithio)ethylamine. The new F108, modified to contain a reactive disulfide (F108–PDS), can be stably stored in solid form at room temperature in a desiccator over P_2O_5 for months. As described by Rydén and Carlsson (34), the 2-pyridyl disulfide group is very stable both in solid form and in aqueous solutions under physiological conditions of pH and temperature. We found that each F108 molecule can only be linked with one disulfide active group even though it contains two

Table 3. Surface Density of Pluronics on Polystyrene Latex Particles (0.272 μm) Determined by ^{125}I Labeling or Sedimentation Field-Flow Fractionation

	surface density ($\text{nm}^2/\text{molecule}$)	
	^{125}I labeling	sedFFF
Pluronic P105	7.4	7.8
Pluronic F68	9.4	10.9
Pluronic F88	8.6	9.9
Pluronic F108	7.9	7.0

active *p*-nitrophenyl groups (Table 1). In Table 2, we summarize the degrees of substitution of 2-pyridyl disulfide as well as different amino-containing molecules to Pluronic F108. The reactions of *p*-nitrophenyl-activated F108 with hydrazine, 1,3-diaminopropane, and 2-aminoethanesulfonic acid are all seen to be reasonably efficient. Yet, as in the attachment of disulfide groups to F108, a low degree of substitution was obtained when the dipeptide glycyltryptophan was conjugated to F108, whether the coupling was performed with or without the base TEA. The yield in the dipeptide reaction was low, even though a large excess of reagents was used in the reactions. This finding indicated that the introduction of the first amine might alter the availability of the second activated PEO end group in the block copolymer.

Pluronic Adsorption to Polystyrene Latex Particles. Polymeric colloids with a large surface area to volume ratio are commonly used as the solid phase in a variety of fields, especially for therapeutic purposes or as medical diagnostic products. In previous studies, we have found that plasma protein uptake on PS colloids can be minimized by using a Pluronic coating and that the blood circulation half-life of such treated colloids can be more than 13 h compared to just a few minutes for the corresponding uncoated particles (4, 5). Quantification of adsorbed surfactants on colloidal particles is one of the important tasks to provide an understanding of their surface properties and their relationship to biocompatibility. By using monodispersed PS latex particles as the adsorbent, we have determined the surface density of several Pluronic surfactants by using both ^{125}I labeling and sedFFF methods as shown in Table 3. In addition, to a good agreement with an independent measurement based on the use of fluorescent probes (5), modification of the triblock copolymers appears not to have altered the characteristics of the surfactants in terms of their adsorption on PS colloids.

Protein Coupling to Pluronic F108-PDS-Coated Surfaces. As mentioned before, there is always a risk of denaturation associated with the use of hydrophobic materials for protein immobilization, although these solid surfaces may provide many conveniences. In addition, a nonspecific protein binding, such as is usually observed on hydrophobic surfaces, may produce misleading results when these solid phases are used in quantitative assays based on specific binding. This leads to the idea of covering the hydrophobic surfaces with biocompatible polymers, while also providing functional groups for the binding of specific biological molecules (35). Our study has shown that by coating a Pluronic surfactant, for example F108, onto hydrophobic materials such as PS colloids, these materials will turn highly hydrophilic and become virtually resistant to protein uptake (4, 5). The reduced protein adsorption is predominantly due to steric repulsion and polymer chain dynamics (36). Here, we extend this approach by examining the attachment of a model protein, namely the naturally thiol-containing β -galactosidase, to Pluronic-coated highly porous PS particles. β -Galactosidase from *E. coli* is one of the most

Table 4. Porous Polystyrene Particles (10 μm) Coated with F108-2-Pyridyl Disulfide and Its Specific Binding of β -Galactosidase

amount of bound F108-2-pyridyl disulfide ($\mu\text{mol/g}$ of particle)	45
thiol content of β -galactosidase (mol/mol of protein)	12
amount of bound β -galactosidase (mg of protein/g of particle)	
on particles coated with F108-2-pyridyl disulfide	34
on particles coated with unmodified F108	<1

extensively investigated enzymes and is a commonly used component in enzyme-linked immunosorbent assays (13).

Monodisperse porous PS particles (10 μm in diameter) provide both an extremely large surface area of approximately 350 m^2/g and the advantage of easy sedimentation for removal of unbound proteins. By measuring released 2-thiopyridone, the amount of F108-PDS adsorbed to these particles, and the resulting thiol content, can be determined as listed in Table 4. Compared to the F108-PDS coating on other particles with similar hydrophobicity (37), the surface concentration found for these particles is very similar, and the coating procedure can therefore be considered useful also for other hydrophobic materials. It was not surprising to find an extremely small amount of β -galactosidase adsorbed on the porous PS particles when coated with the unmodified F108 (see Table 4). By contrast, results in Table 4 demonstrate that the protein binding increased significantly when the same PS particles were coated by F108-PDS. From this it is clear that β -galactosidase can be specifically immobilized on the particles through the thiol-disulfide exchange between F108-PDS and proteins.

Potentially, one of the most interesting applications of this F108-PDS coating is its use in enzyme-linked immunosorbent assay (ELISA), where it may enhance the immunoaffinity of immobilized antibody by site-specific chemical coupling. Although many factors are important in the preparation procedure, the sensitivity and the specificity of an assay are usually enhanced by a robust solid phase capture surface. Because of partial inactivation of the antibody or the shielding of its antigen binding sites as a result of the surface attachment, antibody immobilization through passive adsorption or random chemical coupling always leads to a reduced capacity to bind antigen. To overcome these drawbacks, an orientation control of the immobilization has been studied by many authors, exemplified by the improved specific binding through immobilization of Fab' fragments (38) and the alteration of the chemical nature of the solid phase to promote a directional antibody immobilization (39). In recent work, we have also demonstrated the potential of the present approach by immobilizing thiolated anti-IgE to F108-PDS-coated PS microtiter plates (33). Preliminarily, our data indicate that antigen binding by the site-specifically immobilized antibody is 8 times higher than that of passively coated antibody. Another potential approach is to utilize the free thiol of the antigen binding Fab' fragment for direct attachment at the surface. Although other ligands of interest may not contain thiol groups, a number of techniques, e.g. modification through a nonessential amino group, have been derived to thiolate proteins and peptides without changing their activity or specificity (32). With respect to preparation of the substrate, we have demonstrated that the content of adsorbed F108-PDS on the PS surface can be controlled by incubating the solid phase with mixtures of modified and unmodified Pluronic F108 in different stoichiometric ratios (34). This approach can be used to create a suitable spacing between the im-

mobilized biological molecules to give an optimal microenvironment for their biological function. It is generally very difficult to create such an adjustable distribution of the reactive molecules by direct chemical modification of hydrophobic materials. In summary, surface coating with Pluronic containing these reactive disulfide groups is a general, yet specific, immobilization technique that may prove to be a promising approach in developing stationary phases for affinity chromatography, cell separation, DNA binding, heterogeneous testing assay, immunonephelometry, etc.

CONCLUSION

By employing a reaction of the hydroxyl ends of Pluronic surfactants with 4-nitrophenyl chloroformate, we can efficiently introduce reactive carbonate groups to such PEO/PPO/PEO triblock copolymers. These activated Pluronic surfactants are stable in an organic milieu and in a dry state but react relatively easily with primary amines. Hydrazine, 2-pyridyl disulfides, peptides, and other amino-containing chemicals were thus conjugated to the activated Pluronic. Using hydrazino groups as the bridge, tyrosyl groups for radioisotope labeling purpose were subsequently coupled to the Pluronic by a reaction with the Bolton–Hunter reagent.

On the basis of the specific thiol–disulfide reaction and the well-known surfactant properties of Pluronic, the coating of hydrophobic surfaces with 2-pyridyl disulfide conjugated Pluronic provides a new technique to render hydrophobic surfaces much more protein compatible while at the same time allowing the immobilization of desired molecules with a controlled spacing. As mentioned above, the rate of hydrolysis of the 2-pyridyl disulfide groups at physiological pH is almost negligible in comparison with the rate of the thiol–disulfide exchange reaction, and therefore such surface coatings can be sensitively activated at very low concentrations of thiol-containing biomolecules such as proteins. Since the thiol–disulfide exchange is a reversible reaction, the bound molecules can subsequently be released from the solid phase by adding thiol-containing reagents of low molecular weight. Thiol groups can be substituted into biomolecules without inactivation by a number of general methods, and the solid phase can be a hydrophobic surface in the form of particles (porous or nonporous) or plates. This surface coating technique is therefore very general in nature and has potential for use in diversified fields; it is a particularly attractive alternative when covalent immobilization techniques are complicated to carry out because of a need for prior surface activation.

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LITERATURE CITED

- (1) BASF (1990) *Pluronic & Tetronic Surfactants*, BASF Co., Mount Olive, NJ.
- (2) Amiji, M., and Park, K. (1992) Prevention of protein adsorption and platelet adhesion on surfaces by PEO/PPO/PEO triblock copolymers. *Biomaterials* 13, 682–692.
- (3) Lee, J. H., Kopecek, J., and Andrade, J. D. (1989) Protein-resistant surfaces prepared by PEO-containing block copolymer surfactants. *J. Biomed. Mater. Res.* 23, 351–368.
- (4) Li, J.-T., Caldwell, K. D., and Tan, J. S. (1991) Size analysis of a block copolymer-coated polystyrene latex. *Particle Size Distribution II: Assessment and Characterization* (T. Provder, Ed.) pp 247–262, American Chemical Society, Washington, DC.
- (5) Tan, J. S., Butterfield, D. E., Voycheck, C. L., Caldwell, K. D., and Li, J.-T. (1993) Surface modification of nanoparticles by PEO/PPO block copolymers to minimize interactions with blood components and prolong blood circulation in rats. *Biomaterials* 14, 823–833.
- (6) Gombotz, W. R., Guanghui, W., Horbett, T. A., and Hoffman, A. S. (1992) Protein adsorption to and elution from polyether surfaces. In *Poly(Ethylene Glycol) Chemistry: Biotechnical and Biomedical Applications* (J. Milton Harris, Ed.) pp 247–261, Plenum Press, New York.
- (7) Bridgett, M. J., Davis, M. C., and Denyer, S. P. (1992) Control of staphylococcal adhesion to polystyrene surface by polymer surface modification with surfactants. *Biomaterials* 13, 411–416.
- (8) Watrous-Peltier, N., Uhl, J., Steel, V., Brophy, L., and Merisko-Liversidge, E. (1992) Direct suppression of phagocytosis by amphipathic polymeric surfactants. *Pharm. Res.* 9, 1177–1183.
- (9) Illum, L., Jacobsen, L. O., Müller, E. M., and Davis, S. S. (1987) Surface characteristics and the interaction of colloidal particles with mouse peritoneal macrophages. *Biomaterials* 8, 113–117.
- (10) Davis, S. S., and Illum, L. (1988) Polymeric microspheres as drug carriers. *Biomaterials* 9, 111–117.
- (11) Cappel, M. J., and Kreuter, J. (1991) Effect of nonionic surfactants on transdermal drug delivery: II. Poloxamer and poloxamine surfactant. *Int. J. Pharm.* 69, 155–168.
- (12) Müller, R. H. (1991) *Colloidal Carriers for Controlled Drug Delivery and Targeting*, CRC Press, Boca Raton, FL.
- (13) Villa, A. E., Guzman, L. A., Poptic, E. J., Labhasetwar, V., D'Souza, S., Farrell, C. L., Plow, E. F., Levy, R. J., DiCorleto, P. E., and Topol, E. J. (1995) Effects of antisense c-myc oligonucleotides on vascular smooth muscle cell. *Circ. Res.* 76 (4), 505–513.
- (14) Schaer, G. L., Hursey, T. L., Abrahams, S. L., Buddemeier, K., Ennis, B., Rodriguez, E. R., Hubbell, J. P., Moy, J., and Parrillo, J. E. (1994) Reduction in reperfusion-induced myocardial necrosis in dogs by RheothRx injection (poloxamer 188 N.F.), a hemorheological agent that alters neutrophil function. *Circulation* 90 (6), 2964–2975.
- (15) Tijssen, P. (1985) *Laboratory Techniques in Biochemistry and Molecular Biology: Practice and Theory of Enzyme Immunoassays*, Elsevier, New York.
- (16) Brillhart, K. L., and Ngo, T. T. (1991) Use of microwell plates carrying hydrazide groups to enhance antibody immobilization in enzyme immunoassays. *J. Immunol. Methods* 144, 19–25.
- (17) Sandwick, R. K., and Schray, K. J. (1987) Inactivation of enzymes upon interaction with a hydrophobic latex surface. *J. Colloid Interface Sci.* 115, 130–138.
- (18) Zalipsky, S. (1995) Functionalized poly(ethylene glycol) for preparation of biological relevant conjugates. *Bioconjugate Chem.* 6, 150–165.
- (19) Woghiren, C., Sharma, B., and Stein, S. (1993) Protected thiol-polyethylene glycol: a new activated polymer for reversible protein modification. *Bioconjugate Chem.* 4, 314–318.
- (20) Harris, J. M., Sedaghat-Herati, M. R., Sather, P. J., Brooks, D. E., and Fyles, T. M. (1992) Synthesis of new poly(ethylene glycol) derivatives. *Poly(Ethylene Glycol) Chemistry: Biotechnical and Biomedical Applications* (J. M. Harris, Ed.) pp 371–381, Plenum Press, New York.
- (21) Lääne, A., Haga, M., Aaviksaar, A., Chytry, V., and Kopecek, J. (1983) Activation of poly[N-(2-hydroxypropyl)-methacrylamide] for the binding of bioactive molecules: 1. Activation with 4-nitrophenyl chloroformate. *Makromol. Chem.* 184, 1339–1344.
- (22) Miron, T., and Wilchek, M. (1987) Immobilization of proteins and ligand using chlorocarbonates. *Methods Enzymol.* 135, 84–90.
- (23) Bolton, A. E., and Hunter, W. M. (1973) The labelling of proteins to high specific radioactivities by conjugation to a ¹²⁵I-containing acylating agent. *Biochem. J.* 133, 529–539.
- (24) Raja, R. H., LeBoeuf, R. D., Stone, G. W., and Weigel, P. H. (1984) Preparation of alkylamine and ¹²⁵I-radiolabelled derivatives of hyaluronic acid uniquely modified at the reducing end. *Anal. Biochem.* 139, 168–177.

- (25) Sober, H. A. (1968) *Handbook of Biochemistry Selected Data for Molecular Biology*, The Chemical Rubber Co., Cleveland, OH.
- (26) Carlsson, J., Drevin, H., and Axén, R. (1978) Protein thiolation and reversible protein-protein conjugation, *N*-succinimidyl 3-(2-pyridyldithio)propionate: a new heterobifunctional reagent. *Biochem. J.* 173, 723–737.
- (27) Li, J.-T., and Caldwell, K. D. (1991) Sedimentation field-flow fractionation in the determination of surface concentration of adsorbed materials. *Langmuir* 7, 2034–2039.
- (28) Li, J.-T., Caldwell, K. D., and Rapoport, N. (1994) Surface properties of Pluronic-coated polymeric colloids. *Langmuir* 10, 4475–4482.
- (29) Attia, Y. A., and Rubio, J. (1975) Determination of very low concentrations of polyacrylamide and polyethyleneoxide flocculants by nephelometry. *Br. Polym. J.* 7, 135–138.
- (30) Regoeczi, E. (1984) *Iodine-Labelled Plasma Proteins*, Vol. 1, CRC, Boca Raton, FL.
- (31) Brocklehurst, K., Carlsson, J., Kierstan, M. P. J., and Crook, E. M. (1973) Covalent chromatography: preparation of fully active papain from dried papaya latex. *Biochem. J.* 133, 573–584.
- (32) Jönsson, U., Malmqvist, M., Olofsson, G., and Rönnerberg, I. (1988) Surface immobilization techniques in combination with ellipsometry. *Methods Enzymol.* 137, 381–388.
- (33) Janson, J.-C., and Rydén, L. (1989) *Protein Purification: Principles, High Resolution Methods, and Applications*, VCH, New York.
- (34) Rydén, L., and Carlsson, J. (1989) Covalent chromatography. *Protein Purification: Principles, High Resolution Methods, and Applications* (J.-C. Janson and L. Rydén, Eds.) pp 252–274, VCH, New York.
- (35) Caldwell, K. D., Carlsson, J., and Li, J.-T. (1993) Product for coating of hydrophobic surfaces to render them protein-resistant while permitting covalent attachment of specific ligands. U.S. Pat. Approved, International Publication No. WO 95/06251.
- (36) Li, J.-T., Carlsson, J., Huang, S. C., and Caldwell, K. D. (1996) Adsorption of poly(ethylene oxide)-containing block copolymers: a route to protein resistance. In *Hydrophilic Polymers; Performance with Environmental Acceptability* (J. E. Glass, Ed.) Advances in Chemistry Series 248, pp 61–78, American Chemical Society, Washington, DC.
- (37) Li, J.-T. (1993) Plasma Interactions with Copolymer Stabilized Colloids, Doctoral Dissertation, University of Utah, Salt Lake City.
- (38) Prisyazhnoy, V. S., Fusek, M., and Alakhov, Y. B. (1988) Synthesis of high-capacity immunoaffinity sorbents with oriented immobilized immunoglobulins or their Fab' fragments for isolation of proteins. *J. Chromatogr.* 424, 243–253.
- (39) Lin, J.-N., Chang, I. N., Andrade, J. D., Herron, J. N., and Christensen, D. A. (1991) Comparison of site-specific coupling chemistry for antibody immobilization on different solid supports. *J. Chromatogr.* 542, 41–54.

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Interactions of Cytosine Derivatives with T·A Interruptions in Pyrimidine·Purine·Pyrimidine DNA Triplexes

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The ability of triplex-forming oligopyrimidines to interact with duplex targets which contain a single **pyr·pur** interruption in their homopurine triplex binding site was studied. These oligonucleotides contain either *N*⁴-(3-carboxypropyl)deoxycytidine (**3**) or *N*⁴-(5-carboxytriazolyl)deoxycytidine (**4**) to target the **pyr·pur** interruption. The 3-carboxypropyl and the 5-carboxytriazolyl groups of these cytosine derivatives are designed to span the major groove of the duplex target. Molecular models suggest that the carboxyl group of **3** or **4** can serve as a hydrogen bond acceptor for the *N*⁶-amino hydrogen of **A** in a T·A base pair. Additional contacts are possible between the *N*⁴-amino hydrogen of **3** or **4** and the *O*⁴-carbonyl oxygen of T. UV melting experiments showed that a 15-mer, **I(3)**, containing nucleoside **3** formed a stable triplex with duplex targets containing a single T·A interruption. The melting temperature of this triplex was 7 °C higher than that of a similar triplex containing a single G·T·A triad when a Tris buffer was employed. Oligomer **I(3)** also formed a triplex of lower stability with a target containing a G·C base pair but not with targets containing C·G, U·A, or A·T base pairs. A similar 15-mer, **II**, containing nucleoside **4** was found to be less selective than **I(3)** in its interaction with duplex targets. Thus, gel mobility shift experiments showed that in addition to interacting with T·A, oligomer **II** also formed triplexes with duplex targets containing U·A and C·G interruptions. These studies suggest that nucleoside derivatives which can potentially contact both bases of a **pyr·pur** interruption might provide a means to extend the range of sequences which can be recognized by triplex-forming oligonucleotides.

INTRODUCTION

Pyrimidine-rich oligodeoxyribonucleotides can interact with a purine-rich sequence of a DNA duplex to form triple helical complexes through the formation of stable C⁺·G·C and T·A·T base triads (1–5). However, the ability of natural bases to form stable triads in conventional triplex motifs is greatly reduced by the occurrence of pyrimidine interruptions in an otherwise homopurine strand of a target duplex (4, 5). Several strategies employing natural and non-natural nucleosides have been applied to overcome the recognition barrier posed by these interruptions. In the case of the **pyr·pur·pyr** triplex motif, structural studies show that G can recognize a T·A base pair (6–9) and T can recognize a C·G base pair through the formation of a single hydrogen bond with the pyrimidine of the base pair (10). Another approach utilizes synthetic non-natural nucleoside analogs whose structures are designed to position hydrogen bond donor and/or acceptor groups to interact with such inversions. For example, interaction of 1-(2-deoxy-β-D-ribofuranosyl)-4-(3-benzamidophenyl)imidazole with duplexes containing **pyr·pur** interruption has been described (11). It appears that this analog interacts with T·A and C·G base pairs as a result of intercalation (12).

We have previously reported triplex formation between oligodeoxyribonucleotides that contain the derivatized nucleosides *N*⁴-(3-acetamidopropyl)deoxycytidine (**1**) and *N*⁴-(6-amino-2-pyridinyl)deoxycytidine (**2**) shown in Figure 1 and target duplexes that contain a single C·G base pair interruption (13, 14). Studies of molecular models suggested that the acetamidopropyl or aminopyridinyl group could extend across the major groove of the duplex.

As a result, the amide or amino hydrogen could provide a hydrogen bond contact with the guanine O-6 of the C·G base pair. Although both analogs support triplex formation, the *N*⁴-(3-acetamidopropyl)deoxycytidine substitution displayed better binding specificity for the C·G base pair than did *N*⁴-(6-amino-2-pyridinyl)deoxycytidine.

These studies and examination of molecular models suggested that a similar strategy could be employed in which a hydrogen bond acceptor group could be used to contact the *N*⁶-amino group of a T·A interruption. To test this hypothesis, we have synthesized oligopyrimidines containing *N*⁴-(3-carboxypropyl)deoxycytidine (**3**) or *N*⁴-(5-carboxytriazolyl)deoxycytidine (**4**) and have studied their ability to interact with duplex targets. Molecular models suggest that the carboxylate group of **2** or **3** could be suitably positioned as an acceptor for the hydrogen of the *N*⁶-amino group of adenine in a T·A base pair.

EXPERIMENTAL PROCEDURES

Protected deoxyribonucleoside phosphoramidites and controlled pore glass supports were purchased from Glen Research. All chemicals used were of reagent grade or better. γ-[³²P]ATP was purchased from Amersham Inc., and T4 polynucleotide kinase was purchased from United States Biochemical Corp. Polyacrylamide gel electrophoresis was carried out on 20 × 20 × 0.75 cm gels containing 20% acrylamide and 7 M urea (denaturing conditions) or 15% acrylamide (nondenaturing conditions). The running buffers were either TBE, which contained 89 mM Tris, 89 mM boric acid, and 0.2 mM ethylenediaminetetraacetate buffered at pH 8, or MES, which contained 90 mM 4-morpholineethanesulfonic acid monohydrate (MES monohydrate) and 10 mM magnesium chloride buffered at pH 6.2. The gel loading buffer contained 90% formamide, 0.05% xylene cyanol, and 0.05% bromophenol blue. Reversed phase HPLC was carried out on Microsorb C₁₈ columns purchased from

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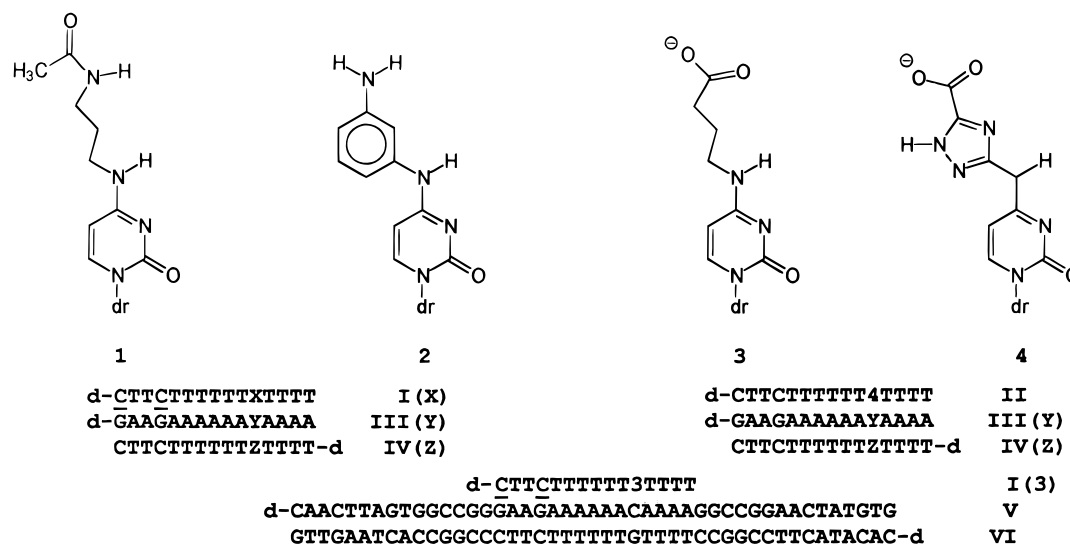


Figure 1. Structures of derivatized deoxycytidines and oligonucleotides. C is 5-methyldeoxycytidine, X is nucleoside 1, 2, or 3, and Y·Z is C·G, G·C, T·A, U·A, or A·T.

Rainin Instruments using a linear gradient of acetonitrile in 50 mM sodium phosphate buffered at pH 5.8.

Synthesis of *N*⁴-(5-Carbomethoxytriazolyl)-3',5'-*O*-(1,1,3,3-tetraisopropyl-1,3-disiloxanediyl)deoxycytidine (7a). 2'-Deoxyuridine (0.98 g, 4.27 mmol) was dried by coevaporating it with anhydrous pyridine (2 × 10 mL). To the dry nucleoside was added anhydrous pyridine (10 mL), and the solution was cooled on ice. A solution of 1,3-dichloro-1,1,3,3-tetraisopropylidisiloxane (1.4 g, 4.46 mmol) in anhydrous dichloromethane was added (10 mL) over a 15 min period with continuous stirring under a nitrogen atmosphere. The reaction was stirred at room temperature for 3 h, at which time analytical TLC showed complete disappearance of the starting material (chloroform/ethanol 9/1). The reaction was quenched with methanol (1 mL) and was evaporated using an oil pump to remove excess pyridine. The resulting glassy solid was dissolved in dichloromethane (25 mL) and washed with 1 M NaHCO₃ solution (2 × 15 mL). The organic layer was separated and dried over anhydrous MgSO₄ and filtered. The solvents were evaporated to give 3',5'-*O*-(1,1,3,3-tetraisopropyl-1,3-disiloxanediyl)deoxycytidine as a white foam in 95% yield.

The TIPS-protected 2'-deoxyuridine (8 g, 17 mmol) was dried by coevaporating twice with anhydrous pyridine and was dissolved in anhydrous dichloromethane (80 mL). Triethylamine (30 mL) was added followed by mesitylenesulfonyl chloride (10 g, 46 mmol) and 4-(dimethylamino)pyridine (0.52 g, 4 mmol). The reaction was stirred for 2 h under nitrogen at room temperature, at which time analytical TLC (ethyl acetate/hexane 8/2) indicated that the reaction was complete. Excess solvents were evaporated, and the resulting solid was coevaporated with anhydrous pyridine (3 × 25 mL) followed by drying under vacuum to give nucleoside 6.

Nucleoside 6 (11.0 g, 17 mmol) was dissolved in anhydrous pyridine (80 mL). 3-Amino-1,2,4-triazole-5-carboxylic acid methyl ester (23.4 g, 165 mmol) was added, and the solution was refluxed for 3 h, followed by overnight stirring at 90 °C. Excess pyridine was removed under vacuum, and the resulting brown gummy mass was extracted with dichloromethane (5 × 200 mL). Any remaining insolubles were discarded at this stage, and the organic phase was washed successively with 1 M NaHCO₃ (5 × 200 mL) and water (5 × 100 mL). The combined aqueous washings were extracted with dichloromethane (200 mL). The combined organic extract was

dried over anhydrous Na₂SO₄. After evaporation of solvents, nucleoside 7a was purified by silica gel chromatography using chloroform/methanol (7:3) as eluting solvent (47% yield): ¹H NMR (CDCl₃) δ 7.83 (H-6, d, *J* = 7.77 Hz), 6.11 (H-5, d, *J* = 6.06 Hz), 5.72 (H-1', d, *J* = 7.71 Hz), 4.40 (H-3', m), 4.06 (H-5',5'', m), 3.77 (H-4', -COCH₃, m), 2.54 (H-2', m), 2.35 (H-2'', m), 1.35 (TIPS, m).

Synthesis of *N*⁴-(5-Carbomethoxytriazolyl)-5'-*O*-(dimethoxytrityl)-3'-(2-cyanoethyl-*N,N*-diisopropylamino)deoxycytidine Phosphoramidite (8b). A solution of nucleoside 7a (2 g, 3.37 mmol) and tetrabutylammonium fluoride (8 mL) in anhydrous tetrahydrofuran (15 mL) was stirred at room temperature for 20 min under a nitrogen atmosphere. A solution of pyridine/water/methanol (3/1/1, 8 mL) was added, and the reaction mixture was poured into a stirred solution of Dowex 50WX4-200 (200 mL, pyridinium form) and gently swirled for 30 min. The resin was filtered and was washed with pyridine/water/methanol (3/1/1, 5 × 150 mL). The combined washings were evaporated under reduced pressure, and the resulting yellow residue was coevaporated with anhydrous methanol. *N*⁴-(5-Carbomethoxytriazolyl)deoxycytidine was precipitated from its methanolic solution (20 mL) by ether. The resulting yellow powder was dried under vacuum over P₂O₅.

Nucleoside 7b (0.65 g, 1.85 mmol) was coevaporated with anhydrous pyridine (3 × 5 mL), and the residue was redissolved in anhydrous pyridine (15 mL). 4,4'-Dimethoxytrityl chloride (0.75 g, 2.21 mmol) and DMAP (0.025 g, 0.18 mmol) were added, and the reaction was stirred at room temperature under a nitrogen atmosphere for 2 h. Additional 4,4'-dimethoxytrityl chloride (0.035 g, 0.1 mmol) was added, and stirring was continued overnight at room temperature. Methanol (10 mL) was added, and solvents were evaporated under reduced pressure. The resulting gummy mass was dissolved in dichloromethane (45 mL). This solution was washed with 1 M NaHCO₃ (4 × 20 mL) followed by water (2 × 25 mL), and the combined aqueous extracts were extracted with dichloromethane (25 mL). The combined organic extract was dried over anhydrous Na₂SO₄. The solvents were evaporated, and the tritylated nucleoside 7a was precipitated from anhydrous tetrahydrofuran by addition of hexanes. This procedure was repeated three times and gave pure 8a in 83% yield: ¹H NMR (CDCl₃) δ 8.48 (H-6, d, *J* = 7.38 Hz), 7.27 (DMTr, m), 6.85 (DMTr, d, *J* = 8.76 Hz),

6.78 (H-5, d, $J = 7.35$ Hz), 6.25 (H-1', t), 4.54 (H-3', m), 4.17 (H-4', m), 3.99 ($-\text{COCH}_3$, s), 3.80 ($-\text{OCH}_3$, s), 3.51 (H-5', s), 2.71 (H-2', m), 2.33 (H-2'', m).

Nucleoside **8a** (1 g, 1.5 mmol) was dried and then dissolved in anhydrous dichloromethane (10 mL). *N,N*-Diisopropylethylamine (0.5 mL, 3 mmol) was added under a constant stream of nitrogen, and the solution was cooled in an ice bath. 2-Cyanoethyl *N,N*-diisopropylchlorophosphoramidite (0.5 mL, 1.8 mmol) was added, and the reaction mixture was stirred for 10 min at 0 °C and then at room temperature for 1 h. Additional *N,N*-diisopropylethylamine (150 μL) and 2-cyanoethyl *N,N*-diisopropylchlorophosphoramidite (100 μL) were added, and stirring was continued for another 1.5 h. The reaction was quenched by addition of methanol (1 mL), and the solvents were evaporated. A gummy mass was obtained which was extracted by ethyl acetate (20 mL). The organic layer was washed with 1 M NaHCO_3 (3×25 mL) followed by water (3×20 mL), and the combined aqueous extracts were extracted with ethyl acetate (2×25 mL). The combined organic extracts were dried over anhydrous Na_2SO_4 . The phosphoramidite **8b** was purified by silica gel chromatography using a gradient of 0–3% ethanol in dichloromethane containing 0.1% triethylamine. The phosphoramidite was precipitated twice from its ethyl acetate solution by addition of pentanes at -40 °C (47% yield): ^{31}P NMR (CDCl_3 , 85% phosphoric acid in D_2O as external standard) δ 150.07 and 149.80.

Syntheses of Oligodeoxyribonucleotides. Oligodeoxyribonucleotides **I(C)** and **II–VI** (see Figure 1) were synthesized on nucleoside-derivatized controlled pore glass supports using an Applied Biosystems Model 392 DNA/RNA synthesizer. The oligonucleotides were prepared on 1 μmol scales using commercially available protected 5'-*O*-dimethoxytrityldeoxyribonucleoside 3'-*O,N,N*-bis(diisopropylamino)- β -cyanoethyl phosphoramidites following standard procedures. The oligomers were deprotected by overnight treatment of the supports with a solution of 50% ammonium hydroxide in pyridine at 55 °C and were purified by C_{18} reversed phase HPLC using a 0–20% linear gradient of acetonitrile in 50 mM sodium phosphate, pH 5.8. The extinction coefficients of the oligomers were determined after digestion of a known amount of oligomer by snake venom phosphodiesterase as previously described (9).

Synthesis of Oligodeoxyribonucleotide I(3). Oligomer **I(C)** (10 A_{260} units) was treated with 96 μL of a solution containing 1 M sodium bisulfite and 3 M 4-aminobutyric acid, pH 7.0, at 50 °C for 48 h as previously described (15). Transamination was complete as indicated by reversed phase HPLC analysis. The oligomer was purified by C_{18} reversed phase HPLC using a 5–15% linear gradient of acetonitrile in 50 mM sodium phosphate, pH 5.8. The oligomer was desalted on a C_{18} reversed phase cartridge. It was characterized by hydrolysis with a combination of snake venom phosphodiesterase and alkaline phosphatase followed by analysis of the resulting nucleosides by C_{18} reversed phase HPLC. The expected ratio of nucleosides was obtained, and no deamination of C was observed as evidenced by the lack of deoxyuridine in the digest.

Synthesis of Oligodeoxyribonucleotide II. Oligomer **II** was synthesized using 5'-*O*-dimethoxytrityl-protected 3'-*O*- β -cyanoethyl *N,N*-diisopropylphosphoramidites of thymidine, N^4 -isobutryldeoxycytosine, and N^4 -(5-carbomethoxytriazolyl)deoxycytidine. A double coupling cycle (10 min/cycle) was used to introduce the latter nucleoside phosphoramidite. The oligomer was deprotected by first treating the support for 15 min with 1 mL of 2 M aqueous sodium carbonate solution. The support

was then treated with 1 mL of 5 M aqueous ammonium hydroxide solution at room temperature for 15 min. The solvents were evaporated, and the crude oligomer was partially purified by C_{18} reversed phase HPLC using a 2–15% linear gradient of acetonitrile in 50 mM sodium phosphate, pH 5.8. The oligomer was then desalted using a C_{18} Sep-Pak cartridge and phosphorylated using γ - ^{32}P -ATP and T4-polynucleotide kinase. The phosphorylated oligomer migrated as a single band on a 20% polyacrylamide gel. The portion of the gel containing the oligomer was excised, crushed, and extracted by incubation overnight with 0.1 M aqueous sodium chloride solution at 37 °C. The phosphorylated oligomer was desalted on a pre-equilibrated C_{18} Sep-Pak cartridge.

Melting Experiments. Melting experiments were carried out in one of the following buffers: 0.1 M sodium chloride, 20 mM magnesium chloride, and 50 mM 4-morpholinepropanesulfonic acid (MOPS), pH 6.2 or 7.0; or 0.1 M sodium chloride, 20 mM magnesium chloride, and 50 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris), pH 7.0. A solution containing 2.0 μM preformed duplex was mixed with an equal volume of 2.0 μM triplex-forming oligomer at room temperature. The solutions were then incubated overnight at 4 °C. These solutions were loaded into cuvettes at 0 °C, and the absorbance versus temperature profile was recorded using a Cary 3E UV–vis spectrophotometer fitted with a thermostated cell block and temperature controller. The cell block was continuously purged with dry nitrogen to prevent moisture condensation at low temperatures. The solutions were heated at a rate of 0.5 °C/min, and the absorbance at 260 nm was measured.

Gel Mobility Shift Analysis. Gel mobility shift experiments were performed on 15% polyacrylamide gels under nondenaturing conditions using 90 mM 4-morpholineethanesulfonic acid (MES), pH 6.2, and 10 mM magnesium chloride as the running buffer. The concentrations of labeled oligomer and the target duplexes were fixed at 1 and 10 μM , respectively, in a total reaction volume of 5 μL . A 1 μL aliquot of 50% aqueous glycerol solution was added just before the samples were loaded onto the pre-equilibrated gels. The gels were run at a constant voltage of 150 V and at constant temperature. The gels were dried, autoradiographed, and scanned on an Epson scanner using the Scantastic program.

RESULTS

Triplex Formation by an Oligonucleotide Containing N^4 -(3-Carboxypropyl)-2'-deoxycytidine. The interaction of N^4 -(3-carboxypropyl)deoxycytidine (**3**) with **pyr·pur** interruptions was studied using oligonucleotide **I(3)**, the sequence of which is shown in Figure 1. This oligomer was prepared by bisulfite-catalyzed transamination of oligomer **I(C)** in the presence of 4-aminobutyric acid. As previously reported (15), this reaction selectively modifies cytosine but not 5-methylcytosine residues in oligonucleotides. The reaction was carried out at pH 7.2 at 50 °C. Under these conditions essentially no deamination takes place and the conversion of **I(C)** to **I(3)** is almost quantitative.

The interaction of **I(3)** with duplex target **III·IV** (Y·Z) was studied by UV melting experiments. In this target system, the base pairs at position Y·Z were varied to assess the sequence specificity of the triplex-forming oligomer. As shown in Figure 2 oligomer **I(3)** forms a stable triplex with **III·IV** (T·A) in Tris buffer at pH 7.0 as indicated by the presence of two transitions. The first transition corresponds to dissociation of **I(3)** from the triplex **I·III·IV** (3·T·A) and the second to dissociation of

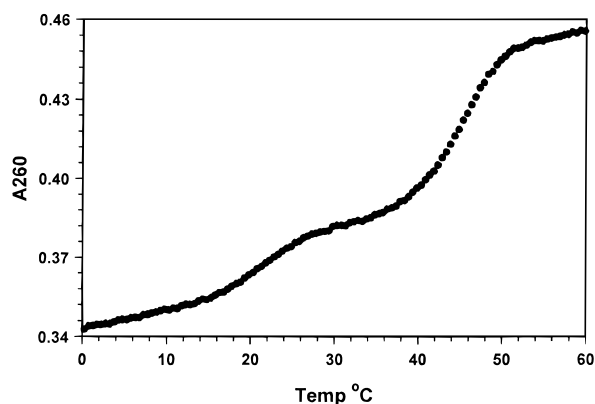


Figure 2. Melting curve of triplex **I-III-IV** (3·T·A). The melt was carried out in a buffer containing 0.1 M sodium chloride, 20 mM magnesium chloride, and 50 mM Tris at pH 7.0. The concentration of each oligomer was 1 μ M.

Table 1. Triplex Melting Temperatures

X	Y	Z	$T_m, ^\circ\text{C}^a$	
			Tris	MOPS
Triplex I·III·IV (X·Y·Z)				
3	T	A	22	<0
3	U	A	<0	<0
3	C	G	<0	<0
3	A	T	<0	<0
3	G	C	18	18
Triplex I·V·VI (X·Y·Z)				
3	T	A	28	nd ^b

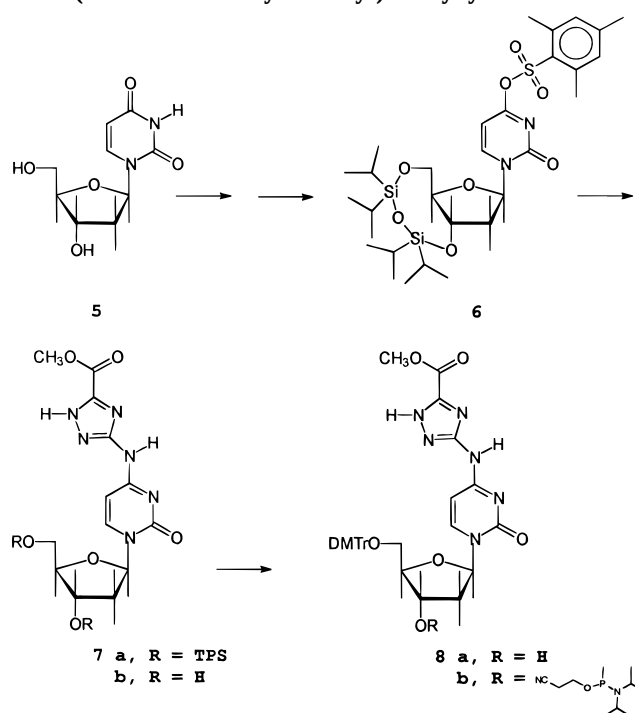
^a Melting temperatures were determined in buffer containing 0.1 M sodium chloride, 20 mM magnesium chloride, and 50 mM Tris, pH 7.0, or 50 mM MOPS, pH 7.0. The concentration of each oligomer strand was 1 μ M. All measurements were carried out in duplicate. ^b Not determined.

the target duplex. The melting temperature of the former transition is 22 °C, whereas the duplex melts at 45 °C.

Triplex formation involving **I(3)** shows selectivity for the T·A interruption. As shown in Table 1, stable triplexes were not observed for the other two possible **pyr·pur** interruptions, C·G or U·A nor was triplex formation observed when Y·Z was A·T. A triplex whose third strand T_m is 18 °C was observed for the interaction of **I(3)** with duplex **III·IV** (G·C). The stability of this triplex may be compared with that of **I·III·IV** (C·G·C), the third strand of which melts at 32 °C (13). Oligomer **I(3)** also forms a stable triplex with the longer duplex target **V·VI** (T·A). In this case the T_m of the third strand dissociation is 28 °C.

In contrast to its behavior in the Tris buffer system, oligomer **I(3)** formed a stable triplex only with duplex target **III-IV** (G•C) in MOPS buffer. The T_m of this triplex is 18 °C, which is identical to the T_m observed in Tris buffer.

Triplex Formation by an Oligonucleotide Containing *N*⁴-(5-Carboxytriazolyl)-2'-deoxycytidine. The interaction of *N*⁴-(5-carboxytriazolyl)deoxycytidine (**4**) with **pyr·pur** interruptions was studied using oligomer **II** and duplex target **III·IV** (Y·Z). The protected phosphoramidite derivative, **8b**, of nucleoside **4** was prepared as shown in Scheme 1. The 3'- and 5'-hydroxyl groups of deoxyuridine were protected with 1,3-dichloro-1,1,3,3-tetraisopropylidisiloxane, and the resulting protected nucleoside was converted to the *O*⁴-mesitylenesulfonate derivative, **6**, by reaction with mesitylenesulfonyl chloride in the presence of triethylamine. Reaction of **6** with the 3-amino-1,2,4-triazole-5-carboxylic acid methyl ester gave



protected nucleoside **7a**. Removal of the tetraisopropylsiloxane group by reaction with tetra-*n*-butylammonium fluoride produced nucleoside **7b**. Nucleoside **8a** was obtained by selective reaction of the 5'-hydroxyl group of **7b** with dimethoxytrityl chloride. This nucleoside was then converted to phosphoramidite **8b** by standard procedures.

Preliminary experiments with nucleoside **7b** showed that it was unusually sensitive to strong basic conditions. Thus, treatment of **7b** with concentrated ammonium hydroxide at 50 °C for 3 h, which is the standard condition for deprotection of oligonucleotides, resulted in nearly quantitative conversion of **7b** to deoxycytidine along with minor amounts of 2'-deoxyuridine. Even very brief treatment with concentrated ammonium hydroxide at room temperature resulted in production of significant amounts of deoxycytidine in addition to the desired product, *N*⁴-(5-carboxytriazolyl)deoxycytidine.

After experimenting with several conditions, we found that **7b** could be smoothly converted to **4** by reaction with 2 M aqueous sodium carbonate at room temperature. We further found that once the methoxy group had been removed, the resulting *N*⁴-(5-carboxytriazolyl)deoxycytidine was less susceptible to conversion to deoxycytidine when exposed to 5 M ammonium hydroxide at room temperature.

On the basis of these observations, oligonucleotide **II** was synthesized using phosphoramidite **8b** and phosphoramidite of 5'-dimethoxytrityl-*N*⁴-isobutyryldeoxycytidine. The latter phosphoramidite was chosen because the isobutyryl protecting group does not require strong basic conditions for removal and thus was compatible with the mild conditions required to deprotect **7a**. Thus, the oligomer was successfully deprotected by first treating the controlled pore glass support with 2 M aqueous sodium bicarbonate for 15 min at room temperature. This resulted in hydrolysis of the methyl ester of **7b** but did not cleave the oligomer from the support. Further deprotection and cleavage from the support was then achieved by treatment with 5 M ammonium hydroxide for 15 min at room temperature. The resulting oligomer was then partially purified by C₁₈ reversed phase HPLC.

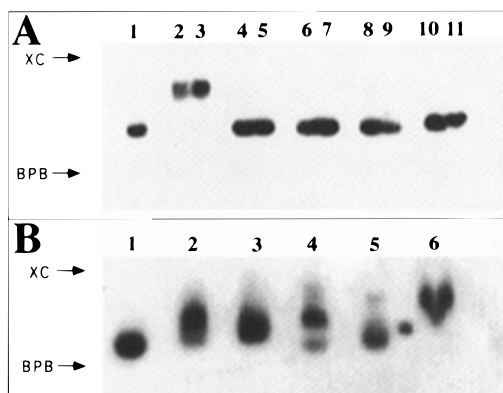


Figure 3. Gel mobility shift detection of triplex formation. Experiments were carried out on 15% polyacrylamide gels run under nondenaturing conditions in buffer containing 90 mM MES, pH 6.2, and 10 mM magnesium chloride. (A) [^{32} P]-**I**(C) alone (lane 1) or run in the presence of 1 or 5 μ M **III·IV** (G·C) (lanes 2 and 3), 1 or 5 μ M **III·IV** (U·A) (lanes 4 and 5), 1 or 5 μ M **III·IV** (T·A) (lanes 6 and 7), 1 or 5 μ M **III·IV** (C·G) (lanes 8 and 9), or 1 or 5 μ M **III·IV** (A·T) (lanes 10 and 11) at 15 $^{\circ}$ C. (B) [^{32} P]-**II** alone (lane 1) or run in the presence of 10 μ M **III·IV** (U·A) (lane 2), 10 μ M **III·IV** (T·A) (lane 3), 10 μ M **III·IV** (G·C) (lane 4), 10 μ M **III·IV** (A·T) (lane 5), or 10 μ M **III·IV** (C·G) (lane 6) at 4 $^{\circ}$ C.

Finally, oligomer **II** was phosphorylated using polynucleotide kinase and γ -[^{32} P]ATP, and the phosphorylated oligomer was purified by polyacrylamide gel electrophoresis. The electrophoretic mobility of **II** was slightly less than that of 5'-phosphorylated oligomer **I**(C), and none of this C-containing oligomer appeared on the gel, suggesting that quantitative conversion of the N^4 -(5-carboxymethoxytriazolyl)deoxycytidine to N^4 -(5-carboxytriazolyl)deoxycytidine had been successfully carried out at the oligomer level.

Because only small quantities of oligomer **II** were available, the interaction of **II** with its duplex DNA target was studied by polyacrylamide gel mobility shift experiments. These experiments were carried out using 1 μ M [^{32} P]-labeled **II** and 10 μ M unlabeled duplex **III·IV** (Y·Z). Because oligomer **II** contains two deoxycytidines, electrophoresis was carried out in MES buffer at pH 6.2 to ensure adequate stability of the triplexes. To verify that the gel electrophoresis could detect sequence-specific triplex formation under these conditions, control experiments involving interaction of **I**(C) with duplex **III·IV** (Y·Z) were first carried out. Oligomer 5'- ^{32} P-labeled **I**(C) at a concentration of 1 μ M was mixed with either 1 or 5 μ M **III·IV** (Y·Z) in 90 mM MES buffer containing 10 mM magnesium chloride. After overnight incubation at room temperature, the solutions were electrophoresed on a 15% polyacrylamide gel at 15 $^{\circ}$ C. As shown in Figure 3a, **I**(C) only interacted with duplex **III·IV** (G·C) as evidenced by the reduced mobility of **I**(C). These results demonstrate that this procedure can detect sequence-specific triplex formation by **I**(C).

Similar experiments were carried out with 5'- ^{32} P-labeled oligomer **II** at 4 and 20 $^{\circ}$ C. In addition to interacting with the duplex target containing a G·C base pair, oligomer **II** also formed triplexes with targets containing T·A, U·A, and C·G base pairs as shown in Figure 3b. Triplex formation was not observed for the target containing an A·T base pair. Similar results were obtained at 20 $^{\circ}$ C, although the extent of triplex formation was significantly reduced (data not shown).

DISCUSSION

Oligomer **I**(3) forms stable triplexes with duplex targets that contain a single T·A interruption in Tris

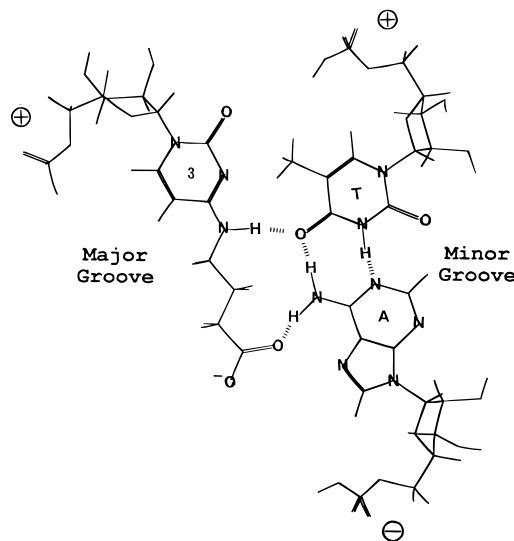


Figure 4. Potential hydrogen bonding scheme for the triad formed by 3·T·A.

buffer at pH 7.0. Triplex formation is also observed when the target base pair is G·C, although the T_m for dissociation of the third strand is 4 $^{\circ}$ C less than that for the triplex formed with T·A and 14 $^{\circ}$ C lower than that of the triplex formed by **I·III·IV** (C·G·C). Because nucleoside **3** is an alkylated derivative of C, the N-3 protonated form of nucleoside **3** could be expected to interact with the G·C base pair in a manner analogous to that of C by formation of Hoogsteen hydrogen bonds with the N-7 and O ^{6} -carbonyl groups of G. The reduced stability of the 3·G·C triad may reflect an increase in the apparent pK_a of the base relative to that of C as a result of alkylation or steric effects caused by the alkyl side chain on formation of a hydrogen bond between the N^4 -amino hydrogen of **3** and the O ^{6} -carbonyl of G.

The N^4 -(3-carboxypropyl)deoxycytidine in oligomer **I**(3) could interact with the target T·A base pair as shown in Figure 4. In this structure, the cytosine of **3** resides in the major groove and the carboxypropyl side chain reaches across the major groove. This configuration would position the carboxyl group near the N ^{6} -amino group of A, where it could serve as a hydrogen bond acceptor. An additional interaction between the N^4 -amino group of **3** and the O ^{4} -carbonyl of the T residue of the target base pair also appears to be possible. Consistent with this scheme is the observation that oligomer **I**(X), where X is N^4 -(*n*-butyl)deoxycytidine, a derivative similar to nucleoside **3** but lacking the carboxyl group, failed to form a triplex with **III·IV** (T·A) (data not shown).

Previous studies have demonstrated that G can recognize a single T·A base pair in target duplexes (6–9). Formation of a G·T·A triad has been shown to involve the formation of a single hydrogen bond between a hydrogen on the N ^{2} -amino group of G and the O ^{4} -carbonyl of T (16–18). It appears that the triplex formed by **I**(3) is more stable than that formed by G under the conditions used in our experiments. Thus, the T_m for the third strand dissociation of **I·III·IV** (3·T·A) is 7 $^{\circ}$ C higher than that of **I·III·IV** (G·T·A), which is 15 $^{\circ}$ C (9). This higher T_m may reflect additional hydrogen bonding interactions between the carboxypropyl side chain of **3** and sites on the T·A base pair as shown in Figure 4.

It is interesting to note that **I**(3) did not form a triplex with the target containing a U·A base pair. Similar behavior was previously observed for **I**(G) (9). This result suggests that the 5-methyl group of T of the T·A base pair plays an important role in stabilizing the interaction

of **3** or of G during triplex formation. In the case of **3**, the 5-methyl group may force the base slightly out and across the major groove to avoid steric clashes with the 5-methyl group. As a consequence, this could help position the carboxyl group within hydrogen bonding distance of the N⁶-amino of A of the T·A base pair.

Although **I(3)** interacted with **III·IV** (T·A) in Tris buffer, no interaction was observed with duplex target in MOPS buffer at pH 7.0. However, a triplex was formed with **III·IV** (G·C). This result suggests that the Tris cation may play a role in the interaction of **3** with T·A, possibly through formation of an ion pair with the carboxyl group of **3**. Such ion-pair formation might facilitate positioning of the carboxypropyl side chain in the more hydrophobic environment of the major groove (**I**) leading to subsequent interaction with the N⁶-amino group of A. It appears unlikely that interactions of the Tris cation with phosphate groups of the oligomer influence the overall stability of the triplex, because **I·III·IV** (**3**·G·C) has identical *T_m* values in both Tris and MOPS buffer at pH 7.0.

Oligomer **II**, which contains a single N⁴-(5-carboxytriazolyl)deoxycytidine (**4**), also forms triplexes with duplex target **III·IV** (Y·Z). As was the case with oligomer **I(3)**, oligomer **II** forms a triplex with the target duplex containing a G·C base pair. Thus, it appears that nucleoside **4** can also act like C in its interaction with a G·C base pair. In contrast to the behavior of **I(3)**, oligomer **II** forms triplexes with targets containing either a T·A or a U·A base pair and in addition interacts with a C·G-containing target. Examination of molecular models suggests that **4** could interact with the T·A or U·A base pair in a manner analogous to that shown for nucleoside **3** in Figure 4. The carboxytriazole group of **4** is expected to have fewer degrees of freedom than the more flexible carboxypropyl side chain of nucleoside **3**. This and the greater likelihood to stack with neighboring bases may account for the ability of this nucleoside analog to interact with the **pyr·pur** interruption of duplex **III·IV**.

Although the results described above are promising, further work is required to improve the specificity and stability of triplex-forming oligomers. It appears, however, that strategies which employ nucleoside derivatives which can potentially contact both bases of a **pyr·pur** interruption might provide a means to extend the range of sequences which can be recognized by triplex-forming oligonucleotides.

ACKNOWLEDGMENT

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LITERATURE CITED

- (1) Radhakrishnan, I., and Patel, D. J. (1994) DNA Triplexes: Solution Structures, Hydration Sites, Energetics, Interactions, and Function. *Biochemistry* 33, 11405–11416.
- (2) Frank-Kamenetskii, M. D., and Mirkin, S. M. (1995) Triplex DNA Structures. *Annu. Rev. Biochem.* 64, 65–95.
- (3) Plum, G. E., Pilch, D. S., Singleton, S. F., and Breslauer, K. J. (1995) Nucleic Acid Hybridization: Triplex Stability and Energetics. *Annu. Rev. Biophys. Biomol. Struct.* 24, 319–350.
- (4) Maher, L. J., III (1996) Prospects for the Therapeutic Use of Antigene Oligonucleotides. *Cancer Invest.* 14, 66–82, and references cited therein.
- (5) Miller, P. S. (1996) Development of Antisense and Antigene Oligonucleotide Analogs. *Prog. Nucleic Acid Res. Mol. Biol.* 52, 261–291, and references cited therein.
- (6) Griffin, L. C., and Dervan, P. B. (1989) Recognition of Thymine-Adenine Base Pairs by Guanine in Pyrimidine Triple Helix Motif. *Science* 245, 967–971.
- (7) Mergny, J.-L., Sun, J.-S., Rougae, Montenay-Garestier, T., Barcelo, F., Chomilier, J., Helene, C. (1991) Sequence Specificity in Triple-Helix Formation: Experimental and Theoretical Studies of the Effect of Mismatches on Triplex Stability. *Biochemistry* 30, 9791–9798.
- (8) Yoon, K., Hobbs, C. A., Koch, J., Sardaro, M., Kutny, R., and Weis, A. L. (1992) Elucidation of the Sequence-Specific Third Strand Recognition of Four Watson-Crick Base Pairs in a Pyrimidine Triple-Helix Motif: T·AT, C·GC, T·CG, and G·TA. *Proc. Natl. Acad. Sci. U.S.A.* 89, 3840–3844.
- (9) Miller, P. S., and Cushman, C. D. (1993) Triplex Formation by Oligodeoxyribonucleotides Involving the Formation of X·UA Triads. *Biochemistry* 32, 2999–3004.
- (10) Radhakrishnan, I., and Patel, D. J. (1994) Solution Structure and Hydration Patterns of a Pyrimidine·Purine·Pyrimidine DNA Triplex Containing a Novel T-CG Base Triple. *J. Mol. Biol.* 241, 600–619.
- (11) Griffin, L. C., Kiessling, L. L., Beal, P. A., Gillespie, P., and Dervan, P. B. (1992) Recognition of All Four Base Pairs of Double-Helical DNA by Triple-Helix Formation: Design of Nonnatural Deoxyribonucleosides for Pyrimidine·Purine Base Pair Binding. *J. Am. Chem. Soc.* 114, 7976–7982.
- (12) Koshlap, K. M., Gillespie, P., Dervan, P. B., and Feigon, J. (1993) Nonnatural Deoxyribonucleoside D₃ Incorporated in an Intramolecular DNA Triple Helix Binds Sequence-Specifically by Intercalation. *J. Am. Chem. Soc.* 115, 7908–7909.
- (13) Huang, C.-Y., Cushman, C. D., and Miller, P. S. (1993) Triplex Formation by an Oligonucleotide Containing N⁴-(Acetamidopropyl)cytosine. *J. Org. Chem.* 58, 5048–5049.
- (14) Huang, C.-Y., and Miller, P. S. (1993) Triplex formation by an Oligodeoxyribonucleotide Containing N⁴-(6-Aminopyridinyl)-2'-deoxycytidine. *J. Am. Chem. Soc.* 115, 10456–10457.
- (15) Miller, P. S., and Cushman, C. D. (1992) Selective Modification of Cytosines in Oligodeoxyribonucleotides. *Bioconjugate Chem.* 3, 74–79.
- (16) Radhakrishnan, I., Gao, X., de los Santos, C., Live, D., and Patel, D. J. (1991) NMR Structural Studies of Intramolecular (Y⁺)_n·(R⁺)_n·(Y⁻)_n DNA Triplexes in Solution: Imino and Amino Proton and Nitrogen Markers of G·TA Base Triple Formation. *Biochemistry* 30, 9022–9030.
- (17) Wang, E., Malek, S., and Feigon, J. (1992) Structure of a G·TA Triplet in an Intramolecular DNA Triplex. *Biochemistry* 31, 4838–4846.
- (18) Radhakrishnan, I., Patel, D. J., Veal, J. M., and Gao, X. (1992) Solution Conformation of a G·TA Triple in an Intramolecular Pyrimidine·Purine·Pyrimidine DNA Triplex. *J. Am. Chem. Soc.* 114, 6913–6915.

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Improved Characteristics of a Human β -Glucuronidase–Antibody Conjugate after Deglycosylation for Use in Antibody-Directed Enzyme Prodrug Therapy

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Antibody-directed enzyme prodrug therapy (ADEPT) aims at the specific activation of relatively nontoxic prodrugs into active drugs at the tumor site. One of the enzymes described to be useful in ADEPT is human β -glucuronidase (GUS), which is expected to have low immunogenicity in patients. A major obstacle for the use of GUS, however, is its rapid glycan-specific hepatic clearance. The carbohydrates of GUS have been modified by subsequent treatment with NaIO_4 and NaBH_4 to improve its retention in the circulation. The modification of GUS did not decrease the enzyme activity. In vitro it was demonstrated that a conjugate prepared with a pancarcinoma specific monoclonal antibody (mAb) 323/A3 and the modified enzyme (mGUS), when bound to tumor cells, was capable of complete prodrug activation. In vivo, the 323/A3–mGUS conjugate was cleared faster from the circulation of BALB/c mice ($t_{1/2} = 9$ h) than mAb 323/A3 ($t_{1/2} = 32$ h), but it was retained in the circulation much longer than an immunoconjugate prepared with native GUS ($t_{1/2} = 24$ min). In nude mice bearing subcutaneous OVCAR-3 tumors the distribution of 323/A3–mGUS was qualitatively comparable to that of mAb 323/A3. The 323/A3–mGUS conjugate showed specific localization in the tumor but to a lesser extent than mAb 323/A3 (2.7% vs 6.4% injected dose per gram at 1 day after iv injection). A favorable tumor-to-blood ratio of >2 was observed for the conjugate at 7 days after administration, which is necessary for tumor-specific prodrug activation.

INTRODUCTION

The efficacy of drugs used in chemotherapy is hampered by the lack of selectivity, as these drugs do not distinguish neoplastic cells from normal cells. One method to increase the selectivity of cytostatic agents is to target the drugs to the tumor site. Antibody-directed enzyme prodrug therapy (ADEPT)¹ uses this strategy (1, 2). First, an enzyme immunoconjugate or fusion protein is administered. After localization of the enzyme in the tumor, a relatively nontoxic prodrug is given. The prodrug is then selectively activated to the drug at the tumor site by enzymatic hydrolysis. Ideally, the prodrug is not activated in the circulation or normal tissues but only by the enzyme moiety of the immunoconjugate localized in the tumor.

For use in ADEPT we have chosen human β -glucuronidase (GUS) as the enzyme for the following reasons. First, an enzyme of human origin is expected to be less immunogenic than enzymes from other species. This is

important, because repeated administration of the enzyme immunoconjugate/prodrug combination may prove necessary for successful treatment of patients. The second reason is that the enzyme is localized intracellularly in microsomes and lysosomes (3) and enzyme activity is not detectable in human blood at neutral pH (4). Thus, untimely activation of prodrug is limited as hydrophilic glucuronide prodrugs do not diffuse into the cell (5). A disadvantage of GUS is its rapid clearance from the circulation and accumulation in the liver (6). The hepatic clearance is caused by glycan-specific recognition of the enzyme. It may be expected that GUS immunoconjugates would likewise be cleared from the circulation, hampering tumor localization. Modification of the carbohydrate side chains of the enzyme might prevent hepatic clearance and improve the retention in the circulation, as was shown for ricin immunotoxins (7).

The selected mAb 323/A3 (mouse IgG1) (8) is reactive with a pancarcinoma antigen epithelial transmembrane glycoprotein with a molecular mass of 40 kDa (9). EGP40 is normally present on the basolateral surface of epithelial cells in secretory tissues and is highly expressed on the surface of most carcinomas, including breast, ovary, lung, and colon tumors. The basal membrane, which is disrupted in tumors, prevents 323/A3 from reaching the antigen on normal epithelial cells. Radiolabeled 323/A3 has recently been shown to be useful for the detection of head and neck squamous cell carcinoma in patients (10). Adjuvant therapy with another mAb directed against EGP40, 17-1A, has been shown to prolong remission-free survival and overall survival in colorectal cancer patients (11).

Anthracyclines were chosen as drugs in ADEPT because they have a broad spectrum of activity in cancer (12). Prodrugs prepared from anthracyclines and glucuronic acid are very hydrophilic and cannot diffuse through the cell membrane. Therefore, they are not

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¹ Abbreviations: ADEPT, antibody-directed enzyme prodrug therapy; BSA, bovine serum albumin; DHFR, dihydrofolate reductase; DNR, daunorubicin; DNR-GA3, *N*-[4-daunorubicin-*N*-carbonyl(oxymethyl)phenyl] *O*- β -glucuronyl carbamate; FCS, fetal calf serum; GUS, human β -glucuronidase; mGUS, NaIO_4 - and NaBH_4 -treated human β -glucuronidase; HPLC, high-performance liquid chromatography; % ID/g, percent injected dose per gram; IC_{50} , concentration that gives 50% growth inhibition when compared with control cell growth; mAb, monoclonal antibody; MTX, methotrexate; PBS, phosphate-buffered saline, pH 7.4; SATA, succinimidyl *S*-acetylthioacetate; SDS–PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SMCC, succinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate; SRB, sulforhodamine B.

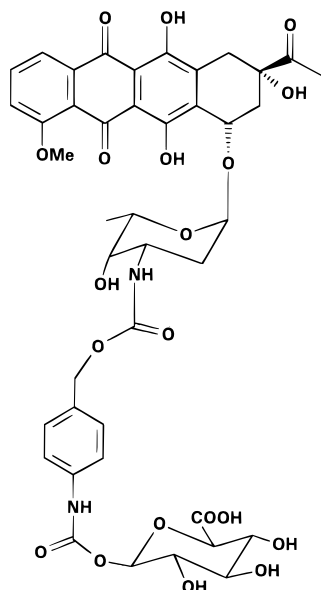


Figure 1. Chemical structure of prodrug *N*-[4-daunorubicin-*N*-carbonyl(oxymethyl)phenyl] *O*- β -glucuronide (DNR-GA3).

activated by GUS present in the microsomes and lysosomes. After activation by the enzyme immunoconjugate bound to the cell surface antigen, the hydrophobic anthracyclines easily diffuse into the cell and cause cell death (5, 13). Other anthracycline prodrugs are being investigated for use in ADEPT, in which the drug is activated by β -glucuronidase (14) or different enzymes, such as β -lactamase (15) or penicillin G amidase (16).

In the present experiments carbohydrates in GUS were modified with NaIO_4 and NaBH_4 to improve the retention of the enzyme in the circulation. After modification, mGUS was analyzed for enzyme activity and in vitro stability. Conjugates prepared from 323/A3 and mGUS or native GUS were characterized for their enzyme activity and immunoreactivity. A selected prodrug, *N*-[4-daunorubicin-*N*-carbonyl(oxymethyl)phenyl] *O*- β -glucuronide (DNR-GA3, Figure 1), prepared by Leenders et al. (17), was examined for its antiproliferative effects before and after activation by the cell-bound enzyme immunoconjugates in vitro. The pharmacokinetics of mGUS, native GUS, and the enzyme immunoconjugates were analyzed in BALB/c mice. The tumor-localizing properties of 323/A3-mGUS in nude mice bearing human OVCAR-3 xenografts were determined and compared with those of 323/A3.

EXPERIMENTAL PROCEDURES

Materials. The human ovarian cancer cell line NIH: OVCAR-3 (OVCAR-3) (18) was grown as a monolayer in Dulbecco's modified Eagle's medium (DMEM; Gibco Life Technologies, Paisley, Scotland) supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mM L-glutamine, 50 IU/mL penicillin (ICN, Costa Mesa, CA), and 50 $\mu\text{g}/\text{mL}$ streptomycin (ICN) in a humidified atmosphere containing 5% CO_2 at 37 $^\circ\text{C}$. The mouse L-MPR 13-2-1 MTX 3.2 cell line (kindly provided by Prof. W. S. Sly, St. Louis University, St. Louis, MO) (19) contains the genes for GUS and for dihydrofolate reductase (DHFR) integrated in its genome. The GUS gene is coamplified with DHFR and overexpressed in the presence of methotrexate. These cells overproduce the enzyme that is secreted into the culture medium. The L-MPR cells were grown in the same culture medium as the OVCAR-3 cell line except for 5% heat-inactivated FCS that was dialyzed

against phosphate-buffered saline (PBS) to reduce the folate content. Methotrexate (Lederle, Etten-Leur, The Netherlands) was added to the medium to provide a final concentration of 3.2 μM .

GUS was isolated from the culture medium of L-MPR cells as described (5). In short, the medium was acidified to pH 4.5, centrifuged, filtered, and applied to a HiTrapSP column (Pharmacia, Uppsala, Sweden). The bound GUS was eluted with 25 mM phosphate buffer, pH 6.0, using a salt gradient (0–1 M NaCl). The enzyme-containing fractions were pooled and diluted 10-fold with 25 mM phosphate buffer, pH 6.0, and applied to a HiTrapQ column (Pharmacia). The peak fractions were collected, brought to pH 4.5, and reloaded onto a HiTrapSP column and eluted with 0.2 M NaCl in 25 mM phosphate buffer at pH 6.0.

Purified murine mAb 323/A3 (8) was kindly provided by Prof. S. O. Warnaar, Centocor Europe, Leiden, The Netherlands. ^{125}I -Labeled 323/A3 was produced using the one-vial iodogen method (20). Precipitation with 10% (w/v) trichloroacetic acid indicated that >95% of the radioactivity was bound to protein after labeling. The specific activity of the radiolabeled 323/A3 was approximately 0.7 mCi/mg.

Daunorubicin (DNR, Société Parisienne d'expansion chimique, Paris, France) was purchased as a powder. DNR-GA3 has been characterized (5, 17). In this prodrug, the anthracycline moiety is linked to the glucuronide via a carbamate spacer with an aromatic center. Stock solutions of drug and prodrug were prepared in sterile water and stored at $-20\text{ }^\circ\text{C}$. HPLC analysis revealed no deterioration of DNR or DNR-GA3 after storage for at least 6 months.

Modification of Human β -Glucuronidase (mGUS).

Purified GUS (1–2 mg/mL) was incubated with 40 mM NaIO_4 (Sigma, St. Louis, MO) in 50 mM sodium acetate, pH 4.5. After 2 h of incubation at 4 $^\circ\text{C}$, the reaction was stopped by addition of excess ethylene glycol (Baker, Deventer, The Netherlands). Unreacted reagents were removed with a Sephadex G-25M column (PD10; Pharmacia) equilibrated with PBS, pH 7.5. The formed aldehydes were reduced to alcohols by incubation with 40 mM NaBH_4 (Sigma) in PBS at pH 7.5 at 37 $^\circ\text{C}$ for 3 min and at 4 $^\circ\text{C}$ for 60 min. The mixture was loaded onto a Sephadex G-25M column and eluted in PBS at pH 7.5 to stop the reaction.

The completion of deglycosylation of the enzyme was checked by 6% nondenaturing PAGE. The gel was stained for GUS activity using naphthol AS-BI β -D-glucuronic acid (Sigma) as described (21).

Preparation and Characterization of Conjugates.

The enzyme immunoconjugates 323/A3-GUS, 323/A3-mGUS, and [^{125}I]-323/A3-mGUS were prepared by using stable thioether bonds as described (13). Succinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (SMCC; Pierce, Oud-Bijerland, The Netherlands) was allowed to react with 323/A3 or with 323/A3 and a tracer dose of [^{125}I]-323/A3 to produce maleimide-activated antibody. GUS or mGUS was reacted with succinimidyl *S*-acetylthioacetate (SATA; Pierce) to introduce sulfhydryl groups. The substituted enzyme and antibody were combined at a 1:1 weight ratio, and the conjugation reaction was started by the addition of hydroxylamine (Janssen, Geel, Belgium) at pH 7.0. After 60 min at room temperature, the reaction was stopped by gel filtration on Sephadex G-25M equilibrated with PBS.

The enzyme immunoconjugates were concentrated by ultrafiltration using a 10PM30 filter (Amicon, Danvers, MA) and purified by gel filtration on a Superdex 200 preparative grade column (10 $\times$ 600 mm, Pharmacia) in

PBS. The fractions containing conjugates with 1:1 antibody/enzyme ratios were pooled. BSA was added to a final concentration of 0.05% (w:v) to prevent loss of the enzyme activity in PBS. The immunoreactivity of the radiolabeled enzyme immunoconjugate was calculated from the binding to OVCAR-3 cells at infinite antigen excess as described by Lindmo et al. (22).

The purity of proteins was checked by subjecting samples to 10% SDS-PAGE under reducing conditions or to 7.5% SDS-PAGE under nonreducing conditions (23). The purity of proteins was further analyzed by HPLC, using a Superdex 200 HR gel filtration column (10 × 300 mm, Pharmacia) and 0.05 M sodium phosphate/0.15 M sodium salt, pH 6.8, as eluent. With a flow rate of 0.5 mL/min the retention time was 24 min for mGUS, 26 min for 323/A3, and 29 min for BSA as determined by reading the extinction at a wavelength of 280 nm. Enzyme activity was assessed with 4-nitrophenyl β -D-glucopyranosiduronic acid as described (13). One unit (U) of enzyme activity is defined as the quantity of GUS needed for the conversion of 1 μ mol of 4-nitrophenyl β -D-glucopyranosiduronic acid to 4-nitrophenol. Protein concentrations were determined with the Micro BCA protein assay, according to the manufacturer's instructions using BSA as a standard (Pierce).

In Vitro Antiproliferative Effects. The antiproliferative effects of drugs and prodrugs were determined with the use of OVCAR-3 cells. OVCAR-3 cells were incubated with the various enzyme immunoconjugates (10 μ g/mL) at 4 °C for 60 min. Thereafter, cells were washed in PBS, resuspended in culture medium (supplemented DMEM), and seeded in triplicate in 96-well culture plates (20 000 cells/well, 10 μ L/well). Drug or prodrug was added (10 μ L/well) to give final concentrations ranging from 1 nM to 10 μ M. After 24 h, 200 μ L of culture medium was added and the cells were incubated for an additional 72 h. After staining with sulforhodamine B (SRB), the absorbance at 492 nm was read. The antiproliferative effects were expressed as IC_{50} values, which are the (pro)drug concentrations that give 50% growth inhibition when compared with control cell growth.

In Vivo Studies. Pharmacokinetic studies were carried out in BALB/c mice (Harlan CPB, Zeist, The Netherlands). The animals were given iv injections of [^{125} I]-323/A3, GUS, mGUS, 323/A3-GUS, 323/A3-mGUS, or [^{125} I]-323/A3-mGUS in 100–200 μ L of PBS. From groups of three mice per time point serial blood samples were collected from the eye plexus with the use of heparinized glass capillaries over a 24 h period. Whole blood samples were weighed and counted in a γ -counter for the determination of ^{125} I activities. Samples were centrifuged at 16000g for 4 min to prepare plasma. The GUS activity in plasma was measured to determine enzyme or enzyme immunoconjugate concentrations.

Distribution studies were performed in athymic nude mice (Harlan CPB) bearing OVCAR-3 sc tumors with a weight of 100–300 mg. Groups of three mice per time point were injected iv with [^{125} I]-323/A3-mGUS or [^{125} I]-323/A3 at a dose of 20 μ g/mouse in PBS/0.05% (w:v) BSA. At 1, 2, 3, 5, 7, and 10 days after injection, the mice were bled and sacrificed. Tissues were collected, weighed, and immediately counted in a γ -counter. Results were expressed as the percent injected dose per gram of tissue (% ID/g).

RESULTS

Modification of Human β -Glucuronidase and Preparation of Conjugates. The recovery of enzyme from the supernatant of L-MPR cells was 70% after

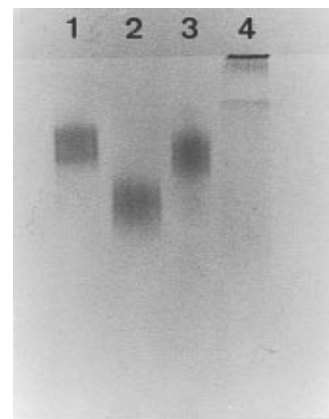


Figure 2. Nondenaturing PAGE (6%) stained for enzyme activity: lane 1, native GUS; lane 2, NaIO₄-treated GUS, freshly prepared; lane 3, NaIO₄- and NaBH₄-treated GUS; lane 4, NaIO₄-treated GUS 3 weeks after preparation.

purification. Purified GUS had a specific activity of 7–10 U/mg (pH 6.8, 37 °C), which remained stable for more than 3 weeks at 37 °C. Denaturing SDS-PAGE showed a major protein band with an apparent molecular mass of 75 kDa. To improve the retention of GUS in the circulation, the enzyme was deglycosylated by successive treatment with NaIO₄ and NaBH₄.

The completion of the modification of GUS was determined by comparing the electrophoretic mobility of GUS, NaIO₄-treated GUS, and NaBH₄-treated GUS (mGUS) by nondenaturing PAGE. NaIO₄ treatment resulted in an increase of the electrophoretic mobility when compared with the mobility of the native enzyme (lanes 2 and 1, Figure 2). When stored at 4 °C, large aggregates developed (lane 4, Figure 2). Successive treatment with NaBH₄ to convert the aldehydes formed by NaIO₄ to alcohols resulted in mGUS with lower electrophoretic mobility (lane 3, Figure 2). In this preparation no aggregates were detectable even after storage at 4 °C for 3 weeks.

The recovery of the enzyme activity was >95% after modification. After treatment with NaIO₄, or NaIO₄ and NaBH₄, the specific activity of the enzyme was approximately 8 U/mg (pH 6.8, 37 °C).

The enzyme immunoconjugates 323/A3-GUS and 323/A3-mGUS were prepared by linking SMCC-pretreated antibody and SATA-pretreated enzyme. The overall protein yield after purification of the enzyme immunoconjugates was approximately 20%. Conjugates retained >95% of the expected GUS specific activity, approximately 4 U/mg. The enzyme activity was preserved in the conjugates and was not diminished after storage at 4 °C for 7 weeks. The immunoreactivity of the conjugates was determined with the use of OVCAR-3 cells and found to be >95% of that of mAb 323/A3. Nonreducing SDS-PAGE combined with autoradiography of [^{125} I]-323/A3-mGUS showed one major band for the conjugate. All radioactivity was associated with the conjugate, and the molecular mass was higher than that found for [^{125} I]-323/A3 in the control lane. HPLC analysis of [^{125} I]-323/A3-mGUS showed indeed one peak with a retention time of 19 min corresponding to a molecular mass of 430 kDa.

In Vitro Antiproliferative Effects. The antiproliferative effects of DNR and DNR-GA3 were determined by measuring the growth of OVCAR-3 cells with a protein dye assay. DNR (IC_{50} = 1.2 μ M) was 18 times more toxic than the prodrug when cells were exposed to drugs for 24 h. Pretreatment of the cells with 323/A3-mGUS conjugate prior to prodrug exposure resulted in an increase in the antiproliferative effects reaching the same

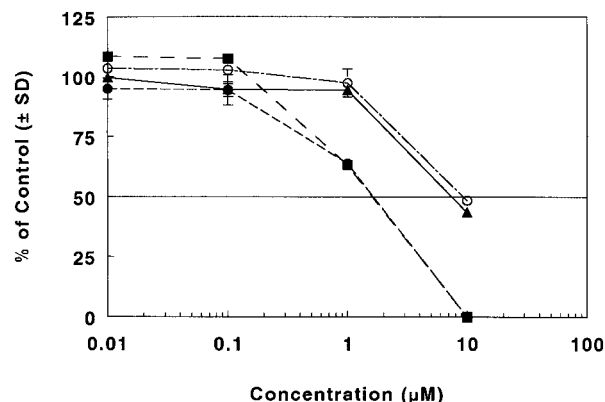


Figure 3. In vitro prodrug activation. OVCAR-3 cells were exposed to 323/A3-mGUS conjugate, washed, and treated with various concentrations of DNR or DNR-GA3. Cell growth was measured by SRB staining and is expressed as the percentage of PBS-treated control cells. (■) PBS, DNR; (▲) PBS, DNR-GA3; (●) 323/A3-mGUS, DNR-GA3; (○) 323/A3-mGUS preceded by an excess of 323/A3 (100 $\mu\text{g}/10^6$ cells/10 μL), DNR-GA3.

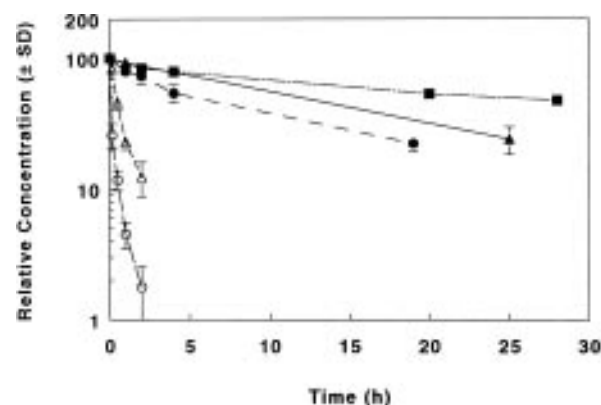


Figure 4. Pharmacokinetics in BALB/c mice. Mab 323/A3 was labeled with ^{125}I , and a [^{125}I]-323/A3-mGUS conjugate was prepared. At different time points after injection of antibody, enzyme, or conjugate, 50 μL of blood was obtained by retroorbital puncture and assayed for enzyme activity [(Δ) GUS; (▲) mGUS; (○) 323/A3-GUS; (●) 323/A3-mGUS] or analyzed for radioactivity [(■) [^{125}I]-323/A3 ($n = 3$)].

IC_{50} as for DNR (Figure 3). Similar results were found for 323/A3-GUS pretreatment. OVCAR-3 cells preincubated with excess 323/A3 followed by exposure to the enzyme immunoconjugates were not affected in growth by DNR-GA3 (Figure 3), indicating the specificity of the conjugate. In a separate experiment prodrug was added 24 h after the cells with bound 323/A3-mGUS conjugate were plated to study the retention of enzyme activity. No reduction of prodrug activation was observed, which suggests that the conjugate was not rapidly internalized or inactivated (data not shown).

In Vivo Studies. The pharmacokinetics of the mAb, the enzyme (GUS and mGUS), and the conjugates (323/A3-GUS and 323/A3-mGUS) were determined in BALB/c mice (Figure 4). GUS cleared rapidly from the blood with a $t_{1/2}$ ($\pm$ SD) of 30 ± 3 min ($n = 4$), whereas the radiolabeled 323/A3 showed a half-life of approximately 32 h ($n = 1$). A conjugate prepared from native GUS and 323/A3 was very rapidly removed from the blood, more rapidly than GUS, with a $t_{1/2}$ of 24 ± 2 min ($n = 3$). Modification of the enzyme resulted in a longer retention in the circulation, with a $t_{1/2}$ for mGUS of 14.4 ± 0.9 h ($n = 5$). Conjugates prepared from mGUS and 323/A3 showed a considerably increased retention time with a $t_{1/2}$ of 8.6 ± 1 h ($n = 6$) when compared with the short $t_{1/2}$ for 323/A3-GUS.

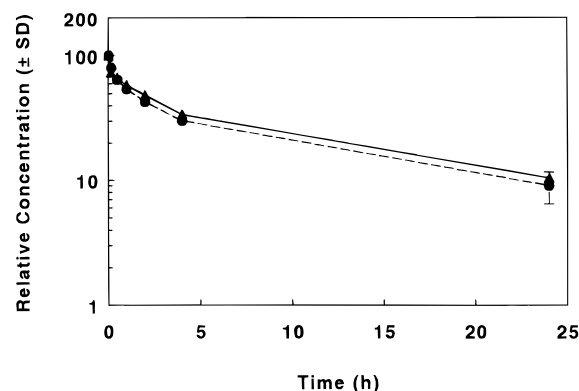


Figure 5. Pharmacokinetics of radiolabeled enzyme immunoconjugate in BALB/c mice. Mab 323/A3 was labeled with ^{125}I , and a [^{125}I]-323/A3-mGUS conjugate was prepared. At different time points after injection of the conjugate, 50 μL of blood was obtained by retroorbital puncture and assayed for enzyme activity (●) and radioactivity (▲) ($n = 3$).

To determine if a radiolabeled enzyme immunoconjugate can be used for measuring the enzymatically active concentration, we compared the pharmacokinetics of the radioactivity and the enzyme activity of [^{125}I]-323/A3-mGUS. Both clearance curves were found to be superimposable (Figure 5) and similar to that of unlabeled 323/A3-mGUS (data not shown). This indicated that the enzyme activity was retained and that the linkage between mGUS and 323/A3 remained intact.

The distribution and tumor localization of 323/A3-mGUS were studied in nude mice bearing OVCAR-3 xenografts. The localization of [^{125}I]-323/A3 and [^{125}I]-323/A3-mGUS at 1, 3, and 7 days after injection is illustrated in Figure 6. The distribution of the enzyme immunoconjugate in the different tissues was qualitatively comparable to that of 323/A3, also in lung, heart, ileum, stomach, and skin (tissues not shown). The absolute uptake in tissues was 2- and 10-fold lower at days 1 and 7, respectively. The liver uptake of the conjugate at day 1 (2.9% ID/g) was relatively higher than that of 323/A3 when the values of the uptake were normalized for blood levels. At later time points the liver uptake of the conjugate was similar to that of the antibody. The concentration of both the conjugate and the antibody in the tumor exceeded that of all tissues at all time points, except for blood. SDS-PAGE combined with autoradiography of homogenized tumor tissue samples showed that all radioactivity was associated with the conjugate and that no free antibody was present.

Tumor-to-tissue ratios of the enzyme immunoconjugate concentrations were calculated from the uptake expressed as the % ID/g (Table 1). The tumor-to-tissue ratios rose steadily for all organs, indicating the specific retention of 323/A3-mGUS in tumors. At 10 days after administration of the conjugate, the tumor-to-blood ratio was >4 . Liver and all other organs showed values >5 at day 7. The tumor-to-tissue ratios of 323/A3-mGUS were grossly comparable to those of 323/A3.

DISCUSSION

Deglycosylation of GUS was successfully accomplished and resulted in a remarkable increase of the $t_{1/2}$ of the enzyme immunoconjugate in the circulation of BALB/c mice. Native GUS was rapidly cleared from the circulation after iv administration to BALB/c mice ($t_{1/2} = 30$ min). The conjugate prepared from native GUS and 323/A3 (323/A3-GUS) showed a half-life similar to that of native GUS, making it ineffective for use in ADEPT. In comparison to 323/A3-GUS the clearance of the 323/A3-

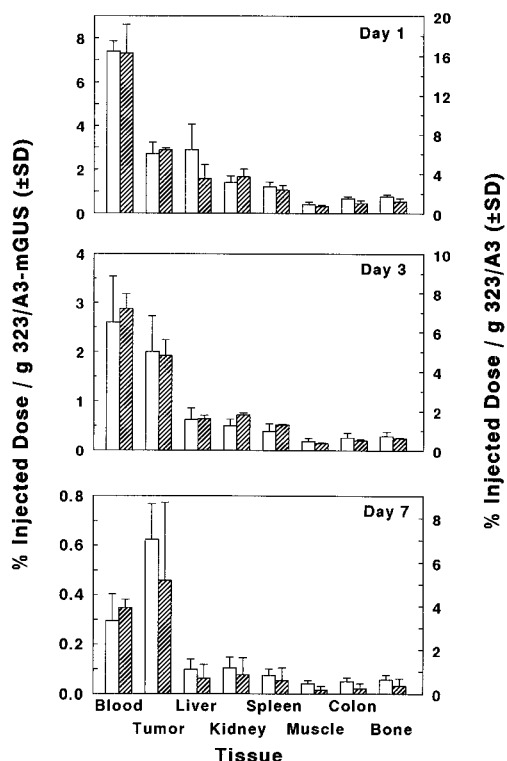


Figure 6. In vivo distribution. MAb 323/A3 was labeled with ^{125}I , and a ^{125}I -323/A3-mGUS conjugate was prepared. Radiolabeled antibody and conjugate were administered to nude mice bearing subcutaneous OVCAR-3 xenografts, and mice were bled and sacrificed on several days after administration. Blood and tissue distribution is shown on days 1, 3, and 7. The uptake was calculated as the % ID/g of tissue for ^{125}I -323/A3-mGUS (open bars) and ^{125}I -323/A3 (hatched bars) ($n = 3$ except for tumor, $n = 6$).

Table 1. Tumor to Nontumor Ratios of ^{125}I -323/A3-mGUS Conjugate and ^{125}I -323/A3 in Nude Mice Bearing OVCAR-3 Xenografts^a

tissue	1 day		3 days		7 days	
	conjugate	323/A3	conjugate	323/A3	conjugate	323/A3
blood	0.4	0.6	0.8	0.7	2.11	1.30
liver	1.0	2.9	3.3	3.0	6.41	7.75
spleen	2.3	4.2	5.2	3.7	8.48	8.68
kidney	1.9	2.7	4.1	2.7	6.00	6.01
lung	1.5	2.7	3.1	2.7	5.85	5.15
muscle	6.5	14.5	11.3	13.2	15.5	29.6

^a Tumor to nontumor ratios were derived from the uptakes of the antibody and conjugate, expressed as % ID/g of tissue (three mice per group; SD < 50%).

mGUS conjugate was greatly improved with a half-life of 8.6 h. In nude mice bearing subcutaneous OVCAR-3 tumors the distribution of 323/A3-mGUS was qualitatively comparable to that of mAb 323/A3.

Native GUS is rapidly cleared from the circulation by receptor-specific recognition, and it mainly localizes in the liver (6). The pharmacokinetics may be improved if these recognition sites on the enzyme are removed. This can be achieved in several ways. Glycosylation inhibitors may be added to the culture medium of the GUS-producing L-MPR cells, and a completely unglycosylated enzyme may be produced. This approach is not applicable for GUS, because four glycosylated monomers are necessary for the formation of an enzymatically active homotetrameric human GUS (24). Another possibility for deglycosylation is the use of *N*-glycosidases that cleave the covalent linkage between sugar moieties, which results in the removal of these carbohydrate structures from the protein. These enzymes are most effective

under denaturing conditions, however, and large quantities are needed for enzymatic deglycosylation of native GUS. This method is therefore not cost-effective (7).

We have deglycosylated GUS chemically by oxidation with NaIO_4 followed by reduction of the formed aldehydes to alcohols with NaBH_4 . This treatment destroyed the recognition sites, sugar moieties, for the receptor-specific recognition. Incubation with NaIO_4 resulted in ring opening of the sugars and in the formation of aldehydes. The formed aldehydes, however, may react with the free amino groups present on the enzyme to form Schiff base linkages, which results in cross-linking and aggregation. Conversion of the aldehydes to alcohols by NaBH_4 treatment may prevent aggregation (7). The completion of the treatment of GUS with NaIO_4 and with NaBH_4 could be monitored by changes in the electrophoretic mobility of the enzyme on a native acrylamide gel. The modified enzyme, mGUS, was enzymatically stable, and the modification procedure gave reproducible results.

The conjugate 323/A3-mGUS as well as 323/A3-GUS when bound to OVCAR-3 cells could completely convert the prodrug DNR-GA3 to DNR. The increase of the antiproliferative effects of DNR-GA3 on cells pretreated with 323/A3-GUS or 323/A3-mGUS was completely blocked when the cells were treated with an excess of 323/A3 before the addition of conjugate, indicating the antigen specificity of the enzyme immunoconjugates. The conjugate 323/A3-mGUS remained bound to the outside of the cells, as was shown by in vitro experiments in which the addition of prodrug was delayed for 24 h. The prodrug was completely activated to DNR, which indicated that the conjugate was not rapidly internalized or inactivated. This is of importance, because for in vivo therapy the time delay between administration of an enzyme immunoconjugate and a prodrug will be several days.

We were not able to measure the enzyme activity of the enzyme immunoconjugate in tissues after iv administration because the sample preparation resulted in the release of intracellular GUS. It was also not possible to first prepare the conjugate and then label it with ^{125}I according to the iodogen method, because this resulted in the complete loss of enzyme activity (data not shown). Therefore, radiolabeled enzyme immunoconjugate was prepared using ^{125}I -323/A3 in the conjugation reaction. The clearance from the circulation of 323/A3-mGUS and ^{125}I -323/A3-mGUS was similar when determined from the enzyme activity or when measured by radioactivity. We thus concluded that the thioether linkage in the enzyme immunoconjugate remained intact and that this radiolabeled conjugate was suitable for distribution studies. In addition, tumor tissue samples up to day 7, analyzed by nonreducing SDS-PAGE combined with autoradiography, showed that all radioactivity was associated with the conjugate. Because storage of purified GUS at 37 °C for 3 weeks did not diminish enzyme activity, we assumed that ^{125}I -323/A3-mGUS localized in tumors represented enzymatically active conjugate.

The distribution of the 323/A3-mGUS conjugate in tumor-bearing nude mice showed that tumor uptake exceeded that of all tissues from day 1 onward. During the time of observation the OVCAR-3 tumors increased in size. The absolute concentration of the conjugate in tumor decreased, but considering the tumor growth the enzyme immunoconjugate was well retained. A favorable tumor-to-blood ratio of >2 was reached at 7 days after injection. Ratios of >5 were measured for other tissues. The tumor-to-blood ratios are comparable to those found by other investigators. Eccles et al. (25) have reported a ratio of 1.5 at 11 days, whereas Sharma et al. (26) have

measured a ratio of 2.9 at 72 h after administration of an enzyme immunoconjugate. The latter group has improved the tumor-to-blood ratio for their conjugate from 2.9 to 7.1 by the use of a conjugate-clearing antibody. For the 323/A3-mGUS conjugate we have also a clearing antibody available (27).

The combination of enzyme immunoconjugate and DNR-GA3 is now being evaluated in nude mice bearing human ovarian cancer xenografts. The advantage of the use of deglycosylated GUS resulting in favorable tumor-to-blood and tumor-to-tissue ratios will be of benefit for increased tumor-selective therapy.

ACKNOWLEDGMENT

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LITERATURE CITED

- (1) Bagshawe, K. D., Springer, C. J., Searle, F., Antoni, P., Sharma, S. K., Melton, R. G., and Sherwood, R. F. (1988) A cytotoxic agent can be generated selectively at cancer sites. *Br. J. Cancer* 58, 700–703.
- (2) Senter, P. D., Wallace, P. M., Svensson, H. P., Vruthula, V. M., Kerr, D. E., Hellstrom, I., and Hellstrom, K. E. (1993) Generation of cytotoxic agents by targeted enzymes. *Bioconjugate Chem.* 4, 3–9.
- (3) Levvy, G. A., and Conchie, J. (1966) β -glucuronidase and the hydrolysis of glucuronides. In *Glucuronic acid, free and combined* (G. J. Dutton, Ed.) pp 301–364, Academic Press, New York.
- (4) Sedlacek, H.-H., Seemann, G., Hoffman, D., Czech, J., Lorenz, P., Kolar, C., and Bosslet, K. (1992) *Antibodies as carriers of cytotoxicity*, Karger, Basel.
- (5) Houba, P. H. J., Leenders, R. G. G., Boven, E., Scheeren, J. W., Pinedo, H. M., and Haisma, H. J. (1996) Characterization of novel anthracycline prodrugs activated by human β -glucuronidase for use in ADEPT. *Biochem. Pharm.* (in press).
- (6) Schlesinger, P. H., Rodman, J. S., Doebber, T. W., Stahl, P. D., Lee, Y. C., Stowell, C. P., and Kuhlenschmidt, T. B. (1980) The role of extra-hepatic tissues in the receptor-mediated plasma clearance of glycoproteins terminated by mannose or N-acetylglucosamine. *Biochem. J.* 192, 597–606.
- (7) Thorpe, P. E., Detre, S. I., Foxwell, B. M., Brown, A. N. F., Skilleter, D. N., Wilson, G., Forrester, J. A., and Stirpe, F. (1985) Modification of the carbohydrate in ricin with meta-periodate-cyanoborohydrate mixtures. *Eur. J. Biochem.* 147, 197–206.
- (8) Edwards, D. P., Grzyb, K. T., Dressler, L. G., Mansel, R. E., Zava, D. T., Sledge, G. W., and McGuire, W. L. (1986) Monoclonal antibody identification and characterization of a Mr 43,000 membrane glycoprotein associated with human breast cancer. *Cancer Res.* 46, 1306–1317.
- (9) Litvinov, S. V., Velders, M. P., Bakker, H. A. M., Fleuren, G. J., and Warnaar, S. O. (1994) Ep-CAM: a human epithelial antigen is a homophilic cell-cell adhesion molecule. *J. Cell. Biol.* 125, 437–446.
- (10) De Bree, R., Roos, J. C., Quak, J. J., den Hollander, W., Snow, G. B., and van Dongen, G. A. M. S. (1994) Clinical screening of monoclonal antibodies 323/A3, cSF-25 and K928 for suitability of targeting tumors in the upper aerodigestive and respiratory tract. *Nucl. Med. Commun.* 15, 613–627.
- (11) Riethmüller, G., Schneider-Gädick, E., Schlimok, G., Schmiegel, W., Raab, R., Höffken, K., Gruber, R., Pichlmaier, H., Hirche, H., Pichlmayr, R., Buggisch, P., and Witte, J. (1994) Randomised trial of monoclonal antibody for adjuvant therapy of resected Dukes' C colorectal carcinoma. *Lancet* 343, 1177–1183.
- (12) Weiss, R. B. (1992) The anthracyclines: will we ever find a better doxorubicin? *Semin. Oncol.* 19, 670–686.
- (13) Haisma, H. J., Boven, E., van Muijen, M., de Jong, J., van der Vijgh, W. J. F., and Pinedo, H. M. (1992) A monoclonal antibody- β -glucuronidase conjugate as activator of the pro-drug epirubicin-glucuronide for specific treatment of cancer. *Br. J. Cancer* 66, 474–478.
- (14) Bosslet, K., Czech, J., and Hoffmann, D. (1994) Tumor-selective prodrug activation by fusion protein-mediated catalysis. *Cancer Res.* 54, 2151–2159.
- (15) Meyer, D. L., Law, K. L., Kindle Payne, J., Mikolajczyk, S. D., Zarrinmayeh, H., Jungheim, L. N., Kling, J. K., Shepherd, T. A., and Starling, J. J. (1995) Site-specific prodrug activation by antibody- β -lactamase conjugates: pre-clinical investigation of the efficacy and toxicity of doxorubicin delivered by antibody directed catalysis. *Bioconjugate Chem.* 6, 440–446.
- (16) Vruthula, V. M., Senter, P. D., Fischer, K. J., and Wallace, P. M. (1993) Prodrugs of doxorubicin and melphalan and their activation by a monoclonal antibody-penicillin-G amidase conjugate. *J. Med. Chem.* 36, 919–923.
- (17) Leenders, R. G. G., Ruytenbeek, R., Damen, E. W. P., and Scheeren, J. W. (1996) Highly diastereoselective synthesis of anomeric β -O-glycopyranosyl carbamates from isocyanates. *Synthesis* (in press).
- (18) Hamilton, T. C., Young, R. C., McKoy, W. M., Grotzinger, K. R., Green, J. A., Chu, E. W., Whang-Peng, J., Rogan, A. M., Green, W. R., and Ozols, R. F. (1984) Characterization of a human ovarian carcinoma cell line (NIH:OVCA-3) with androgen and estrogen receptors. *Cancer Res.* 43, 5379–5389.
- (19) Watanabe, H., Grubb, J. H., and Sly, W. S. (1990) The overexpressed human 46-kDa mannose-6-phosphate receptor mediates endocytosis and sorting of β -glucuronidase. *Proc. Natl. Acad. Sci. U.S.A.* 87, 8036–8040.
- (20) Haisma, H. J., Hilgers, J., and Zurawski, V. R., Jr. (1986) Iodination of monoclonal antibodies for diagnosis and radiotherapy using a convenient one vial method. *J. Nucl. Med.* 27, 1890–1895.
- (21) Kyle, J. W., Grubb, J. H., Galvin, N., and Vogler, C. (1992) β -Glucuronidase (GUS) assay in animal tissue. *GUS protocols: using the GUS gene as a reporter of gene expression* (S. R. Gallagher, Ed.) pp 189–203, Academic Press, New York.
- (22) Lindmo, T., Boven, E., Cuttitta, F., Fedorko, J., and Bunn, P. A. (1984) Determination of the immunoreactive fraction of radiolabeled monoclonal antibodies by linear extrapolation to binding at infinitive antigen excess. *J. Immunol. Methods* 72, 77–89.
- (23) Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the bacteriophage T4. *Nature* 227, 680–685.
- (24) Shipley, J. M., Grubb, J. H., and Sly, W. S. (1993) The role of glycosylation and phosphorylation in the expression of active human β -glucuronidase. *J. Biol. Chem.* 268, 12193–12198.
- (25) Eccles, S. A., Court, W. J., Box, G. A., Dean, C. J., Melton, R. G., and Springer, C. J. (1994) Regression of established breast carcinoma xenografts with antibody-directed enzyme prodrug therapy against c-erbB2 p185. *Cancer Res.* 54, 5171–5177.
- (26) Sharma, S. K., Bagshawe, K. D., Burke, P. J., Boden, R. W., and Rogers, G. T. (1990) Inactivation and clearance of an anti-CEA carboxypeptidase-G2 conjugate in blood after localisation in a xenograft model. *Br. J. Cancer* 61, 659–662.
- (27) Haisma, H. J., Van Muijen, M., Scheffer, G., Schep, R. J., Pinedo, H. M., and Boven, E. (1986) A monoclonal antibody against human β -glucuronidase for application in antibody-directed enzyme prodrug therapy. *Hybridoma* 14, 377–382.

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TECHNICAL NOTES

Introduction of the Maleimide Function onto Resin-Bound Peptides: A Simple, High-Yield Process Useful for Discriminating among Several Lysines

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Incorporation of ϵ -Adpoc-lysine as a residue in solid phase peptide synthesis allows selective deprotection of this residue on the resin-bound peptide relative to other acid labile groups such as Boc. Premature resin cleavage is avoided. A maleimide group, a useful thiol-capture reagent, was readily introduced by reacting the liberated amino function with an acylating agent containing the maleimide functionality. Acidic cleavage from the resin, with an appropriate scavenging system, afforded peptides that are derivatized with a maleimide functionality on a specific lysine. This is advantageous for producing peptide-carrier conjugates of defined specificity, useful as immunogens, by maleimide-thiol coupling. The derivatization and resin removal chemistries appear to proceed in excellent yield with respect to the maleimide group. The structures were confirmed by tandem mass spectrometry.

INTRODUCTION

Addition of a sulfhydryl group to a maleimido function is a method widely used to conjugate two molecules (1). The reaction is rapid and forms a stable adduct which makes it suitable for preparing an immunogen comprised of an antigen and a carrier molecule such as a protein. Generally, maleimide functionality is introduced onto one of the reaction partners by acylating a pendant amine with one of several commercially available reagents such as the *N*-hydroxysuccinimide ester of β -maleimidopropionic acid (MPS).^{1,2} Thiols are appended on the other reaction partner by a variety of reagents or are synthesized as an integral part of the molecule, such as a cysteine residue in a peptide. The preferred direction of the reaction (i.e., thiolated carrier with maleimide-containing antigen or vice versa) will depend on the structural attributes of the two partners. Reasons why introduction of the maleimido group by MPS onto a protein might be disfavored include the following. (a) The change of charge type and increase of hydrophobicity can cause the protein to precipitate. (b) Characterization of

the extent of the maleimide reaction with the protein is more difficult than an Ellman assay for protein thiol groups. This is important because the maleimide functionality may hydrolyze to a maleamic functionality which does not add thiol readily. (c) Metastable acylation of protein hydroxyls by MPS can be a complicating factor. (d) Excision of β -alanine from the 3-maleimidopropionyl moiety on acid hydrolysis provides a marker amino acid if it is incorporated into the peptide allowing definition of peptide levels in the conjugate. This option is not available if the maleimide group is initially on the protein. These problems are readily avoided if a pure maleimide-containing peptide is isolated and characterized by NMR, mass spectroscopy, and amino acid analysis.

There has been increasing interest in the past 30 years in using small peptides to generate antibodies that recognize congruent sequences in intact proteins (2, 3). The applications for such systems are vaccines, affinity columns, and analytical technologies such as the ELISA method. Chemosterilants have been prepared by conjugating the peptide hormone GnRH to a toxin (4). Such peptidyl antigens are commonly prepared by solid phase synthesis using Fmoc or Boc chemistries (5). They can be made to contain amino groups capable of acylation, either in lysine moieties or in N-terminal residues, and thus are amenable to maleimide introduction by acylation with the aforementioned *N*-hydroxysuccinimide ester. In general, this has been accomplished by solution chemistry after the peptide has been released from the solid support and purified. This approach involves a number of shortcomings, such as the need, generally, for two chromatographies with the accompanying losses in yield and time. Of more importance is the case which involves peptides with more than one amino group. Since the antigenic nature of a peptide will vary with the specific residue in its chain that is modified, maleimiding a particular residue can be critical to raising the desired antibodies. The usual protection schemes and the acid-

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¹Abbreviations: Adpoc, 1-(1'-adamantyl)-1-methylethoxycarbonyl; Boc, *tert*-butoxycarbonyl; DIEA, diisopropylethylamine; DMF, *N,N*-dimethylformamide; Fmoc, 9-fluorenylmethyloxycarbonyl; GnRH, gonadotropin-releasing hormone; HPLC, high-pressure liquid chromatography; MeCN, acetonitrile; MPS, 3-maleimidopropionic acid *N*-hydroxysuccinimide ester; LC-MS, liquid chromatography-mass spectrometry; MS, mass spectrometry; NMP, *N*-methylpyrrolidinone; Pgl, pyroglutamic acid; Pmc, 2,2,5,7,8-pentamethylchroman-6-sulfonyl; TFA, trifluoroacetic acid.

²Other available maleimido-containing acylating agents include the acetic, butyric, caproic, 4-methylenecyclohexane-1-carboxylic, and 3-benzoic acid analogs.

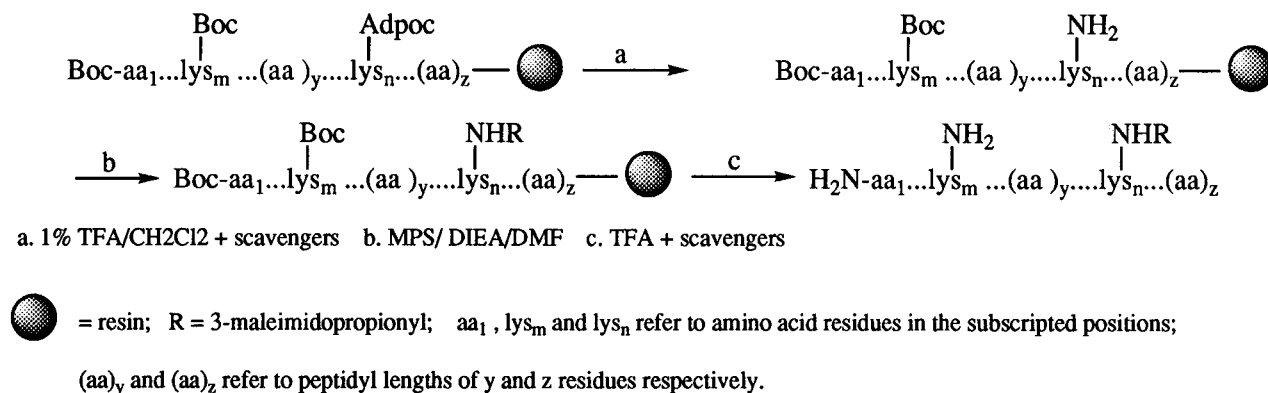


Figure 1. Reaction sequence for preparing a peptide with a specific maleimidolysine.

effected resin cleavages involved in the Fmoc and Boc chemistries are not compatible with generating a unique amino group that can be acylated to form a stable maleimide function.³ As a consequence, one is faced with effecting a partial acylation and a difficult separation to obtain a peptide with a particular lysine that is derivatized.

We report herein conditions which allow acylation with MPS of a resin-bound peptide, affording excellent yields of the cleaved, maleimide-containing product, and a method using the very acid sensitive 1-(1'-adamantyl)-1-methylethoxycarbonyl (Adpoc) (6) ϵ -aminolysine protection. This lysine derivative, which is commercially available, allows selective introduction of the maleimide group when employed in the synthesis of a peptide containing multiple lysines. Figure 1 outlines an example of the multiple lysine-Adpoc scenario.

EXPERIMENTAL PROCEDURES

Preparation of [Lys⁶]GnRH (I) on Resin. The peptide was synthesized on an Applied Biosystems (ABI, Foster City, CA) 431 peptide synthesizer using Fmoc chemistry on a 0.25 mmol scale. Fmoc amide resin (ABI) was used to allow for a C-terminal amide upon cleavage. Prepacked amino acid cartridges (ABI) were used directly, except for pyroglutamic acid (Sigma Chemical Co., St. Louis, MO) and Fmoc-Lys(Adpoc) (Bachem Bioscience Inc., King of Prussia, PA) which were packed by hand. The synthesizer was programmed for synthesis using SynthAssist v1.0.0 (ABI). All residues were programmed for single coupling, using appropriate single-couple cycles included in SynthAssist. The exceptions include Gly¹⁰ and Arg⁸, which were programmed for double coupling using appropriate double-couple cycles included in SynthAssist and Pgl which was programmed using a modified final single-couple cycle in which the eleventh and twelfth modules in the SynthAssist cycle (calling for N-terminal Fmoc deprotection by piperidine and NMP washes) were deleted. After the synthesis, the resin was washed twice with methanol and then dried while on the synthesizer.

Selective Deprotection and Maleimide Introduction onto [Lys⁶]GnRH (I) on Resin. To remove the Adpoc protecting group of Lys⁶, the peptide-loaded resin was covered with 20 mL of 5% TFA in CH₂Cl₂ (v/v) and stirred for 30 min. The resin was then filtered, washed

with CH₂Cl₂, and then transferred to a fritted reaction vessel. The resin was washed three times with DMF in the reaction vessel and then covered with 15 mL of DMF. DIEA (10 equiv) was added to the solution, followed by MPS (4 equiv). This was allowed to agitate by N₂ sparging for 3.5 h, following which an additional 1 equiv of MPS was added. This was allowed to react for 1 h, and then the reaction solution was drained and the resin washed three times with DMF followed by three washes with CH₂Cl₂.

Cleavage and Purification of Unmodified and Modified [Lys⁶]GnRH (I and I-6). The respective peptide was cleaved using 10 mL of a 90% TFA/5% thioanisole/3% H₂O/2% anisole (v/v/v/v) solution. After being stirred for 2 h, the resin was filtered, the filtrate concentrated under high vacuum, and the residue triturated by stirring with 30 mL of diethyl ether for 30 min. The crude peptide was purified by HPLC [flow rate of 13.0 mL/min, monitor = 230 nm, gradient of 20 to 40% MeCN_{aq}/0.1% TFA over 40 min, Deltapak RCM 2 × (25 mm × 10 cm) C₁₈ column]. The isolated peptide was characterized by MS, analytical HPLC (which showed a single peak), and amino acid analysis on a Beckman 6300 amino acid analyzer.

Preparation of FEMAKIDVKT Peptides (II, II-5, and II-9) on Resin. The peptides were synthesized as above except Fmoc-Thr (tBu)-Wang resin (Bachem California, Torrance, CA) was used. Prepacked amino acid cartridges (ABI) were used directly, except for Fmoc-Lys(Adpoc) (Bachem Bioscience Inc.) which was packed by hand. This residue was used in the 5 and 9 position for the respective peptides that were to be modified. It was also used only in the 9 position for the peptide that was not to be modified. Most residues were programmed using the appropriate SynthAssist single-couple cycles. The exceptions were Met³, Ala⁴, Lys⁵, and Ile⁶ which were programmed using the appropriate SynthAssist double-couple cycles. The N-terminal Phe used was α -N-Boc-protected, and this residue was programmed using the aforementioned modified final single-couple cycle to eliminate the final piperidine deprotection and NMP wash modules. After synthesis, the resins were washed twice with methanol and then dried while on the synthesizer.

Selective Deprotection and Maleimide Introduction onto FEMAKIDVKT (II-5 and II-9) on Resin. The Adpoc protecting group of the particular Lys residue was selectively removed by covering the respective resin with 10 mL of 1% TFA (the use of this level of TFA is effective in this case and should preempt acid-effected reactions which are undesirable) in CH₂Cl₂ (v/v) to which 1% 1,2-ethanedithiol (v/v) and 1% anisole (v/v) had been added. After being stirred 30 min, the resin was filtered

³It has been demonstrated that the maleimide function is rapidly destroyed by piperidine which is commonly used to remove Fmoc (M. Rivetna, private communication). Hydrogenolytic removal of the usual mild-acid stable groups such as benzyloxycarbonyl is incompatible with the maleimide functionality.

in a fritted reaction vessel and washed three times with CH_2Cl_2 . The resin was then washed twice with DMF and then covered with 10 mL of DMF. To this was added DIEA (10 equiv) followed by MPS (4 equiv), and the solution was allowed to react with N_2 sparging for 1 h. The reaction solution was then drained and the resin washed three times with DMF. The resin was then again covered with 10 mL of DMF, and DIEA (10 equiv) and MPS (4 equiv) were added. After aging for 1 h, the solution was drained and the resin washed twice with DMF and then twice with CH_2Cl_2 .

Cleavage and Purification of Unmodified FEMAKIDVKT (II). This peptide, which had been synthesized with Lys(Adpoc) in the 9 position, was subjected to an Adpoc deprotection scheme similar to the one used for the peptides that were to be further modified. However, after Adpoc deprotection, the peptide was then carried directly through the cleavage–purification procedure rather than the modification protocol. The peptide was cleaved by covering the resin with 10 mL of a cleavage solution consisting of 90% TFA/5% thioanisole/3% 1,2-ethanedithiol/2% anisole (v/v/v/v) and stirring for 2 h. The resin was then separated by filtration, and the filtrate was concentrated. The crude peptide was triturated by stirring in 30 mL of diethyl ether for 30 min and then collected by filtration. It was purified by HPLC (flow rate of 10.0 mL/min, monitor = 230 nm, gradient of 10 to 20% MeCN_{aq} /0.1% TFA over 30 min, Deltapak RCM 25 mm $\times$ 10 cm C_{18} column). The isolated peptide was characterized by MS, analytical HPLC (which showed a single peak), and amino acid analysis on a Beckman 6300 amino acid analyzer.

Cleavage and Purification of Modified FEMAKIDVKT Peptides (II-5 and II-9). The particular peptide was cleaved from its resin by stirring for 2 h in 10 mL of a cleavage solution consisting of 90% TFA/5% thioanisole/3% H_2O /2% anisole (v/v/v/v). The resin was separated by filtration, and the filtrate was concentrated. The crude peptide was triturated by stirring in 30 mL of diethyl ether for 30 min and then collected by filtration. It was purified by HPLC [flow rate of 13.0 mL/min, monitor = 230 nm, gradient of 20 to 40% MeCN_{aq} /0.1% TFA over 30 min, Deltapak RCM 2 $\times$ (25 mm $\times$ 10 cm) C_{18} column] and then characterized by MS, analytical HPLC (which showed a single peak), and amino acid analysis. Sequence characterization to determine the site of maleimide introduction was effected by tandem mass spectrometry.

Yield Determinations. Amino acid analysis was used to determine the peptide content of all purified and characterized peptide materials (modified and unmodified samples of both [Lys⁶]GnRH and FEMAKIDVKT). These materials were used to generate standard curves for each material using an HPLC-peak height method. Peptide yield in the crude cleavage mixtures of all the peptide entities was determined by this method. Calculations using these data and the theoretical yields as predicted by using the manufacturer's stated resin loading were made to determine overall yields for each peptide.

Mass Spectrometry. All mass spectra were recorded on a Finnigan MAT (San Jose, CA) TSQ-700 triple-stage quadrupole mass spectrometer as described below and previously (7). Peptides were analyzed by LC–MS and LC–MS/MS as follows. Aliquots of sample were injected onto an ABI 130 HPLC column (ABI) and loaded onto a Brownlee 2.1 $\times$ 100 mm octyl (C_8) reverse phase LC column. A gradient was used to elute the peptide from the column through an in-line UV detector (220 nm) and into the electrospray ionization source of the spectrom-

Table 1. Yields of Peptides

peptide	% peptide in crude ^{a,b}	% yield ^c
Pgl-HWSYKLRPG-NH ₂ (I)	35.6	31.2
Pgl-HWSYK*LRPG-NH ₂ (I-6) ^d	50.4	30.9
FEMAKIDVKT (II)	56.7	45.2
FEMAK*IDVKT (II-5)	70.4	81.2
FEMAKIDVK*T (II-9)	59.7	67.3

^a Mass of peptide, determined using HPLC-peak height method, per unit mass of crude $\times$ 100. ^b Obtained by single extraction of resin using the TFA scavenger system. ^c Mass of peptide in crude divided by mass expected based on manufacturer's listed loading of resin $\times$ 100. ^d K* = ϵ -maleimidopropionyllysine.

eter. The gradient was as follows: 2 to 70% B in 20 min [buffer A, water (0.1% TFA); buffer B, MeCN (0.085% TFA)]. For LC–MS, the first quadrupole of the mass spectrometer was scanned over a mass to charge ratio (m/z) range of 350–2000 in 2 s, and the second and third quadrupoles were operated to pass all ions. For LC–MS/MS, precursor ions were selected in the first quadrupole and focused into the second quadrupole containing 5 mTorr argon. Collision energies ranging from 25 to 35 eV were used. Product ions were mass separated in the third quadrupole of the instrument over a m/z range of 50–1400 in 2 s. Data for LC–MS and LC–MS/MS were acquired in centroid mode and analyzed using Finnigan ICIS software and the Finnigan BioMass software package. Typically, mass spectra were generated from the summation of 10–30 scans. The instrument was calibrated over the mass range of interest using a mixture of apomyoglobin and a tetrapeptide (Met-Arg-Phe-Ala). For LC–MS, typical mass accuracies were 0.01%. For LC–MS/MS, mass accuracies were 0.5%.

RESULTS AND DISCUSSION

The work addresses the following issues: (a) efficacy (i.e., yield) of the process, (b) compatibility of the process with the common functionalities and protecting groups found in peptidyl residues, and (c) confirmation of the identity of the products showing an absence of rearrangements. Two systems were used to define these parameters. One was the [Lys⁶]GnRH analog Pgl-HWSYKLRPG-NH₂⁴ (**I**) using Fmoc chemistry and Adpoc lysine protection. It has been shown by Voelter and his associates that GnRH can be synthesized on an acid labile resin using Adpoc protection chemistry (8), indicating that premature resin cleavage would not be a problem. The other system was the model peptide FEMAKIDVKT (**II**) which was selectively derivatized on Lys⁵ and Lys⁹, affording the maleimidopropionyl products **II-5** and **II-9**, respectively.

(a) Calculation of the percent yield based on the isolated yield and the manufacturer's resin loading titer would not take into account nonoptimized cleavage and purification procedures and loss of stated resin loading titers by aging. Therefore, the isolated and characterized peptide and maleimide-functionalized peptide materials were quantitated for their peptide content by amino acid analysis. They were then used as references for yield determinations in the crude cleavage products using an HPLC assay. Although only 31.2% (based on the theoretical loading of the resin) of the peptide (**I**) (which does not contain a maleimide group) was present in its crude, 30.9% of the analog containing the maleimide group was present in its crude, indicating that the maleimide introduction on the resin was about 99% efficient for the GnRH peptide. Table 1 indicates yields determined by

⁴The conventional one-letter symbols for the amino acids are used.

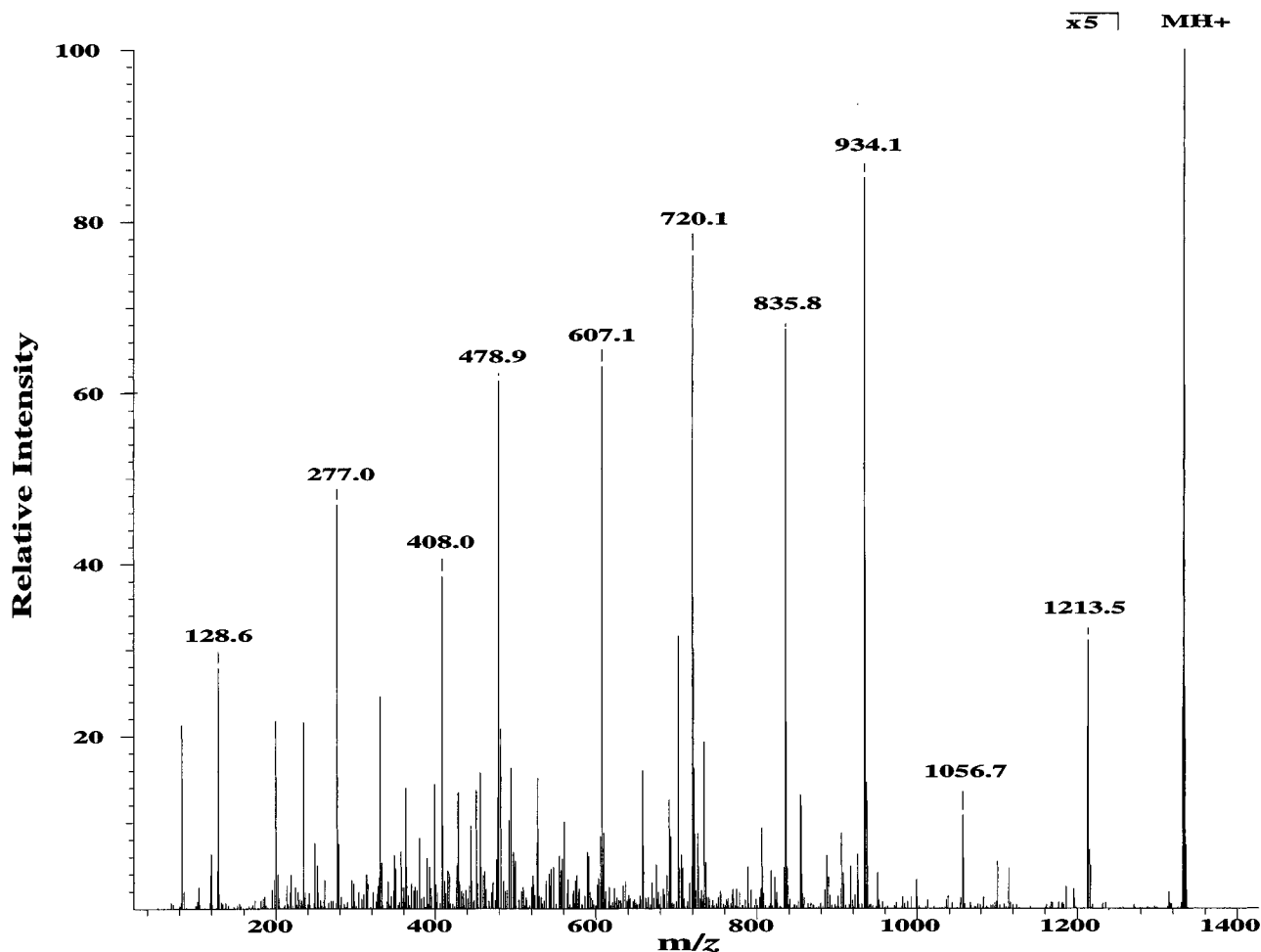


Figure 2. Product ion spectrum of **II-9**.

HPLC, and it is probable that increases in the yield can be effected by engineering optimal cleavage scenarios and synthetic protocols. It is, however, clear that the process of introducing a maleimide is a high-yield step.

(b) Together, peptide substrates **I** and **II** encompass all the natural amino acids except for N, Q, and C. Cysteine would be internally incompatible with a maleimide function. *N*-Boc, *O*-*tert*-butyl, guandino-Pmc, and *N*_{im}-trityl protections were employed in the syntheses. The commonly employed TFA cleavage system using a 5% thioanisole/3% H₂O/2% anisole scavenger system, which avoids a thiol component, seems effective. Therefore, a reasonable cross section of peptide protecting group types seems amenable to the method sequence: Adpoc deprotection (1% TFA/CH₂Cl₂), maleimide introduction, and resin cleavage.

(c) **II-5** and **II-9** have distinct retention volumes in the HPLC, and it was found that maleimidopropionyl crossover products do not occur in crude **II-5** and **II-9**. The structural identity of each of these was established as follows. The isolated material was shown by LC-MS to have the correct mass of 1332.9. For LC-MS/MS analysis, singly charged ions at *m/z* 1332.9 were mass selected in the first quadrupole of the instrument and collisionally activated with argon. The resultant fragmentation spectrum is displayed in Figure 2 for **II-9**, and Table 2 summarizes the results from these experiments. A rich series of B-type fragment ions is seen for both **II-5** and **II-9** (9). For **II-9**, the presence of ions at *m/z* 607.8, 720.9, 836.0, and 935.1 and the absence of ions at *m/z* 758.8, 871.9, 987.0, and 1086.1 demonstrate that this peptide was modified at Lys⁹ and not at Lys⁵.

Table 2. Fragmentation of Lysine-Modified Peptides **II-5 and **II-9** by MS/MS^a**

II-5			II-9		
	predicted mass for B-type fragment ion	observed mass (Da)		predicted mass for B-type fragment ion	observed mass (Da)
B1	148.2	ND ^b	B1	148.2	ND ^b
B2	277.3	277.0	B2	277.3	276.9
B3	408.5	408.0	B3	408.5	408.0
B4	479.6	479.0	B4	479.6	478.9
B5	758.8	758.4	B5	607.8	607.1
B6	871.9	872.5	B6	720.9	720.1
B7	987.0	986.5	B7	836.0	835.8
B8	1086.1	1085.5	B8	935.1	934.4
B9	1214.3	1213.7	B9	1214.3	1214.2
B10	1315.4	1314.7	B10	1315.4	1314.9

^a MH⁺ ions of mass 1332.9.

A similar set of experiments with **II-5** showing the presence of ions at *m/z* 758.8, 871.9, 987.0, and 1086.1 and the absence of ions at *m/z* 607.8, 720.9, 836.0, and 935.1 demonstrate that this peptide was modified at Lys⁵ and not at Lys⁹.

In summary, a synthetic protocol has been developed which allows the deprotection of a specific ϵ -amino group on a lysine residue incorporated into a resin-bound peptide. The freed amino group can be reacted with an acylating agent which incorporates a maleimido group and the resulting derivative cleaved from the resin in high yield. The product maleimidepeptide can be conjugated to a thiol-containing molecule such as a protein, affording a material useful as an immunogen, or a thiol-containing resin, affording an affinity column.

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LITERATURE CITED

- (1) Peeters, J. M., et al. (1989) Comparison of four bifunctional reagents for coupling peptides to proteins and the effect of the three moieties on the immunogenicity of the conjugates. *J. Immunol. Methods* 120, 133–143.
- (2) Moser, R., et al. (1985) Applications of Synthetic Peptides. *Angew. Chem., Int. Ed. Engl.* 24, 719–727.
- (3) Walter, G. (1986) Production and use of antibodies against synthetic peptides. *J. Immunol. Methods* 88, 149–161.
- (4) Rusiecki, V. K., et al. (1995) GnRH-toxin chimera as chemosterilants: Synthesis and conjugation of GnRH analogs to truncated bacterial toxins. In *Peptides 1994* (H. L. S. Maia, Ed.) pp 755–766, Escom Press, Leiden.
- (5) Atherton, E., and Sheppard, R. C. (1989) *Solid Phase Peptide Synthesis-A Practical Approach*, IRL Press at Oxford University Press, Oxford, England.
- (6) Kalbacher, H., and Voelter, W. (1978) 1-(1-Adamantyl)-1-methylethoxycarbonyl: A New protecting Group for Peptide Synthesis. *Angew. Chem., Int. Ed. Engl.* 12, 944–945.
- (7) Griffin, P. R., Coffman, J. A., Hood, L. E., and Yates, J. R., III (1991) Structural Analysis of Proteins by Capillary HPLC Electrospray Tandem Mass Spectrometry. *Int. J. Mass Spectrom. Ion Processes* 111, 131–149.
- (8) Shao, J., et al. (1991) A Test Case for the 1-(1-Adamantyl)-1-methylethoxycarbonyl (Adpoc) Group: Solid Phase Synthesis of LH-RH using N^α-Adpoc Protection and an Acid Labile Handle. *Tetrahedron Lett.* 32, 345–346.
- (9) Roepstorff, P., and Fohlman, J. (1984) Proposal for a common nomenclature for sequence ions in mass spectra of peptides. *Biomed. Mass Spectrom.* 11, 601.

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ARTICLES

Syntheses of Functionalized Indium Porphyrins for Monoclonal Antibody Labeling

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The syntheses and characterizations of five differently hydrosoluble monosubstituted aryl porphyrins are reported. Their metallation with indium-111 was achieved and provided tracers with strong specific activities. The covalent coupling between indium-111 porphyrins and BSA served as a model reaction for the definition of the best experimental coupling conditions; the transposition to the labeling of anti-CEA monoclonal antibody was realized. The conjugates exhibited an *in vitro* good mAb-labeling efficiency, as well as a good preservation of immunoreactivity.

INTRODUCTION

The attachment of radioactive metal ions to monoclonal antibodies for cancer diagnosis and therapy has gained considerable interest in recent years (1–5). Radionuclides of interest for radioimmunoimaging should emit γ -ray energy of greater than 120 keV (which can be detected by conventional scintillation cameras), have a short half-time (24–72 h), and have a rapid clearance rate in order to obtain the maximum tumor-to-background ratio (6). Indium-111 strictly a γ -emitter, is well-suited for these purposes, and tumor uptake of antibodies labeled with it is generally higher than that with iodine-labeled antibodies (7). Chelators that can hold radio-metals with high stability under physiological conditions

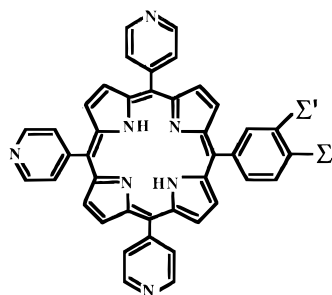
are essential to avoid excessive radiation damage to nontarget cells. This led to the development of bifunctional chelating agents possessing functional groups capable of binding a metal ion and allowing covalent linkage to proteins. In many of the numerous approaches, poly(aminocarboxylic acids) (EDTA, DTPA, and their derivatives) have been covalently bound to mAbs and complexed with indium-111 (8–10). It is essential for effective imaging that the radioactive metal ion remains complexed by the mAb–chelator conjugate. The loss of In-111 from its chelate *in vivo* depends on the rate at which In ligands exchange with other ligands; these are dependent on the metal involved, but they are strongly influenced by the structure of the chelator (11). Chelators containing highly basic groups [e.g. poly(aminocarboxylates)] will bind H^+ vigorously at physiological pH, and this can reduce the effective value of the stability constant by a large factor. Many of the radiometal chelates formed from these chelating agents are not stable enough to prevent transcomplexation to serum proteins such as transferrin or conalbumin (12). Efforts to enhance the stability of these complexes under physiological conditions have afforded new analogues of

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[(3-Σ'-4-Σ)-P]Py₃P H₂

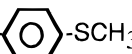
1-	Σ' = NO ₂	Σ = F
2-	Σ' = H	Σ = F
3-	Σ' = H	Σ = O(CH ₂) ₄ NH ₂
4-	Σ' = H	Σ = OCH ₂ COOH
5-	Σ' = H	Σ = OCH ₂ -COO-  -SCH ₃

Figure 1. Structures of porphyrins synthesized in this study.

DTPA. The addition of branching groups to the ethylenediamine backbone of DTPA has been achieved and led to more stable chelates (13–15). Bifunctional derivatives of DTPA have been developed in which the carbon backbone of the chelate has a linking group inserted for attachment to the antibody such that all eight coordination sites on DTPA are preserved (16).

In the same way, there has also been considerable interest in the development of macrocyclic poly(amino-carboxylate) chelators (17, 18). Particularly, derivatives of DOTA form extremely stable complexes with a variety of radiometals (19). This type of macrocyclic agent offers promise for antibody labeling.

As an alternative to the many methods reported in the literature, we have developed bifunctional chelating porphyrinic agents to label antibodies with indium-111. We selected porphyrins as ¹¹¹In chelating agents to be conjugated to antibodies for a main reason. Tetrapyrrolic macrocycles of indium belong to the class III type (20) and are stable *in vivo* against demetallation (21).

We have previously performed model-coupling reactions between symmetrical and unsymmetrical meso-substituted (carboxyphenyl)indium porphyrins and amino acid ester derivatives and defined the best experimental conditions yielding the amide derivatives (22).

Also, several monoclonal IgG and F(ab')₂ fragments were labeled *in vitro* with the indium-111 derivative of meso-5-(p-carboxyphenyl)-10,15,20-tritolylporphyrin (TTCPP¹¹¹InCl) using the ester-activated method [*N*-hydroxysuccinimide/1-ethyl-3-[3-(dimethylamino)propyl]-carbodiimide] (23). Up to 10 molecules of tracer were bonded to mAb and 1.5 to its F(ab')₂ fragment. The absence of any significant differences in the results of ELISA tests carried out with the metallated, unmetallated, and unmodified antibody suggested that immunoreactivity was not altered by covalent linkage between indium porphyrin and mAbs.

In this report, we describe the syntheses of hydro-soluble and monofunctionalized meso-tripyridinylporphyrins (Figure 1), more compatible with the biological medium and possessing various function susceptible to covalent coupling, not only to amino group side chains but also to carboxylic, thiol, alcohol, and phenol groups as well as to aldehyde functions generated by oxidation of carbohydrate moieties. We report the preparation and

characterization of these chelates with indium(III) and finally describe the syntheses of the conjugates of the indium porphyrin derivatives and bovin serum albumin (BSA) and a whole monoclonal antibody, the anticarcinoma embryonic monoclonal antibody (anti-CEA).

MATERIALS AND METHODS

Reagents (analytical grade) were used without further purification.

General Methods. ¹H NMR spectra were recorded at 25 °C on Hitachi Perkin-Elmer R-24B (60 MHz) and Bruker WM 250 spectrometers operating at 200 MHz FT. Unless otherwise specified, the solvent was deuteriochloroform with TMS as the internal standard. The chemical shifts, δ, are given in parts per million, and coupling constants are in hertz. Abbreviations used were as follows: br, broad; d, doublet; dd, doublet of doublet; m, multiplet; o, octet; q, quadruplet; s, singlet; and t, triplet.

Optical spectra in the Soret and visible region were measured on a Beckman-25 spectrophotometer, and infrared spectra were recorded on a Perkin-Elmer 683 spectrometer; the samples were prepared as a 0.5% dispersion in KBr pellets.

Mass spectrometry was carried out using a Micromass 16F instrument for the aldehydes and a Nermag R10-10B instrument for desorption ionization (DI-MS). FAB-MS studies were carried out in the Laboratoire de Spectrométrie de Masse, CNRS, BP 22, F-69399 Vernaison, France; the FAB-MS matrices used were thioglycerol and 3-nitrobenzyl alcohol (NBA).

HPLC analysis and preparations were carried out on a Varian 5000 Liquid Chromatograph coupled with a Varian 2550 multiwavelength detector set at 420 nm or a Waters 590 Programmable HPLC pump. An analytical silica column, Micro Pak Si 10 (4.6 × 250 mm, 20 μm particles, 60 Å pore size, column 1), or a preparative silica column (40 × 200 mm, 20 μm particles, 60 Å pore size, column 2) was used for un-ionized free base porphyrins and their indium derivatives; for the quaternized macrocycles, free bases, and indium derivatives, a Nucleosil C4 column was used (4.6 × 250 mm, 10 μm particles, 300 Å pore size, column 3).

TLC microplates of silica gel were used.

Liquid chromatography was performed on a silica gel column (25 × 200 mm, 230–400 mesh), and anion exchange resin Dowex 1 × 8 (200–400 mesh, chloride form) was used for quaternized free bases and indium derivatives.

Porphyrin Syntheses. meso-10-(3-Nitro-4-fluorophenyl)-5,15,20-tri(4'-N-pyridinyl)porphyrin (1). 3-Nitro-4-fluorobenzaldehyde. Following a published procedure (24), a mixture of concentrated sulfuric acid (94 mL) and nitrite-free nitric acid (12.5 mL) was cooled to –5 °C and under argon was treated dropwise with 4-fluorobenzaldehyde (23.8 g, 191.5 mmol) while the temperature was kept below 5 °C. Then the reaction mixture was allowed to stand at room temperature for 3 h and then poured on ice (500 g). The nitro derivative precipitated and was filtered off through a sintered glass frit. The solid was washed with water and dried. Recrystallization from diethyl ether gave the pure aldehyde (70%, 16.6 g, 134 mmol). Mp: 46 °C [lit. (24) 46.5 °C]. NMR (60 MHz, CDCl₃): 7.5 (q, 1H), 8.4–8.06 (m, 1H), 8.56 (dd, 1H), 10.03 (s, 1H). MS: *m/e* (relative intensity) 170 (MH⁺, 8.5), 168 (M⁺ – 1, 100).

Porphyrin 1. 4-Pyridinecarboxaldehyde (7.13 g, 66.6 mmol) and 3-nitro-4-fluorobenzaldehyde (2.25 g, 13.3 mmol) were added to propionic acid (200 mL), and the mixture was brought to reflux. Pyrrole (5.37 g, 80 mmol)

Table 1. 250 MHz ^1H NMR Chemical Shifts of the Protons in Free Base Porphyrins^a

$\text{A} = \text{C} = \text{D} =$ $; \text{B} = \text{Ph} \Sigma$

compound	Σ	solvent	pyrrole	pyridyl	phenyl	NH	Σ
1	3'-NO ₂ , 4'-F	CDCl ₃	8.89 (d, 2H), $J = 4.9$ Hz 8.87 (s, 4H: 2,3,17,18) 8.82 (d, 2H), $J = 4.9$ Hz	9.07 (d, 6H: 3',5'), $J = 5.5$ Hz 8.18 (d, 6H: 2',6'), $J = 5.3$ Hz	8.92 (m, H ₂) 8.47 (o, H ₆), $J_{\text{HHo}} = 8.5$ Hz $J_{\text{HHm}} = 2.3$ Hz $J_{\text{HFm}} = 4.2$ Hz 7.74 (dd, H ₅ '), $J_{\text{HHo}} = 8.5$ Hz $J_{\text{HFo}} = 10.4$ Hz	-2.92 (s, 2H)	
2	4'-F	CDCl ₃	8.90 (d, 2H), $J = 4.8$ Hz 8.85 (s, 4H: 2,3,17,18) 8.82 (d, 2H), $J = 4.8$ Hz	9.06 (d, 6H: 3',5'), $J = 5.9$ Hz 8.18 (d, 6H: 2',6'), $J = 5.9$ Hz	8.15 (m, 2H: 2',6') 7.49 (dd, 2H: 3',5'), $J = 8.8$ Hz	-2.91 (s, 2H)	
6	4'-O(CH ₂) ₄ NC ₈ H ₄ O ₂	CDCl ₃	8.96 (d, 2H), $J = 4.9$ Hz 8.85 (s, 4H: 2,3,17,18) 8.81 (d, 2H), $J = 4.9$ Hz	9.04 (d, 6H: 3',5'), $J = 5.7$ Hz 8.16 (d, 6H: 2',6'), $J = 5.7$ Hz	8.08 (d, 2H: 2',6'), $J = 8.6$ Hz 7.27 (d, 2H: 3',5'), $J = 8.6$ Hz	-2.88 (s, 2H)	phtha: 7.86 (m, 2H), 7.7 (m, 2H) OCH ₂ : 4.28 (m, 2H) NCH ₂ : 3.87 (m, 2H)
3	4'-O(CH ₂) ₄ NH ₂	CDCl ₃	8.96 (d, 2H), $J = 4.8$ Hz 8.85 (s, 4H: 2,3,17,18) 8.81 (d, 2H), $J = 4.8$ Hz	9.03 (d, 6H: 3',5'), $J = 5.8$ Hz 8.15 (d, 6H: 2',6'), $J = 5.8$ Hz	8.08 (d, 2H: 2',6'), $J = 8.6$ Hz 7.27 (d, 2H: 3',5'), $J = 8.6$ Hz	-2.86 (s, 2H)	[CH ₂] ₂ : 2.04 (m, 4H) OCH ₂ : 4.27 (t, 2H) H ₂ NCH ₂ : 2.90 (t, 2H) [CH ₂] ₂ : 2.02 (m, 4H)
7	4'-OCH ₂ COOEt	CDCl ₃	8.95 (d, 2H), $J = 4.9$ Hz 8.85 (s, 4H: 2,3,17,18) 8.82 (d, 2H), $J = 4.9$ Hz	9.05 (d, 6H: 3',5'), $J = 5.9$ Hz 8.16 (d, 6H: 2',6'), $J = 5.9$ Hz	8.13 (d, 2H: 2',6'), $J = 8.7$ Hz 7.32 (d, 2H: 3',5'), $J = 8.7$ Hz	-2.87 (s, 2H)	OCH ₂ CO: 4.93 (s, 2H) CH ₂ Me: 4.42 (q, 2H), $J = 7.1$ Hz CH ₂ CH ₃ : 1.42 (t, 3H), $J = 7.1$ Hz
4	4'-OCH ₂ COOH	CD ₃ COOD	9.00 (m, 8H)	9.29 (m, 6H: 3',5') 8.64 (m, 6H: 2',6')	8.16 (m, 2H: 2',6') 7.36 (m, 2H: 3',5')	—	OCH ₂ : 4.99 (s, 2H)
5	4'-OCH ₂ COOPhSMe	CDCl ₃	8.96 (d, 2H), $J = 4.8$ Hz 8.87 (s, 4H: 2,3,17,18) 8.83 (d, 2H), $J = 4.8$ Hz	9.08 (d, 6H: 3',5'), $J = 5.9$ Hz 8.2 (d, 6H: 2',6'), $J = 5.9$ Hz	8.15 (d, 2H: 2',6'), $J = 8.6$ Hz 7.35 (d, 2H: 3',5'), $J = 8.6$ Hz	-2.89 (s, 2H)	PhSMe: 7.71 (m, 2H), 7.54 (m, 2H) OCH ₂ : 5.06 (s, 2H) SCH ₃ : 2.5 (s, 3H)

^a Residual absorptions from TMS for CHCl₃ (7.25 ppm) and CH₃COOH (2.06 ppm).

was then added, and the mixture was refluxed for a further 1 h and concentrated to dryness. The solid obtained was dissolved in DMF and the resulting solution allowed to cool overnight at 0 °C. The purple slurry of mixed porphyrins (1.46 g) containing the six possible macrocycles was then filtered and fractionated by preparative silica gel liquid chromatography (bed volume, 390 mL) with a gradient elution (CHCl₃ containing 0 to 4% methanol). Porphyrin **1** was eluted with CHCl₃/CH₃OH (97/3) in a 1.9% yield (1.72 mg, 0.25 mmol). NMR (CDCl₃): Table 1. Electronic absorption spectrum (CHCl₃): λ_{max} (ϵ/ϵ_{512}) 417, 512.5 (1), 546 (0.36), 587 (0.39), 643 nm (0.12). FAB-MS: m/e (relative intensity) 681 (MH⁺, 65), 391 (100), 307 (61). IR (KBr): 540 (NO₂), 1130 cm⁻¹ (CF).

meso-10-(4-Fluorophenyl)-5,15,20-tri(4'-*N*-pyridinyl)-porphyrin (**2**). We followed the same protocol as for porphyrin **1**, using 4-fluorobenzaldehyde (1.65 g, 13.3 mmol). In this case, the total crude material was dissolved in the minimum amount of DMF and precipitated by the addition of methanol. Porphyrin **2** was isolated from the six macrocycles (2.5 g) by column chromatography on silica gel (bed volume, 390 mL) with CHCl₃/CH₃OH (97/3) as eluent (2.6%, 222.5 mg, 0.35 mmol). NMR (CDCl₃): Table 1. Electronic absorption spectrum (CHCl₃): λ_{max} (ϵ/ϵ_{511}) 415.5, 511.5 (1), 545.5

(0.32), 586.5 (0.31), 643 nm (0.12). FAB-MS: m/e (relative intensity) 636 (MH⁺, 39), 391 (68), 149 (100). IR (KBr): 1085 cm⁻¹ (CF).

meso-10-[4-(4-Aminobutoxy)phenyl]-5,15,20-tri(4'-*N*-pyridinyl)porphyrin (**3**). 4-(Phthalimidobutoxy)benzaldehyde. 4-Hydroxybenzaldehyde (2.44 g, 0.02 mol), *N*-(4-bromobutyl)phthalimide (5.64 g, 0.02 mol), and anhydrous K₂CO₃ (13.82 g, 0.1 mol) were mixed in anhydrous DMF (100 mL). The mixture was stirred at room temperature for 5 h. After filtration and washings with water, the solution was extracted with chloroform. The organic layer was shaken with 3 M NaOH and dried over NaHCO₃ and the solvent evaporated to give the pure aldehyde (70%, 4.53 g, 14 mmol). Mp: 114–115 °C. NMR (60 MHz, CDCl₃): 1.87 (m, 4H), 3.72 (m, 2H), 4.05 (m, 2H), 6.91 (d, 2H), 7.70 (m, 6H), 9.82 (s, 1H). MS: m/e (relative intensity) 323 (M⁺, 3), 202 [(CH₂)₄NC₈H₄O₂⁺, 44.6], 160 (CH₂NC₈H₄O₂⁺, 100).

meso-10-[4-(4-Phthalimidobutoxy)phenyl]-5,15,20-tri(4'-*N*-pyridinyl)porphyrin (**6**). The same protocol as for porphyrin **1** was followed, using 4-(4-phthalimidobutoxy)benzaldehyde as the functionalized aldehyde (4.3 g, 13.3 mmol). The six macrocycles (1.4 g) were separated by preparative HPLC (column 2; flow rate, 12 mL/min; binary linear gradient of 0 to 3% CH₃OH with chloroform). Combined fractions corresponding to porphyrin

6 were vacuum rotary evaporated to leave the pure porphyrin (2.2%, 239.62 mg, 0.29 mmol). NMR (CDCl_3): Table 1. Electronic absorption spectrum (CHCl_3): λ_{max} (ϵ/ϵ_{516}) 419.5, 516 (1), 552 (0.51), 590 (0.35), 647 nm (0.23). FAB-MS: m/e (relative intensity) 835 (22), 391 (100), 307 (72).

Porphyrin (3). Porphyrin **6** (48.9 mg, 59.7 μmol) was dissolved in THF (50 mL); anhydrous hydrazine (5 mL) was added, and the mixture was brought to reflux for 2 h and then filtered. The solution was extracted with chloroform; the organic layer was washed with KHCO_3 (pH 9) and a saturated ammonium chloride solution and then dried with NaHCO_3 and concentrated to dryness under vacuum. The purity of porphyrin **3** was verified by TLC (silica gel plate; eluent, 97/3 $\text{CHCl}_3/\text{MeOH}$) (95%, 39.9 mg, 56.7 μmol). NMR (CDCl_3): Table 1. Electronic absorption spectrum (CHCl_3): λ_{max} (ϵ/ϵ_{516}) 420, 516.5 (1), 551.5 (0.55), 591 (0.34), 650.5 nm (0.58). IR (KBr): 2960 (CH_2), 1245 cm^{-1} (ether).

meso-10-[4-(Carboxymethoxy)phenyl]-5,15,20-tri(4'-N-pyridinyl)porphyrin (4). 4-Formylphenoxyacetic Acid. Adapting a published procedure (25), 4-hydroxybenzaldehyde (48.85 g, 0.4 mol) and NaOH (16 g, 0.4 mol) were mixed in water (500 mL), and potassium chloroacetate (57.01 g, 0.43 mol) was added. The solution was brought to reflux for 1 h and then left until room temperature was reached. pH was adjusted to 8–9 by addition of sodium hydrogenocarbonate; phenol precipitated and was eliminated by filtration. A subsequent adjustment to pH 1 by concentrated HCl addition precipitated 4-formylphenoxyacetic acid. The pure aldehyde was filtrated, washed with ethanol and ether, and dried under vacuum (21%, 15.13 g, 84 mmol). Mp: 194 °C [lit. (25) 192–195 °C]. NMR (60 MHz, $\text{DMSO}-d_6$): 4.8 (s, 2H), 7.05 (d, 1H), 7.82 (d, 2H), 9.85 (s, 2H). MS: m/e (relative intensity) 180 (M^+ , 19.4), 179 ($\text{M}^+ - 1$, 16.8), 121 ($p\text{-CHOPhO}^+$, 100), 93 (PhO^+ , 43.1), 65 (C_5H_5^+ , 40.7).

Ethyl (4-Formylphenoxy)acetate. *N,N'*-Carbonyldiimidazole (6.81 g, 42 mmol) was dissolved in anhydrous THF (200 mL), and 4-formylphenoxyacetic acid (7.21 g, 40 mmol) was added. The mixture was brought to reflux for 30 min, and then absolute ethanol (1.53 mL, 42 mmol) was added. The resulting solution was further brought to reflux for an additional 1 h. Solvent was evaporated and the resulting solid dissolved in chloroform. The organic solution was washed with dilute HCl and water and dried. Once chloroform evaporated, the crude product was purified by liquid chromatography on silica gel (25 $\times$ 200 mm; eluent, CHCl_3). The aldehyde was then recrystallized in hot ether (80%, 6.66 g, 32 mmol). Mp: 154 °C [lit. (26) 155 °C]. NMR (60 MHz, CDCl_3): 1.27 (t, 3H), 4.25 (q, 2H), 4.70 (s, 2H), 6.96 (d, 2H), 7.80 (d, 2H), 9.90 (s, 1H). MS: m/e (relative intensity) 208 (M^+ , 100), 135 ($\text{M}^+ - \text{COOEt}$, 91.6), 105 (PhOCH_2^+ , 56.9), 77 (C_6H_5^+ , 36.9).

meso-10-[4-(Carboethoxymethoxy)phenyl]-5,15,20-tri(4'-N-pyridinyl)porphyrin (7). The same protocol as for porphyrin **2** was followed to synthesize porphyrin **7** using the aldehyde described above (2.77 g, 13.3 mmol). The six possible macrocycles (2 g) were separated by preparative HPLC (column 2; flow rate, 12 mL/min; gradient elution, CHCl_3 containing 0 to 3% methanol). Porphyrin **7** was isolated after elimination of the solvent under vacuum (5.6%, 535 mg, 0.74 mmol). NMR (CDCl_3): Table 1. Electronic absorption spectrum (CHCl_3): λ_{max} (ϵ/ϵ_{515}) 418.5, 515 (1), 550.5 (0.44), 589 (0.31), 646 nm (0.19). FAB-MS: m/e (relative intensity) 720 (MH^+ , 100), 391 (67), 307 (65). Analytical HPLC (column 1; flow rate, 2 mL/min; gradient elution, CHCl_3 (relative intensity) 0 to 5% MeOH within 2 min); $t_R = 6$ min 50 s.

Porphyrin 4 (27). Porphyrin **7** (51.97 mg, 72.2 μmol) was dissolved in THF (50 mL). Ester hydrolysis was carried out by addition of methanolic sodium hydroxide (1 mL) [NaOH (4 g, 0.1 mol), H_2O (5 mL), EtOH (50 mL)]. The reaction mixture was then refluxed for 1 h; solvent was flashed off and residue dissolved in chloroform. The resulting solution was washed with 0.1 M HCl; precipitate was filtered and dissolved in methanol. Insoluble materials were eliminated, and solvent was evaporated under vacuum. Porphyrin **4** was obtained in a 95% yield (47.45 mg, 68.6 μmol). NMR (CD_3COOD): Table 1. Electronic absorption spectrum (CHCl_3): λ_{max} (ϵ/ϵ_{511}) 413, 511 (1), 546.5 (0.45), 586.5 (0.35), 643 nm (0.22). FAB-MS: m/e (relative intensity) 692 (MH^+ , 25), 391 (90), 307 (100). IR (KBr): 1725 (COOH), 1225 cm^{-1} (ether).

4-(Methylthio)phenyl Ester of meso-10-[4-(Carboxymethoxy)phenyl]-5,15,20-tri(4'-N-pyridinyl)porphyrin (5) (28). To porphyrin **4** (17.29 mg, 25 μmol) and in solution in THF (30 mL) was added DCC (51.58 mg, 0.25 mmol) in DMF (5 mL); the solution was cooled to 0 °C, and 4-(methylthio)phenol (70.1 mg, 0.5 mmol) was added. The mixture was maintained under stirring at room temperature for 24 h. The solvent was evaporated to dryness, and the residue was chromatographed on a silica gel column (bed volume, 390 mL; eluent, 97/3 chloroform/methanol) (78%, 15.87 mg, 19.5 μmol) NMR (CDCl_3): Table 1. Electronic absorption spectrum (CHCl_3): λ_{max} (ϵ/ϵ_{513}) 417, 513 (1), 548 (0.44), 588 (0.32), 644.5 nm (0.22). Analytical HPLC (column 1; flow rate, 2 mL/min; gradient elution, CHCl_3 containing 3 to 5% MeOH within 1 min); $t_R = 1$ min 50 s.

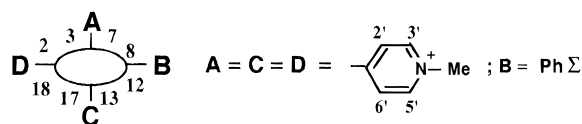
Quaternization of Porphyrins 1, 2, and 4. The following general procedure was followed. The free base porphyrin (0.13 mmol) was dissolved in DMF (10 mL), and an excess of iodomethane (20 mL) was added. The mixture was stirred and heated to 40 °C for 2 h and then cooled to room temperature; porphyrin iodide salt was precipitated by addition of diethyl ether, recovered by filtration, and dried at 130 °C in a vacuum oven. The cationic porphyrin was dissolved in methanol and converted to the chloride form with an anion exchange resin (eluent, methanol). The cationic free base porphyrins were characterized by their UV–visible (in MeOH) and ^1H NMR (in CD_3OD) spectra which were in good agreement with the proposed structures.

meso-10-(3-Nitro-4-fluorophenyl)-5,15,20-tris(4'-N-methylpyridinyl)porphyrin Trichloride Salt (Me-1). Yield: 88% (95.2 mg, 114.4 μmol). Electronic absorption spectrum: λ_{max} (ϵ/ϵ_{513}) 417, 513 (1), 549.5 (0.45), 589 (0.37), 647.5 nm (0.17). NMR: Table 2. IR (KBr): 1505, 1220 cm^{-1} .

meso-10-(4-Fluorophenyl)-5,15,20-tris(4'-N-methylpyridinyl)porphyrin Trichloride Salt (Me-2). Yield: 87% (89.03 mg, 113.1 μmol). Electronic absorption spectrum: λ_{max} (ϵ/ϵ_{514}) 422, 514.5 (1), 551 (0.45), 589.5 (0.36), 646.5 nm (0.14). NMR: Table 2. DI-MS: m/e (relative intensity) 715 ($\text{M}^+ - 2\text{Cl}^-$, 12), 680 ($\text{M}^+ - 3\text{Cl}^-$, 100). IR (KBr): 1110 cm^{-1} .

meso-10-[4-(Carboxymethoxy)phenyl]-5,15,20-tris(4'-N-methylpyridinyl)porphyrin Trichloride Salt (Me-4). Yield: 70% (76.73 mg, 91 μmol). Electronic absorption spectrum: λ_{max} (ϵ/ϵ_{517}) 427.5, 517.5 (1), 556 (0.58), 592 (0.42), 627.5 nm (0.22). NMR: Table 2. FAB-MS: m/e (relative intensity) 842 (MH^+ , 5), 391 (55), 214 (100). IR (KBr): 1760, 1715 (COOH), 1210 cm^{-1} (ether).

Radioactive Indium Derivatives of Cationic Porphyrins Me-1, Me-2, and Me-4 and Free Bases 3 and 5. A solution of 37 MBq $^{111}\text{InCl}_3$ in 0.05 M HCl (370 MBq/mL; specific activity, 17.02 TBq/mg) was placed in a tube and evaporated to dryness under a stream of nitrogen.

Table 2. 250 MHz ^1H NMR Chemical Shifts of the Protons in Quaternized Free Base Porphyrins in CD_3OD^a 

compound	Σ	pyrrole	pyridyl	phenyl	Σ
Me-1	3'-NO ₂ , 4'-F	9.13 (br s, 8H)	9.39 (m, 6H: 3',5') 8.94 (m, 6H: 2',6')	9.07 (m, 2H: 2',6') 7.68 (m, H ₅)	N ⁺ Me: 4.86 (s, 9H)
Me-2	4'-F	9.06 (br s, 8H)	9.40 (m, 6H: 3',5'), $J = 6$ Hz 8.94 (m, 6H: 2',6'), $J = 6$ Hz	8.18 (m, 2H: 2',6') 7.54 (dd, 2H: 3',5'), $J = 8.8$ Hz	N ⁺ Me: 4.85 (s, 9H)
Me-4	4'-OCH ₂ COOH	9.08 (br s, 8H)	9.40 (m, 6H: 3',5') 8.96 (m, 6H: 2',6')	8.14 (m, 2H: 2',6') 7.39 (m, 2H: 3',5')	N ⁺ Me: 4.84 (s, 9H) OCH ₂ : 5.04 (s, 2H)

^a Residual absorption from TMS (3.32 ppm).

Then indium-115 trichloride (1.35×10^{-7} mol) and porphyrin (1.4×10^{-7} mol) in solution in acetic acid containing 3.3% trifluoroacetic acid (250 μL) were added, and the mixture was heated to 140 $^{\circ}\text{C}$ for 3 h.

Porphyrins Me-1, Me-2, and Me-4. Once the reaction was completed, the pH was adjusted to 4–5 by 1 M NaOH. Radiolabeled indium porphyrins were purified on anion exchange resin as described for the quaternization (eluent, distilled water). Fractions (1 mL) were collected, and ^{111}In radioactivity was measured by γ counting. Radioactive indium porphyrin fractions were pooled, and the solvent was evaporated under vacuum.

meso-10-(3-Nitro-4-fluorophenyl)-5,15,20-tris(4'-N-methylpyridinyl)porphyrin Indium(III) Tetrachloride (Me-1-In). Yield: 75%. Electronic absorption spectrum (MeOH): λ_{max} (ϵ/ϵ_{561}) 428.5, 521.5 (0.2), 561 (1), 604.5 nm (0.34). Specific activity A (MBq/mg): 547.6.

meso-10-(4-Fluorophenyl)-5,15,20-tris(4'-N-methylpyridinyl)porphyrin Indium(III) Tetrachloride (Me-2-In). Yield: 85%. Electronic absorption spectrum (MeOH): λ_{max} (ϵ/ϵ_{562}) 429.5, 523 (0.2), 562 (1), 606.5 nm (0.39). Specific activity A (MBq/mg): 573.5.

meso-10-[4-(Carboxymethoxy)phenyl]-5,15,20-tris(4'-N-methylpyridinyl)porphyrin Indium(III) Tetrachloride (Me-4-In). Yield: 87%. UV-visible (MeOH): λ_{max} (ϵ/ϵ_{564}) 433.5, 525 (0.23), 564.5 (1), 609.5 nm (0.48). Specific activity A (MBq/mg): 540.2.

Porphyrins 3 and 5. When the reaction was completed, the pH was adjusted to 6–7 with 1 M NaOH. In order to completely remove unreacted carrier-free $^{111}\text{In}/^{115}\text{In}$, the solution was extracted with chloroform (2 $\times$ 10 mL); chloroformic solutions were combined and washed several times with distilled water to neutrality. The chloroform was then evaporated under vacuum.

meso-10-[4-(4-Aminobutoxy)phenyl]-5,15,20-tris(4'-N-methylpyridinyl)porphyrin Indium(III) Tetrachloride (3-In). Yield: 76%. Electronic absorption spectrum (CHCl_3): λ_{max} (ϵ/ϵ_{559}) 426.5, 520.5 (0.23), 559.5 (1), 600 nm (0.4). Specific activity A (MBq/mg): 629.

meso-10-[4-(Carboxymethoxy)phenyl]-5,15,20-tris(4'-N-pyridinyl)porphyrin Indium Chloride 4'-Methylthio)phenyl Ester (5-In). Yield: 53%. Electronic absorption spectrum (CHCl_3): λ_{max} (ϵ/ϵ_{559}) 424, 522 (0.16), 559 (1), 598.5 nm (0.29). Specific activity A (MBq/mg): 555.

Quaternization of Porphyrin 5-In. The indium porphyrin **5-In** (67.35 mg, 7×10^{-2} mmol) was dissolved in methyl iodide (5 mL), and the mixture was maintained under stirring at 40 $^{\circ}\text{C}$ for 2 h. Then, an excess of methyl iodide was eliminated under vacuum, and the residue was dissolved in methanol (2 mL). The cationic indium porphyrin was converted to the chloride form with an anion exchange resin in the Cl^- form (eluent, methanol). Fractions (1 mL) were collected, and the radioactivity was

measured. Radioactive fractions were pooled, and the solvent was evaporated to dryness.

meso-10-[4-(Carboxymethoxy)phenyl]-5,15,20-tris(4'-N-methylpyridinyl)porphyrin Indium Pentachloride 4'-(Dimethylthio)phenyl Ester (Me-5-In). Yield: 74% (60.3 mg, 5.18×10^{-2} mmol). Specific activity A (MBq/mg): 481.

BSA Labeling. BSA was obtained from Sigma Chimie and was used without further purification.

[$^{111}\text{In}/^{115}\text{In}$]Porphyrins Me-1-In, Me-2-In, and Me-5-In. To 6.9×10^{-2} μmol (37 MBq) of indium porphyrin derivatives in water (200 μL) was added 15 μL of a BSA solution in water (4.6×10^{-4} M). The volume was adjusted to 1 mL by addition of water. The mixture was maintained under stirring at room temperature for 1.5 h and then chromatographed on a Sephadex G75 column (10 mL). Fractions (1 mL) were collected; the conjugate porphyrin–BSA was eluted within the third to eighth fractions. The yields of coupling determined by γ counting of ^{111}In radioactivity for **Me-1-In** and **Me-2-In** were 95 and 97%, respectively, with specific activities A of 66.6 MBq/mg.

In the case of the activated ester **Me-5-In**, the same protocol was used, leading to a porphyrin–BSA conjugate in a 67% yield ($A = 48.1$ MBq/mg).

[$^{111}\text{In}/^{115}\text{In}$]Porphyrins 3-In and Me-4-In: Carbodiimide Activation. The indium derivative (6.9×10^{-2} μmol , 37 MBq) was dissolved in water (200 μL). To this solution were added 30 μL of NHS solution (2.29×10^{-3} M) and 46 μL of EDAC solution (1.5×10^{-3} M) both in 12.5 mM sodium phosphate (pH 6). The mixture was stirred in the dark at room temperature for 1 h before BSA in water was added (6.9×10^{-3} μmol , [porphyrin]/BSA = 10). The stirred reaction mixture was kept at room temperature in the dark for an additional 1 h. The same protocol, as described above, was used to purify the labeled BSA. Using these conditions, the coupling efficiency was 89% for **3-In** ($A = 66.6$ MBq/mg) and 38% for **Me-4-In** ($A = 29.6$ MBq/mg).

Preparation of Anti-CEA mAb–Radioactive Porphyrin Conjugates. A commercially available antibody directed against carcinoembryonic antigen (anti-CEA mAb; CIS-Bioindustries Co., France) was used in the entire mAb form. Experiments were run at this company.

[$^{111}\text{In}/^{115}\text{In}$]Porphyrins Me-1-In, Me-2-In, and Me-5-In. All conjugations were carried out in water at pH 6.5–7 and room temperature for 12 h (**Me-1-In** and **Me-2-In**) and 2 h (**Me-5-In**). Radioactive indium porphyrin derivative (19.5×10^{-2} μmol , 103.6 MBq) was dissolved in water (1 mL), and 2.6×10^{-2} μmol of mAb was added (stoichiometry of chelate–antibody = 7.5). After incubation, the porphyrin–anti-CEA mAb conjugates were further isolated from the uncoupled porphyrins by cen-

trifugation in an Amicon Centriflo CF 50 membrane cone (molecular weight cutoff of 50 000). The labeling yields were 60% for **Me-1-In**, 75% for **Me-2-In**, and 35% for **Me-5-In**, with specific activities of 14.8, 18.5, and 7.4 MBq/mg, respectively.

¹¹¹In/¹¹⁵In]Porphyrins **3-In** and **Me-4-In**. For these two macrocycles, the same protocol as described previously for BSA (carbodiimide activation) was used. After incubation, the porphyrin-anti-CEA mAb conjugates were purified by centrifugation in a Centricon membrane cone and were counted for radioactivity. Labeling efficiencies were ~100% for **3-In** (A = 25.9 MBq/mg) and 91% for **Me-4-In** (A = 22.2 MBq/mg).

HPLC Analysis of Radioactive Porphyrin-mAb Conjugates. The labeled conjugates were analyzed by HPLC using a Nucleosil C4 column (column 3; flow rate, 1.2 mL/min) and detection at 420 nm. The elution medium was 0.1% trifluoroacetic acid (solvent A) and acetonitrile/solvent A (60/40 by volume) (solvent B). A linear gradient of solvent B in solvent A was developed over 10 min. Whatever the conjugate was, retention times were the same for coupled and uncoupled mAbs (10 min 20 s), the nonbonded porphyrins being eluted after the mAb fractions.

Labeling of Anti-ACE mAb without Isotopic Dilution: Derivatives Me-1-In, Me-2-In, and 3-In. For the insertion of 370 MBq indium-111 in these macrocycles (26 × 10⁻² μmol), we followed the same experimental conditions as described before, without addition of ¹¹⁵InCl₃, and obtained for each case a mixture of free base and indium derivative free from any carrier-free ¹¹¹In. The yields of metallation determined by γ counting of ¹¹¹In radioactivity in the extract were 75% for **Me-1-In** (A = 1.28 GBq/mg), 85% for **Me-2-In** (A = 1.54 GBq/mg), and 76% for **3-In** (A = 1.54 GBq/mg).

Conjugations with anti-CEA mAb were carried out in sodium hydrogen carbonate (0.5 M, pH 8.5) at room temperature for 12 h, using an input stoichiometry of chelate/antibody of 10/1 and an antibody concentration of 26 μM. We followed the same protocol as for isotopic dilution labeling. Taking into account the high radioactivity present, after incubation, the porphyrin (¹¹¹In and free base derivative)-anti-CEA mAb conjugates were further isolated from the unreacted porphyrin derivatives on a 10 mL Sephadex G100 column presaturated with HSA. Antibody fractions, eluting in the void volume, were pooled and counted for radioactivity. Coupling efficiencies were ~95% with **Me-1-In** (A = 66.6 MBq/mg) and ~100% with **Me-2-In** (A = 81.4 MBq/mg) and **3-In** (A = 74 MBq/mg).

Immunoreactivity. The immunoreactivity of the radiolabeled conjugates **Me-2-In-mAb** and **3-In-mAb** was determined by passage over a CEA immunoabsorbent in the Laboratoire de Contrôle et Qualité de CIS-Bioindustries Co. The percentage of activity in the absorbed fraction was related to the total activity recovered. Recoveries from the affinity columns were between 55 and 60% for the **3-In-anti-CEA mAb** conjugate and between 40 and 45% for the **Me-2-In-anti-CEA mAb** conjugate.

RESULTS AND DISCUSSION

Syntheses of Substituted Free Base Porphyrins.

Taking into account the numerous functions present in a protein, various reactions can be used to covalently attach compounds. In particular, this attachment can be achieved with the amino acid residues of the lateral chains of proteins, e.g. amino or carboxy groups (lysine and arginine, glutamic and aspartic acid), the sulfhydryl group of cysteine, the hydroxy group of serine and

tyrosine, the imidazole group of histidine, and the various functional groups of the aromatic amino acid residues. The purpose of this investigation was to synthesize a series of differently monosubstituted arylporphyrins possessing at their periphery three pyridinyl groups, whose quaternization gives hydrosoluble molecules, and only one functional phenyl group, to avoid possible ulterior reticulation during the course of coupling reactions (Figure 1).

By analogy with Sanger's reagent (29), which allows the quantitative arylation of N-terminal amines as well as ε-amine groups of lysine, imidazole, histidine, alcohol, and thiol functions of serine, tyrosine, and cysteine residues, fluorine was introduced in the para position of the phenyl group (Figure 1, **1** and **2**). Micheel et al. (24) have shown that the mesomeric effect of the nitro group, in 3-nitro-4-fluorobenzaldehyde, is sufficient to initiate fluoride lability; we synthesized these porphyrins to compare the influence of the porphyrinic macrocycle on the C-F bond with and without the presence of the NO₂ substituent.

To react with the carbohydrate moieties, located outside the antigen binding domain of the antibody molecule and carboxy groups of the Fc region, the free base **3** was synthesized. To maintain a sufficient nucleophilic character of the peripheral nitrogen, four sp³ carbons were introduced.

Since the coupling of a carboxylate to an amine site on a protein is a common strategy, a carboxylic substituent was introduced. A spacer was used to move it away from the macrocycle (porphyrin **4**) since, when it is directly bonded to the phenyl ring, Bedel-Cloutour et al. (23) have shown that reactivity of COOH is decreased and synthesis of the corresponding acid chloride is incomplete.

Finally, porphyrin **5**, derived from the latter, was synthesized; 4-(methylthio)phenol ester presents a great interest (28). In neutral medium, it acts as a nonactivated ester; methylation of the thioether activates the ester, leading to a good leaving benzoate group.

The reaction scheme to produce new cationic periphery porphyrins was based on literature precedents (30–33). The free bases corresponding to the general formula *meso*-(pyridinyl)₃(Σ-phenyl)PH₂ were prepared by condensation of pyrrole with a mixture of 4-pyridinecarboxaldehyde and the corresponding substituted benzaldehyde by a modification of the mixed-aldehyde method described by Little *et al.* (30).

Porphyrins **1** and **2** were obtained by direct synthesis with their corresponding aldehyde: either the commercially available 4-fluorobenzaldehyde or its synthetic nitrated derivative (24) (Figure 2).

The synthetic routes used to obtain free bases **3–5** are shown in Figure 3 for their precursors and in Figure 4.

The initial step, conversion of the commercially available 4-hydroxybenzaldehyde to the corresponding *N*-(4-butyl)phthalimido derivative (34, 35), provides a precursor for condensation with pyrrole and 4-pyridinecarboxaldehyde to produce after isolation and purification *meso*-10-[4-(4-phthalimidobutoxy)phenyl]-5,15,20-tri(4'-*N*-pyridinyl)porphyrin (**6**). The phthalimido group was removed by hydrolysis following the typical method using hydrazine (36), leading to the aminoporphyrin **3**.

In the case of porphyrin **4**, 4-hydroxybenzaldehyde was converted into its acetoxyl derivative, which was subsequently esterified with ethanol in the presence of *N,N'*-carbonyldiimidazole. The condensation of ethyl (4-formylphenoxy)acetate along with 4-pyridinecarboxaldehyde and pyrrole gave, after purification, the

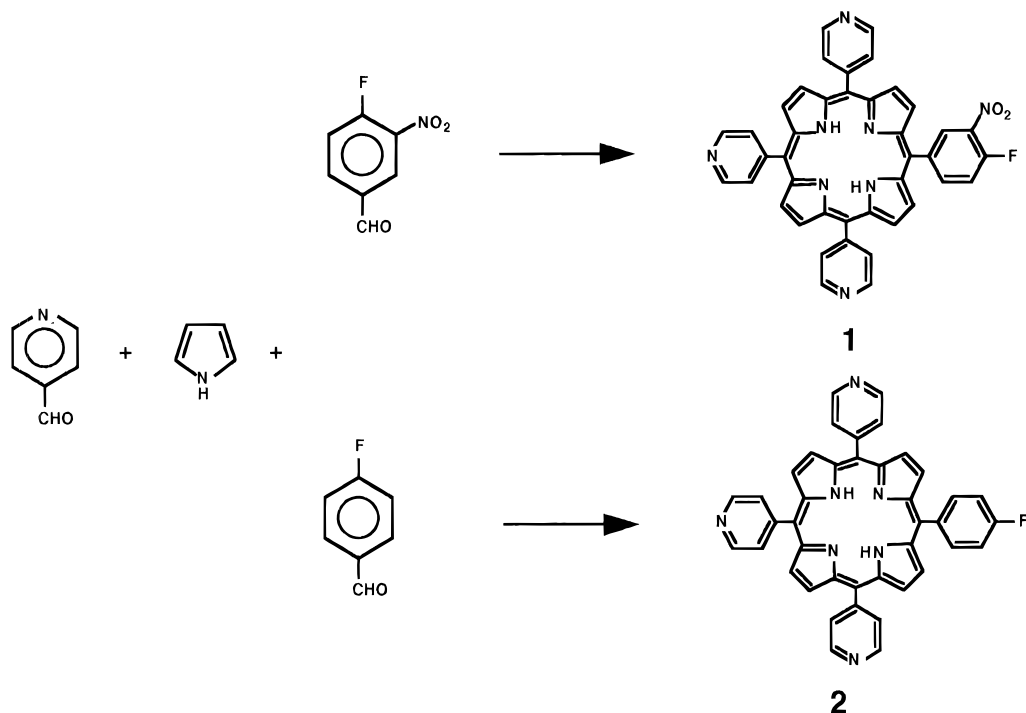


Figure 2. Chemistry for the synthesis of macrocycles **1** and **2**.

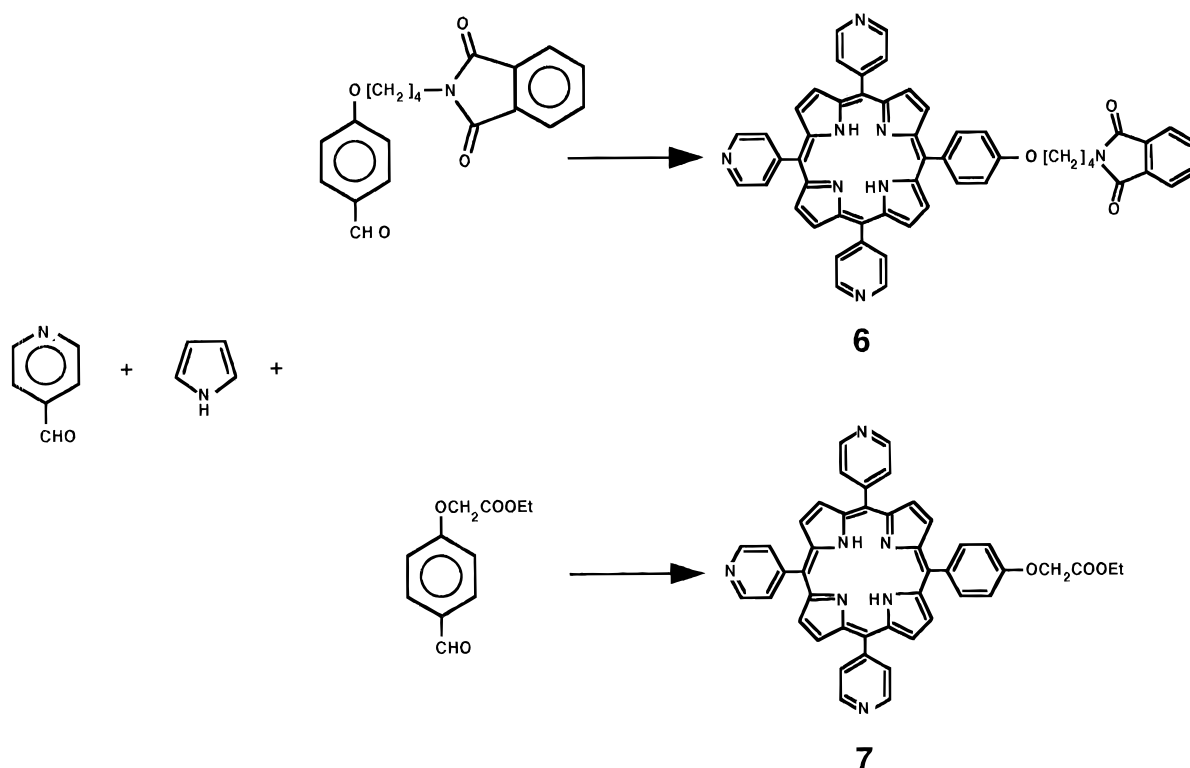


Figure 3. Chemistry for the synthesis of macrocycles **6** and **7**.

esterified porphyrin **7**. Hydrolysis of the ester with alcoholic sodium hydroxide gave the acid **4** (27).

Finally, the synthesis of the activated ester **5** was carried out by esterification of porphyrin **4** with 4-(methylthio)phenol in the presence of dicyclohexylcarbodiimide (**28**).

All free base porphyrins were characterized by UV-visible spectroscopy, mass spectrometry, and ^1H NMR spectroscopy.

Porphyrins **1–7** gave UV–visible spectra typical of *meso*-tetraaryl-substituted porphyrins (37), i.e. a strong

B band (around 418 nm) and four Q bands (510–650 nm), the intensities of which decreased with increasing wavelengths. The spectra reported in Materials and Methods all fit this general pattern. Mass spectra of compounds **1**, **2**, **4**, **6**, and **7** were measured by positive FAB techniques where $M + 1$ peaks appeared as parent ions.

The ^1H NMR spectra of free bases **1–7** were straightforward and were readily assigned on the basis of chemical shift correlations and integration measurements. Proton chemical shifts are reported in Table 1. Our results were in agreement with literature data

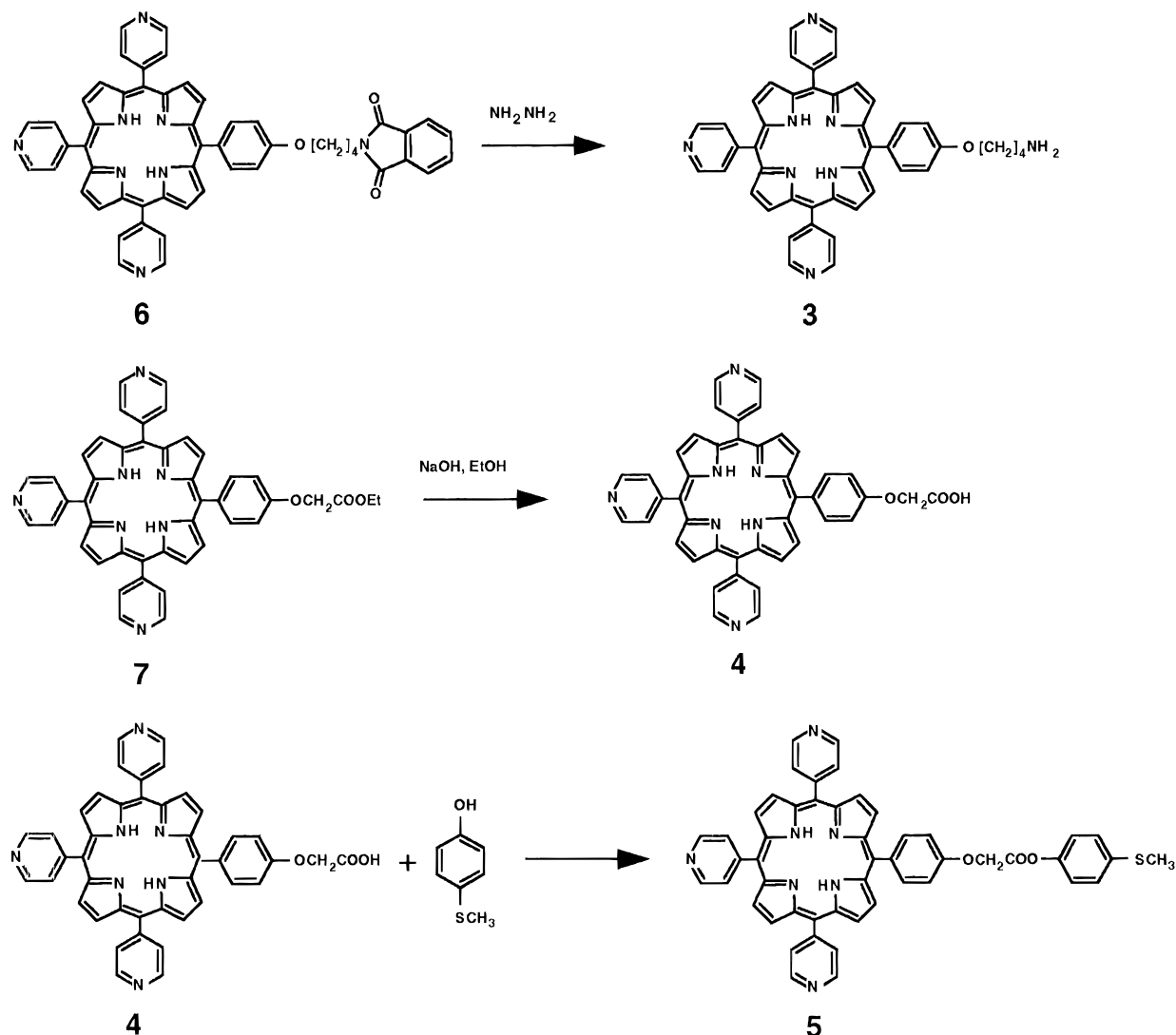


Figure 4. Chemistry for the synthesis of macrocycles 3–5.

reported previously for *meso*-5-phenyl-10,15,20-tri(4'-*N*-pyridinyl)porphyrin (38). The introduction of asymmetry into the molecule leads to an inequivalence of pyrrolic protons; except with compound **4**, a splitting pattern is observed: one singlet corresponding to the four protons H2, H3, H17, and H18 and two asymmetrical doublets, each corresponding to two protons, H7 and H8 being equivalent pairwise to H12 and H13, respectively. They have their proton resonances split into an AB quartet which is reduced almost to first order at 250 MHz ($J \sim 5$ Hz). Pyridinyl ring signals were found to be independent of the substituent present on the molecule; they appear at ~ 9.1 and ~ 8.2 ppm (protons H3' and H5' and H2' and H6', respectively).

For the compounds monosubstituted on the phenyl ring, the *meta* and *ortho* protons appear as doublets at ~ 8.1 and 7.3 ppm. In compound **1**, the *meta* proton adjacent to the fluorine appears as a doublet of doublets ($J_{HH} = 8.5$ Hz, $J_{HF} = 10.4$ Hz), whereas the *ortho* proton H6' appears as an octet at 8.5 ppm and the *ortho* proton H2' as a multiplet at ~ 8.9 ppm.

All the NH resonances appear in the range of -2.9 ppm, except for compound **4** since the NH signal was not visible in CD_3COOD .

For compounds **3–7**, integrations of the aromatic regions relative to the phenyl substituent resonances are diagnostic of the purity of these derivatives.

Quaternization and Metallation of Substituted

Free Base Porphyrins. The corresponding triiodine salts of cationic porphyrins **1**, **2**, and **4**, e.g. **Me-1**, **Me-2**, and **Me-4**, were prepared using a variation of the method reported in the literature (Figure 5) (39).

The trichloride salts were synthesized by passing the cationic porphyrins through an ion exchange column. They were characterized by UV-visible spectroscopy, mass spectrometry, and infrared spectroscopy (see Materials and Methods); proton chemical shifts of cationic porphyrins at ambient temperature are listed in Table 2. The porphyrin ring protons appear at ca. 9.1 ppm as a broad singlet; the monomer-dimer equilibrium could explain the broadening of this NMR signal. Chemical shifts of pyridyl protons were in accordance with data obtained previously for unsubstituted *meso*-5-phenyl-10,15,20-tris(4'-*N*-methylpyridinyl)porphyrin (33).

Quaternization of pyridyl rings does not modify chemical shifts of phenyl protons. Porphyrin **3** was not quaternized since it is impossible to convert it into its trichloride salt without quaternizing the primary amine; the basicities of these various amine functions differ. The pK_a values of the primary amine vary from 10 to 12, whereas it is equal to 5.2 in the case of pyridine (40). In the case of porphyrin **5**, the quaternization was conducted after metallation of the molecule, since the thioether function is also methylated to give the activated ester which must be used immediately for further coupling.

For metallation with indium, we applied the method

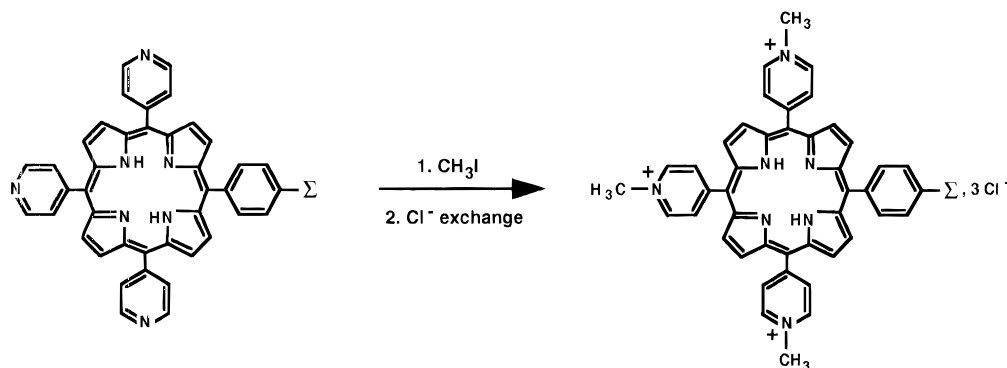


Figure 5. General scheme for the quaternization of free base porphyrins.

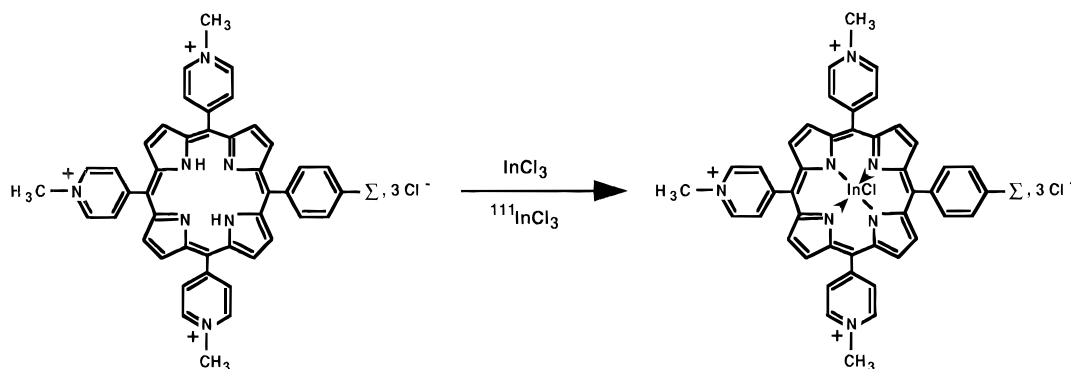


Figure 6. Metallation of the quaternized free base porphyrins.

we have already used for *meso*-substituted phenyl porphyrins (22), by refluxing a slight excess of metal salt, a mixture of $^{111}\text{InCl}_3$ and $^{115}\text{InCl}_3$, with the porphyrin base in glacial acetic acid (Figure 6). To improve the solubilization of the free base, 3.3% trifluoroacetic acid was added.

Hydrosoluble indium derivatives of macrocycles **Me-1**, **Me-2**, and **Me-4**, **Me-1-In**, **Me-2-In**, and **Me-4-In**, respectively, were purified on anion exchange resin; the corresponding indium complexes of porphyrins **3** and **5**, e.g. **3-In** and **5-In**, were purified by chloroformic extraction, a method we perfected earlier (21), suitable for further *in vivo* use. The yields of metallation, determined by γ counting of ^{111}In radioactivity of the different pure derivatives, were between 75 and 90% except for that for compound **5-In** (53%); this lower yield confirms the instability of the ester in acidic medium.

These radioactive derivatives were obtained with a specific activity of ~ 555 MBq/mg, smaller than the one observed with 5-(*p*-carboxyphenyl)-10,15,20-tritylporphyrin $^{111}\text{indium(III)}$ chloride (23) since, in this case, we used an isotopic dilution of indium-115 and -111.

The electronic spectra of metalloporphyrins consist of an intense band near 420 nm (Soret band) and two bands between 500 and 600 nm, α and β bands (37); on metallation of our macrocycles, the four-banded etio-type visible spectra lead to the normal visible spectra for a metalloporphyrin (see Materials and Methods). The absorption bands observed appear at about the same wavelengths irrespective of the macrocycle.

Indium Porphyrin–Bovine Serum Albumin Conjugates. Coupling with BSA served as a model for the optimization of the experimental conditions and was conducted with macrocycles **Me-1-In**, **Me-2-In**, **3-In**, **Me-4-In**, and **Me-5-In**. Just prior to BSA coupling, **5-In** was quaternized with CH_3I and purified by passing it through an anion exchange column in the Cl^- form; **Me-5-In** was obtained in a 74% yield with a specific activity of 481 MBq/mg.

A radioactive porphyrin/BSA molar ratio of 10 was used, and all reactions were conducted in the dark to prevent porphyrin photooxidation.

Labeling of BSA with **Me-1-In**, **Me-2-In**, and **Me-5-In** derivatives was carried out in water, within 1.5 h, and conjugates were further purified by Sephadex G75 size exclusion chromatography, to give preparations in which 95 and 97% of the radiolabel, **Me-1-In** and **Me-2-In**, respectively, was bound to BSA. In the case of the activated ester **Me-5-In**, the coupling yield was lower (67%).

For porphyrin **Me-4-In**, we followed the method described by Lavalley et al. (41) involving formation of the activated carboxylate by reaction with *N*-hydroxysuccinimide (NHS) in the presence of 1-ethyl-3-[3'-(dimethylamino)propyl]carbodiimide hydrochloride (EDAC), followed by conjugation with BSA ([porphyrin]/[BSA] = 10). The conjugate **Me-4-In**–BSA was purified, as previously described, by chromatography of the conjugation mixture on a Sephadex G75 column; 38% of the conjugate was recovered with a specific activity of 29.6 MBq/mg.

An identical protocol was followed to synthesize the conjugate **3-In**–BSA in an 89% yield.

Indium Porphyrin–mAb Conjugates. One area of oncology where radiolabeled monoclonal antibodies are most likely to play an important clinical role is in the evaluation of patients with colorectal carcinomas (4). While carcinoembryonic antigen (CEA) is not specific to tumors, high concentrations of this antigen occur in a variety of tumors, particularly in colorectal cancer (42), and for this reason, it has been used as a target in conjunction with a labelled anti-CEA mAb (43–46).

Taking into account the previous results, we transposed these reactions to label the anticarcinoembryonic antigen monoclonal antibody (anti-CEA mAb).

For all derivatives, we followed an experimental protocol identical to that for BSA, using an input stoichiometry of chelate/antibody of 7.5/1; the first assays were performed with the derivatives metallated with an

isotopic dilution of indium trichloride. Under these conditions, excellent labeling yields were obtained for derivatives **3-In** and **Me-4-In** (~100 and 91%, respectively). In particular, the amino derivative **3-In**, which gave only 38% of the conjugate after 2 h of contact with BSA, required a longer contact time to react; labeling was nearly quantitative. In contrast, with fluoro derivatives **Me-1-In** and **Me-2-In**, a lower labeling efficiency was obtained (60%, 75%). The presence of a nitro substituent did not improve the coupling yield; on the contrary, the influence of its steric hindrance was predominant. With the activated ester **Me-5-In**, as for BSA, labeling efficiency was low. Specific activities were between 7.4 and 25.9 MBq.

Covalent coupling was verified by analytical reversed-phase HPLC. Under the conditions used (see Materials and Methods), no uncoupled radioactive porphyrin was detected whatever the conjugate.

It is noteworthy that porphyrin-mAb conjugates in aqueous solution, kept at room temperature and daylight, were stable; after 48 h, the integrity of the chelates was maintained.

In order to improve the specific activities of the conjugates, we performed the metallation of macrocycles **Me-1**, **Me-2**, and **Me-3** in the presence of a higher radioactivity (370 MBq), without isotopic dilution; after coupling, conjugates were purified on a Sephadex G100 column to eliminate unreacted indium-111-porphyrins. Using these conditions, the coupling efficiency was between 95 and 100%. The number of porphyrin residues per mAb molecule was given by the product of the fraction of counts associated with mAb (mAb-bound radioactivity) (0.95–1) with the total porphyrin/mAb ratio used in the conjugation reaction (10/1); we obtained radioimmunoconjugates with an average number of indium porphyrin groups attached to each mAb molecule of 9.5–10 and an average specific activity of 73 MBq/mg.

Despite the great number of indium porphyrins covalently coupled to the monoclonal antibody, these conjugates displayed a relatively good retention of antibody immunoreactivity. This preliminary result needs more investigations which will be carried out in the near future.

CONCLUSION

The syntheses and characterizations of new functionalized radioactive indium porphyrins, which hold metal with extreme stability, under physiological conditions, are an important step in the field of labeling. The experiments described above demonstrate the feasibility of coupling these radioactive derivatives, with high yield, to a monoclonal antibody in view of tumor detection.

Indium porphyrin-labeled anti-CEA mAbs scanning could significantly impact patient management as a complementary tool to preoperative evaluation of a patient with colorectal cancer.

In vivo delivery studies of these conjugates are currently under way.

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LITERATURE CITED

- (1) Meares, C. F. (1986) Chelating Agents for the Binding of Metal Ions to Antibodies. *Nucl. Med. Biol.* 13, 311–318.
- (2) Hnatowich, D. J. (1990) Antibody Radiolabelling, Problems and Promises. *Nucl. Med. Biol.* 17, 49–55.
- (3) Liu, Y., and Wu, C. (1991) Radiolabelling of Monoclonal Antibodies with Metal Chelates. *Pure Appl. Chem.* 63, 427–463.
- (4) Kramer, E. L., and Larson, S. M. (1991) Tumor Targeting with Radiolabeled Antibody for Diagnosis and Therapy. *Immunol. Allergy Clin. North Am.* 11, 301–339.
- (5) Baranowska-Kortylewicz, J., Dalrympe, G. V., Quadri, S. M., and Harrison, K. A. (1995) Radiolabeling of Antibodies for Therapy and Diagnosis. In *Methods in Molecular Biology*, 51: *Antibody Engineering Protocols* (S. Paul, Ed.) pp 423–439, Humana Press Inc., Totowa, NJ.
- (6) Kozak, R. W., Waldmann, T. A., Atcher, R. W., and Gansow, O. A. (1986) Radionuclide-conjugated Monoclonal Antibodies: a Synthesis of Immunology Inorganic Chemistry and Nuclear Science. *Trends Biotechnol.* 4, 259–264.
- (7) Fairweather, D. S., Bradwell, A. R., Dykes, P. W., Vaughan, A. T., Watson-James, S. F., and Chandler, S. (1983) Improved Tumor Localisation Using Indium-111 Labelled Antibodies. *Br. Med. J.* 287, 167–170.
- (8) Meares, C. F., and Goodwin, D. A. (1984) Linking Radiometals to Proteins with Bifunctional Chelating Agents. *J. Protein Chem.* 3, 215–228.
- (9) Wang, T. S. T., Srivastava, S. C., Fawwaz, R. A., Giacomini, P., Ferrone, S., Richards, P., Hardy, M. A., and Alderson, P. O. (1984) A Comparison of the Cyclic Anhydride and Mixed Anhydride Methods for ¹¹¹In-DTPA Chelation to Monoclonal Antibodies. *Nuklearmedizin* 4, 193–195.
- (10) Fawwaz, R. A., Wang, T. S. T., Estabrook, A., Rosen, J. M., Hardy, M., Alderson, P. O., Srivastava, S. C., Richards, P., and Ferrone, S. (1985) Immunoreactivity and Biodistribution of Indium-111-Labeled Monoclonal Antibody to a Human High Molecular Weight-Melanoma Associated Antigen. *J. Nucl. Med.* 26, 488–492.
- (11) Margerum, D. W., Cayley, G. R., Weatherburn, D. C., and Pagenkopf, G. K. (1978) Kinetics and Mechanisms of Complex Formation and Ligand Exchange. *ACS Monogr.* 174, 1–220.
- (12) Cole, W. C., De Nardo, S. J., Meares, C. F., McCall, M. J., De Nardo, G. L., Epstein, A. L., O'Brien, H. A., and Moi, M. K. (1987) Comparative Serum Stability of Radiochelates for Antibody Radiopharmaceuticals. *J. Nucl. Med.* 28, 83–90.
- (13) Brechbiel, M. W., and Gansow, O. A. (1991) Backbone-Substituted DTPA Ligands for ⁹⁰Y Radioimmunotherapy. *Bioconjugate Chem.* 2, 187–194.
- (14) Kozak, R. W., Raubitschek, A., Mirzadeh, S., Brechbiel, M. W., Junghaus, R., Gansow, O. A., and Waldmann, T. A. (1989) Nature of the Bifunctional Chelating Agent Used for Radioimmunotherapy with Yttrium-90 Monoclonal Antibodies: Critical Factors in Determining *in vivo* Survival and Organ. *Cancer Res.* 49, 2639–2644.
- (15) Cummins, C. H., Rutter, E. W., Jr., and Fordyce, W. A. (1991) A Convenient Synthesis of Bifunctional Chelating Agents Based on Diethylenetriaminepentaacetic Acid and their Coordination Chemistry with Yttrium(III). *Bioconjugate Chem.* 2, 180–186.
- (16) Brechbiel, M. W., Gansow, O. A., Atcher, R. W., Schlom, J., Esteban, J., Simpson, D., and Colcher, D. (1986) Synthesis of 1-(p-Isothiocyanatobenzyl) Derivatives of DTPA and EDTA. Antibody Labeling and Tumor Imaging Studies. *Inorg. Chem.* 25, 2772–2781.
- (17) Moi, M. K., Meares, C. F., and De Nardo, S. J. (1988) The Peptide Way of Macrocyclic Bifunctional Chelating Agents. Synthesis of 2-(p-Nitrobenzyl)-1,4,7,10-tetraazacyclododecane-*N,N',N'',N'''*-tetraacetic Acid and Study of Its Yttrium(III) Complex. *J. Am. Chem. Soc.* 110, 6266–6267.
- (18) Cox, J. P. L., Graig, A. S., Helps, L. M., Jandowski, K. J., Parker, D., Eaton, M. A. W., Millican, A. T., Millar, K., Beeley, N. R. A., and Boyce, B. A. (1990) Synthesis of C- and N-Functionalised Derivatives of 1,4,7-Triazacyclononane-1,4,7-triylacetic Acid (NOTA), 1,4,7,10-Tetraazacyclododecane-1,4,7,10-tetrayltetraacetic Acid (DOTA), and Diethylenetriaminepentaacetic Acid (DTPA): Bifunctional Complexing Agents for the Derivatisation of Antibodies. *J. Chem. Soc., Perkin Trans. 1*, 2567–2576.
- (19) Meares, C. F., Moi, M. K., Diril, H., Kubis, D. L., McCall, M. J., Deshpande, S. V., De Nardo, S. J., Snook, D., and

- Epenetos, A. A. (1990) Macrocyclic Chelates of Radiometals for Diagnosis and Therapy. *Br. J. Cancer, Suppl. 10*, 21–26.
- (20) Buchler, J. W. (1975) Static Coordination Chemistry of Metalloporphyrins. In *Porphyrins and Metalloporphyrins* (K. M. Smith, Ed.) pp 157–231, Elsevier Scientific, Amsterdam.
- (21) Cloutour, C., Ducassou, D., Pommier, J. C., and Vuillemin, L. (1982) Chloro Indium-111 Tetraphenylporphyrin and its Possible Clinical Application. *Int. J. Radiat. Isot.* 33, 1311–1318.
- (22) Bedel-Cloutour, C. H., and Gacherieu, C. (1987) Functionalization of Tetrapyrrolic Macrocycles: Free Bases and Indium Derivatives, Models for Protein Labeling. *Main Group Met. Chem.* 10, 109–128.
- (23) Bedel-Cloutour, C. H., Maneta-Peyret, L., Pereyre, M., and Bezan, J. H. (1991) Synthesis of a Monoclonal Antibody-Indium-111-Porphyrin Conjugate. *J. Immunol. Methods* 144, 35–41.
- (24) Micheel, F., and Noffz, D. (1957) Reactions of 3-Nitro-4-fluorobenzaldehyde with Amino Acids. *Chem. Ber.* 90, 1586–1589.
- (25) Cavallini, G., and Ravenna, F. (1951) Thiosemicarbazones. *Farmaco (1946–1952)* 6, 145–149.
- (26) Elkan, T. (1886) On the Isomers of Aldehydophenoxyacetic Acid. *Berichte XIX*, 3041–3054.
- (27) Molinaro, F. S., Little, R. G., and Ibers, J. A. (1977) Oxygen Binding to a Model for the Active Site in Cobalt-Substituted Hemoglobin. *J. Am. Chem. Soc.* 99, 5628–5632.
- (28) Kouge, K., Koizumi, T., Okaï, H., and Kato, T. (1987) Peptide Synthesis in Aqueous Solution. I. Application of *p*-Dialkyl-sulfoniophenols as a Water-Soluble Coupling Reagent. *Bull. Chem. Soc. Jpn.* 60, 2409–2418.
- (29) Sanger, F. (1945) Free Amino Groups of Insulin. *Biochem. J.* 39, 507–515.
- (30) Little, R. G., Anton, J. A., Loach, P. A., and Ibers, J. A. (1975) The Synthesis of Some Substituted Tetraarylporphyrins. *J. Heterocycl. Chem.* 12, 343–349.
- (31) Little, R. G. (1981) The Mixed-Aldehyde Synthesis of Difunctional Tetraarylporphyrins. *J. Heterocycl. Chem.* 18, 129–133.
- (32) Williams, G. N., Williams, R. F. X., Lewis, A., and Hambright, P. (1979) Synthesis, Characterization and Copper Incorporation into 5-(4-pyridyl)-10,15,20-triphenylporphyrin. *J. Inorg. Nucl. Chem.* 41, 41–44.
- (33) Sari, M. A., Battioni, J. P., Dupre, D., Mansuy, D., and Le Pecq, J. B. (1990) Interaction of Cationic Porphyrins with DNA: Importance of the Number and Position of the Charges and Minimum Structural Requirements for Intercalation. *Biochemistry* 29, 4205–4215.
- (34) Kojima, S., Onishi, A., Tanaka, H., and Itakura, J. (1977) *p*-(Alkoxyalkoxy)-benzylidene-*p*'-cyanoanilines. *Jpn. Kokai Tokkyo Koho* 7787, 139.
- (35) Ito, H., Nitta, A., Tanaka, T., Kamio, H., and Tsuboi, K. (1985) Halo-substituted Aromatic, Alicyclic, and Heterocyclic Ethers. *Jpn. Kokai Tokkyo Koho* 60161, 942.
- (36) March, J. (1992) in *Advanced Organic Chemistry. Reactions, Mechanisms, and Structure*, 4th ed., pp 293–500, Wiley-Interscience, New York.
- (37) Gouterman, M. (1978) Optical Spectra and Electronic Structure. In *The Porphyrins* (D. Dolphin, Ed.) Vol. III, pp 1–165, Academic Press, New York.
- (38) Shamin, A., Worthington, P., and Hambright, P. (1981) Synthesis and Characterization of Phenyl/4-pyridyl *meso* Substituted Porphyrins. *J. Chem. Soc. Pak.* 3, 1–3.
- (39) Hambright, P., and Fleischer, E. B. (1970) The Acid-Base Equilibria, Kinetics of Cu-Ion Incorporation and Acid-Catalyzed Zinc-Ion Displacement from the Water-Soluble Porphyrin $\alpha,\beta,\gamma,\delta$ -Tetra(4-N-methylpyridyl)porphine. *Inorg. Chem.* 9, 1757–1761.
- (40) Gordon, A. J., and Ford, R. A. (1972) in *The Chemist's Companion. A Handbook of Practical Data, Techniques, and References*, pp 1–80, Wiley-Interscience, New York.
- (41) Roberts, J. C., Figard, S. D., Mercer-Smith, J. A., Svitra, Z. V., Anderson, W. L., and Lavalley, D. K. (1987) Preparation and Characterization of Copper-67 Porphyrin-antibody Conjugates. *J. Immunol. Methods* 105, 153–164.
- (42) Gold, P., and Freedman, S. O. (1965) Specific Carcinoembryonic Antigens of the Human Digestive System. *J. Exp. Med.* 122, 461–485.
- (43) Goldenberg, D. M., Wlodkowski, T. J., Sharkey, R. M., Silberstein, E. B., Serafini, A. N., Garty, I. I., Vanheertum, R. L., Higginbothamford, E. A., Kotler, J. A., Balasubramanian, N., Swayne, L. C., Hansen, H. J., and Pinsky, C. M. (1993) Colorectal Cancer Imaging With Iodine-123-Labelled CEA Monoclonal Antibody Fragments. *J. Nucl. Med.* 34, 61–70.
- (44) Webster, W. B., Harwood, S. J., Carroll, R. G., and Morrissey, M. A. (1992) Pharmacokinetics of Indium-111-Labelled B72.3 Monoclonal Antibody in Colorectal Cancer Patients. *J. Nucl. Med.* 233, 498–504.
- (45) Halpern, S. E., Dillman, R. O., Amox, D., Hagan, P. L., Burks, R., Dillman, J., Perdikakis, B., Merchant, B., Frincke, J., and Schweighardt, S. (1992) Detection of Occult Tumor Using Indium-111-Labeled Anticarcinoembryonic Antigen Antibodies. *Arch. Surg. (Chicago)* 127, 1094–1100.
- (46) Curtet, C., Vuillez, J. P., Daniel, G., Aillet, G., Chetanneau, A., Visset, J., Kremer, M., Thedrez, P., and Chatal, J. F. (1990) Feasibility Study of Radioimmunoguided Surgery of Colorectal Carcinomas Using Indium-111-CEA-Specific Monoclonal Antibody. *Eur. J. Nucl. Med.* 17, 299–304.

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Assessment of Radiochemical Design of Antibodies Using an Ester Bond as the Metabolizable Linkage: Evaluation of Maleimidoethyl 3-(Tri-*n*-butylstannyl)hippurate as a Radioiodination Reagent of Antibodies for Diagnostic and Therapeutic Applications

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Reduction of radioactivity levels in nontarget tissues such as the liver and kidney constitutes a problem to be resolved in diagnostic and therapeutic applications of radiolabeled monoclonal antibodies (mAbs). A new radioiodination reagent with an ester bond to liberate *m*-iodohippuric acid from covalently conjugated proteins, maleimidoethyl 3-(tri-*n*-butylstannyl)hippurate (MIH), was recently developed. MIH liberated *m*-iodohippuric acid from galactosylneoglycoalbumin in murine liver, and the radiometabolite was rapidly eliminated from the liver into urine as an intact structure. In this study, intact IgG and Fab fragment of a mAb against osteogenic sarcoma were radioiodinated with MIH to further assess the applicability of MIH to radioimmunomaging and therapy. For comparison, a mAb radioiodinated with *N*-succinimidyl iodobenzoate (SIB) and indium-111 (¹¹¹In)-labeled mAbs with diethylenetriaminepentaacetic dianhydride (cDTPA) or 1-[4-[(5-maleimidopentyl)amino]benzyl]-ethylenediaminetetraacetic acid (EMCS-Bz-EDTA) were used. Size-exclusion HPLC analysis and cell binding assays indicated the preservation of both structure and antigen binding affinity of radioiodinated MIH-OST7 (IgG). In biodistribution studies in mice, [¹²⁵I]MIH-OST7 (IgG) showed faster systemic clearance of radioactivity after 24 h postinjection than did [¹³¹I]SIB- and [¹¹¹In]EMCS-Bz-EDTA-OST7 (IgG). [¹²⁵I]MIH-OST7 (IgG) also exhibited much lower radioactivity levels in nontarget tissues such as the liver and kidney, with higher radioactivity levels in the blood up to 72 h postinjection when compared with [¹¹¹In]cDTPA-OST7 (IgG). Radioactivity excreted from the mice was found in the urine as *m*-iodohippuric acid, following administration of [¹²⁵I]MIH-OST7 (IgG). In athymic mice bearing osteogenic sarcoma, [¹³¹I]MIH-OST7 (IgG) indicated higher tumor-to-nontarget ratios of radioactivity at both 24 and 48 h postinjection than [¹²⁵I]SIB-OST7 (IgG). Although both radioiodinated OST7s showed similar radioactivity levels in the target at 24 h postinjection, a small but significant decrease in the target radioactivity level was observed with [¹³¹I]MIH-OST7 (IgG) at 48 h postinjection. In addition, [¹³¹I]MIH-OST7 (Fab) showed very rapid cleavage of the ester bond both *in vivo* and *in vitro*. These findings indicated that while MIH may be a useful reagent for radioimmunomaging using IgG mAb, its application to smaller molecular weight mAbs and radioimmunotherapy would be hindered due to the labile characteristics of the ester bond in plasma. Thus, while the present study reinforced the usefulness of metabolizable linkages for reducing nontarget radioactivity levels, a development of plasma-stable metabolizable linkages is also warranted for radioimmunotherapy and for smaller molecular weight polypeptides.

INTRODUCTION

Recent advances in genetic and protein engineering have provided monoclonal antibodies (mAbs) with reduced or no immunogenicity (e.g. chimeric or humanized mAbs) or more favorable distribution characteristics to target tissues (e.g. single-chain Fv fragment) (1–3). Radiochemists have developed various reagents that allow stable attachment of radiolabels to mAbs *in vivo*

(4–6). Efforts have also been made to increase the target radioactivity levels by changing the administration routes, by hyperthermia, or by using biological response modifiers with various mechanisms of action (7). These approaches would eliminate problems associated with the clinical application of radiolabeled mAbs. However, localization of radioactivity in nontarget tissues still constitutes a problem to be resolved, and the development of radiochemistry of mAbs that can decrease the undesirable radioactivity localization is highly warranted for both diagnostic and therapeutic applications of the radiopharmaceuticals. Furthermore, the development of such chemistry would also be useful for radiolabeling of smaller molecular weight peptides such as octreotide and chemotactic peptides.

Among the various strategies to decrease the nontarget localization of radioactivity following administration of radiolabeled mAbs, use of a “metabolizable linkage” constitutes one attractive approach (8–14). This radio-

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chemical design of mAbs allows excretion of the radioactivity accumulated in nontarget tissues into the urine through the introduction of a cleavable linkage between the mAb and the radiolabel. The rationale behind the radiochemical design of mAbs is supported by recent metabolic studies of radiolabeled polypeptides, indicating that radiometabolites generated after lysosomal proteolysis of polypeptides play an important role in the radioactivity levels in the liver (15–18). These studies also indicated the importance of the chemical and biological properties of radiometabolites in the radiochemical design of mAbs using metabolizable linkages; even if a suitable metabolizable linkage is placed between the mAb and radiolabel, little or no improvement in the radioactivity levels in the nontarget tissues would be achieved if the radiometabolite is retained in or only slowly excreted from the lysosomal compartment of nontarget tissues. Therefore, metabolizable linkages should be designed to liberate radiometabolites with rapid urinary excretion from covalently conjugated mAbs after lysosomal proteolysis in nontarget tissues.

Recently, we developed a novel radioiodination reagent with an ester bond to liberate *m*-iodohippuric acid, maleimidoethyl 3-(tri-*n*-butylstannyl)hippurate (MIH) (19). Preliminary studies using galactosylneoglycoalbumin (NGA) as a model protein indicated that MIH liberates *m*-iodohippuric acid as the major radiometabolite after lysosomal proteolysis in hepatic parenchymal cells, and the metabolite was excreted rapidly from the hepatocytes into the urine intact. Thus, MIH is a radioiodination reagent that liberates a radiometabolite of known chemical and biological properties after cleavage of the ester bond from the covalently conjugated protein in hepatic parenchymal cells, where nonspecific localization of radioactivity is observed in mice when radiolabeled mAbs are administered (20, 21). Such chemical and biological characteristics make MIH a potentially useful reagent for radioiodination of mAbs for diagnostic and therapeutic purposes. Furthermore, since meta-iodohippuric acid possesses almost ideal chemical and biological characteristics as a radiometabolite, use of MIH would provide insight for estimation of the usefulness of ester bonds as metabolizable linkages.

To assess the applicability of MIH to radioimmunoimaging and therapy, an intact IgG (IgG₁) and Fab fragment of a mAb against osteogenic sarcoma (OST7) were radioiodinated using MIH. Stabilities of the ester bonds in MIH-labeled OST7s were estimated. Biodistributions of radioactivity of MIH-labeled OST7 (IgG) were investigated in normal and athymic mice bearing osteogenic sarcoma. Radioiodinated OST7 (IgG and Fab) with *N*-succinimidyl 3-iodobenzoate (SIB) and indium-111 (¹¹¹In)-labeled OST7s (IgG) with diethylenetriaminepentaacetic dianhydride (cDTPA) or 1-[4-[(5-maleimidopentyl)-amino]benzyl]ethylenediaminetetraacetic acid (EMCS-Bz-EDTA) as bifunctional chelating agents (BCAs) were used as references. Chemical structures of each conjugate are illustrated in Figure 1. SIB generates radioiodinated mAbs with high stability against *in vivo* deiodination and is superior to direct radioiodination of mAbs for both diagnostic and therapeutic purposes (6). The benzyl-EDTA chelating site in EMCS-Bz-EDTA forms an ¹¹¹In chelate of high *in vivo* stability (16, 22) and the chelating agent can be attached to OST7 (IgG) by conjugation procedures similar to those used for radioiodinated MIH. cDTPA has been a widely used BCA for ¹¹¹In radiolabeling of mAbs. Furthermore, radiometabolites generated from the respective reagent after lysosomal proteolysis in murine hepatocytes have been well characterized in previous studies (16–19, 23, 24). The

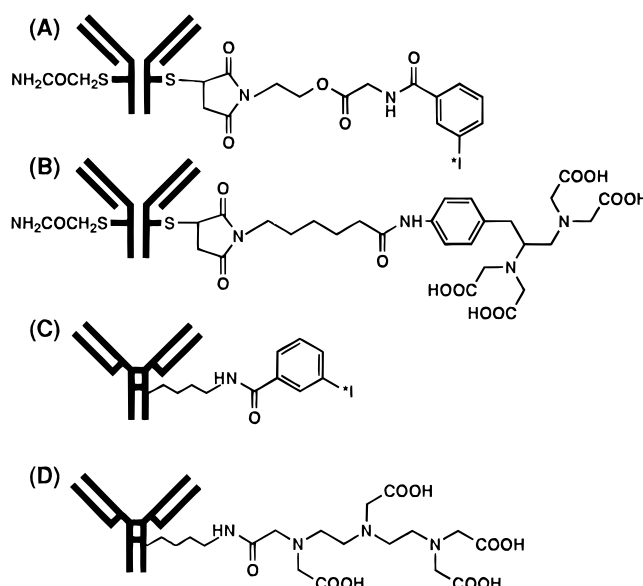


Figure 1. Chemical structures of MIH-labeled OST7 (IgG) (A), 1-[4-[(5-maleimidopentyl)amino]benzyl]ethylenediaminetetraacetic acid (EMCS-Bz-EDTA)-conjugated OST7 (IgG) (B), *N*-succinimidyl 3-iodobenzoate (SIB)-labeled OST7 (IgG) (C), and cDTPA-conjugated OST7 (D). While MIH and EMCS-Bz-EDTA were attached to OST7 by similar conjugation procedures, cDTPA and SIB were attached to amine residues of OST7. MIH and SIB possess a *m*-iodobenzoyl group to stabilize iodine against *in vivo* deiodination.

persistent retention of radiometabolites in hepatocytes derived from cDTPA-¹¹¹In would be useful for estimation of the role played by the ester bond in MIH. The radiochemical design of mAbs using an ester bond as the metabolizable linkage was also evaluated for diagnostic and therapeutic applications in nuclear medicine.

MATERIALS AND METHODS

Reagents and Chemicals. Na[¹³¹I]I and Na[¹²⁵I]I were obtained from Daiichi Radioisotopes Laboratories (Tokyo, Japan) and Daiichi Kagaku (Tokyo), respectively, and were diluted with phosphate buffer (PB; 0.1 M, pH 7.4) to 7.4 MBq (0.2 mCi)/μL and 3.7 MBq (0.1 mCi)/μL, respectively. ¹¹¹InCl₃ (74 MBq (2 mCi)/mL in 0.02 N HCl) was supplied by Nihon Medi-Physics (Takarazuka, Japan). MIH was synthesized as described previously (19). *N*-Succinimidyl 3-(tri-*n*-butylstannyl)benzoate (ATE) was prepared according to the method of Zalutsky et al. (6). Both cDTPA and EMCS-Bz-EDTA were purchased from Dojindo Laboratories (Kumamoto, Japan). Size-exclusion HPLC and reversed-phase (RP) HPLC were performed using Cosmosil Diol-300 (7.5 × 600 mm, Nacalai Tesque, Kyoto, Japan) and Cosmosil 5C₁₈-AR (4.6 × 250 mm, Nacalai Tesque), respectively. Size-exclusion HPLC and RP-HPLC columns were equilibrated and eluted with 0.1 M phosphate buffer (PB; pH 6.8) and with a mixture of 0.1% aqueous phosphoric acid–acetonitrile (7:3), respectively, at a flow rate of 1 mL/min. Other reagents were of reagent grade and used as received.

Tumor and mAbs. KT005-cloned human osteogenic sarcoma was maintained by serial subcutaneous transplantation in athymic mice. Pieces of tumor tissue (1–2 mm³; 0.3–0.5 g) at ca. 2 weeks postplantation were used for the *in vivo* study. Single-cell suspensions from xenografted tumors were used for the *in vitro* study (25, 26). The mAb against osteogenic sarcoma (OST7, IgG₁), generated by standard hybridoma technology, was purified by ammonium sulfate precipitation with subsequent protein A affinity chromatography (Pharmacia Biotech

Co. Ltd., Tokyo) (27). The Fab fragment of OST7 was prepared according to the standard procedure (28).

Preparation of [$^{125/131}$ I]MIH-Labeled OST7s (IgG and Fab). Radioiodination of MIH with Na[^{131} I]I was performed in the presence of *N*-chlorosuccinimide (NCS) used as an oxidant, as described previously (19). Briefly, a 22.0 μ L solution of MIH in methanol containing 1% acetic acid (0.56 mg/mL) was mixed with 6.04 μ L of NCS in methanol (0.5 mg/mL) in a sealed vial, followed by an addition of Na[^{131} I]I (3 μ L). After incubation of the reaction mixture for 45 min at room temperature, the reaction was quenched with aqueous sodium bisulfite (2.92 μ L, 0.72 mg/mL). The radiochemical yields of [^{131} I]-MIH were determined by TLC (Merck Art. 5553) developed with a mixture of chloroform–ether (1:1). The solvent was removed under a flow of N_2 prior to subsequent conjugation reactions with OST7s. Radioiodination of MIH with Na[^{125} I]I was performed as described above except using Na[^{125} I]I in place of Na[^{131} I]I.

Conjugation of [$^{125/131}$ I]MIH with OST7 (IgG) was performed by reducing the disulfide bonds of OST7, as described previously (8). Briefly, OST7 (IgG) (300 μ L, 2.5 mg/mL) in well-degassed PB (0.1 M, pH 7.0) containing 2 mM ethylenediaminetetraacetic acid (EDTA) was allowed to react with a 1000-fold molar excess of 2-mercaptoethanol (2-ME) at room temperature for 30 min. Excess 2-ME was removed by a centrifuged column procedure (29) using Sephadex G-50 (Pharmacia Biotech, Tokyo, Japan) equilibrated and eluted with PB (0.1 M, pH 6.0) containing 2 mM EDTA. Aliquots of this mixture were sampled for estimation of the number of thiol groups with 2,2'-dithiopyridine (30). The filtrate (215 μ L) was then added to a reaction vial containing crude [$^{125/131}$ I]MIH. After the reaction mixture was agitated gently for 2 h at room temperature, 39 μ L of iodoacetamide (10 mg/mL) in PB (0.1 M, pH 6.0) was added. The reaction mixture was further incubated for 30 min to alkylate the unreacted thiol groups (8). [$^{125/131}$ I]MIH-labeled OST7 (IgG) was subsequently purified by the centrifuged column procedure, equilibrated, and eluted with phosphate-buffered saline (PBS; 0.1 M, pH 6.0).

Conjugation of Fab fragment of OST7 with [^{131} I]MIH was performed by reaction of crude [^{131} I]MIH with OST7 (Fab) (410 μ L, 1.4 mg/mL) freshly pretreated with a 1000-fold molar excess of 2-ME to expose thiol groups. [^{131} I]-MIH was prepared according to the procedure described above. After incubation of the reaction mixture for 2 h at room temperature, iodoacetamide (37 μ L; 10 mg/mL) was added. [^{131} I]MIH-labeled OST7 (Fab) was purified according to the centrifuged column procedure as described above.

Preparation of [$^{125/131}$ I]SIB-Labeled OST7 (IgG and Fab). [$^{125/131}$ I]SIB was prepared by the radioiodination of ATE with Na[$^{125/131}$ I]I according to the procedure described above except that 0.45 mg/mL ATE in 1% acetic acid–methanol was used in place of MIH. Radiochemical yields of [$^{125/131}$ I]SIB were determined by TLC developed with a mixture of 30% ethyl acetate in hexane (6).

Conjugation of [$^{125/131}$ I]SIB with OST7s (IgG and Fab) was performed according to the procedure of Zalutsky et al. (6) with slight modifications as follows: A solution of OST7 (IgG; 120 μ L, 2.5 mg/mL) or OST7 (Fab; 200 μ L, 4 mg/mL) in 0.2 M borate buffer (pH 8.5) was added to the dried residue of crude [$^{125/131}$ I]SIB. After gentle incubation for 1 h at room temperature, radioiodinated OST7s were purified according to the centrifuged column procedure, equilibrated, and eluted with PB (0.1 M, pH 7.4).

Preparation of ^{125} I-OST7 (IgG). Direct radioiodination of OST7 (IgG) was performed according to the chloramine T method (31). To 200 μ L aliquots of OST7

(0.25 mg/mL) in 0.3 M PB (pH 7.5) was added 5 μ L of Na[^{125} I]I. Chloramine T (0.1 mg/mL, 25 μ L), freshly prepared in the same buffer, was then added. After incubation of the mixture for 10 min at room temperature, the reaction was terminated by addition of 6 μ L of aqueous sodium bisulfite (0.7 mg/mL). ^{125} I-Labeled OST7 (IgG) was then purified according to the centrifuged column procedure.

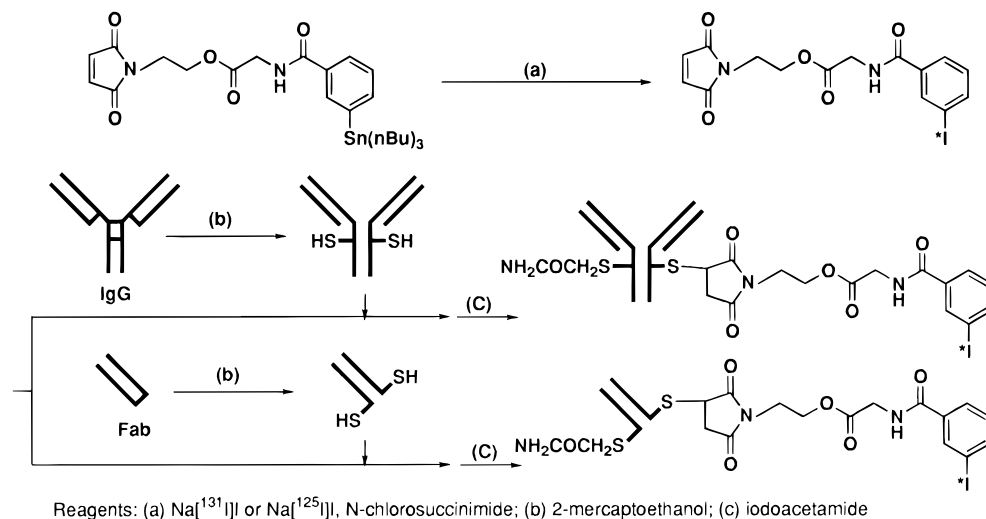
Radiochemical purities of the radioiodinated mAbs were determined by size-exclusion HPLC, TLC, and cellulose acetate electrophoresis (CAE). CAE was run at an electrostatic field of 0.8 mA/cm for 45 min in the veronal buffer ($I = 0.05$, pH 8.6).

Preparation of ^{111} In-Labeled OST7 (IgG). OST7 (IgG) was also labeled with ^{111} In using cDTPA or EMCS-Bz-EDTA as the BCA. cDTPA was conjugated to OST7 according to the procedure of Paik et al. (32) with slight modifications. To a solution of OST7 (400 μ L; 6 mg/mL) in 0.05 M borate-buffered saline (pH 8.5) was added 20 μ L of cDTPA in dry dimethyl sulfoxide (5.7 mg/mL). After gentle stirring at room temperature for 30 min, a small aliquot (10 μ L) was removed for ^{111} In-radiolabeling (see below) to estimate the number of DTPA molecules attached per molecule of OST7. The remainder of the reaction mixture was purified by size-exclusion HPLC, followed by Sephadex G-50 column chromatography (1.8 $\times$ 40 cm), equilibrated, and eluted with 20 mM 2-(*N*-morpholino)ethanesulfonic acid (MES)-buffered saline (pH 6.0). EMCS-Bz-EDTA was conjugated to OST7 (IgG) by reducing the disulfide bonds in OST7 according to a procedure similar to that used for [^{131} I]MIH conjugation. EMCS-Bz-EDTA–OST7 (IgG) was purified from unreacted small molecular weight compounds according to the centrifuged column procedure using Sephadex G-50, equilibrated, and eluted with 20 mM MES-buffered saline (pH 6.0). The number of EMCS-Bz-EDTA introduced per molecule of OST7 was determined by measuring the thiol groups before and after conjugation reaction using 2,2'-dithiopyridine (30), as previously reported (8).

The purified cDTPA–OST7 (IgG) and EMCS-Bz-EDTA–OST7 (IgG) were labeled with ^{111} In according to the procedure of Brechbiel et al. (4) with slight modifications as previously described (15). Briefly, 5 μ L of ^{111} InCl $_3$ in 0.02 N HCl was added to a mixed solution (5 μ L) of 1 M sodium acetate and 1.75 N HCl (4:1). After 3 min, 40 μ L of respective conjugate (1 mg/mL) was added, and the reaction solution was kept at 23 $^{\circ}$ C for 1 h. A 13 μ L aliquot of 2 mM EDTA in 0.1 M acetate buffer (pH 5.0) was then added to each reaction solution. After 30 min of incubation at 23 $^{\circ}$ C, ^{111} In-labeled OST7s were purified according to the centrifuged column procedure using Sephadex G-50, equilibrated with 0.1 M PBS (pH 6.0). cDTPA–OST7 (IgG) was further purified by size-exclusion HPLC under the conditions described above. Radiochemical purity of ^{111} In-cDTPA–OST7 (IgG) was determined by CAE and TLC developed with a mixture of 10 % aqueous ammonium formate–methanol–0.2 M aqueous citric acid (2:2:1). The number of cDTPA per OST7 molecule was also determined by the ^{111} In radiolabeling of an unpurified reaction solution of cDTPA–OST7 (IgG), followed by TLC analysis, as previously described (33, 34). The radiochemical purity of ^{111} In-EMCS-Bz-EDTA–OST7 (IgG) was determined by CAE.

Immunoreactivity Measurement. The immunoreactivity of radioiodinated OST7s (IgG) was determined as described previously (8, 25). Tumor cells (2×10^4 – 1×10^7) suspended in 100 μ L of Dulbecco's PBS were incubated with 100 μ L of radiolabeled OST7 in microcentrifugation tubes (5.7 $\times$ 46 mm) for 2 h at 4 $^{\circ}$ C. After centrifugation at 10000g for 5 min, the supernatant was

Scheme 1



discarded and the radioactivity determined using a well counter (ARC 2000, Aloka, Tokyo, Japan).

Stability Estimation of the Ester Bonds in [¹³¹I]-MIH-OST7s (IgG and Fab). [¹³¹I]MIH-OST7s (IgG and Fab) were diluted to 0.5 mg/mL with 0.1 M PBS (pH 6.0), and 20 μ L of each solution was added to 230 μ L of freshly prepared murine plasma, human serum, or 20 mM PBS (pH 7.4). After incubation for 1, 3, 6, or 24 h at 37 $^{\circ}$ C, samples were taken from the solutions, and the percentages of radioactivity released from each antibody were analyzed by paper chromatography (PC; No. 50, Advantec Toyo, Tokyo, Japan) and TLC. Each value was calculated by dividing the antibody-bound radioactivity at various intervals by the radiochemical purity of freshly prepared respective antibody. PC was developed with a mixture of methanol–water (8:2), and TLC was developed with a mixture of chloroform–methanol–water (15:8:1). Under these analytical conditions, protein-bound radioactivity, free iodine, *m*-iodobenzoic acid, and *m*-iodohippuric acid had *R_f* values of 0, 0.6–0.65, 0.9–0.95, and 0.9–0.95 on PC and 0, 0.1, 0.4, and 0.25 on TLC, respectively. Percentages of radioactivity released from [¹²⁵I]SIB-OST7 (IgG and Fab) were also determined under similar conditions.

In Vivo Studies. Each radiolabeled OST7 was diluted to 200 μ g/mL with 0.1 M PBS (pH 6.0). Two-milliliter aliquots of [¹²⁵I]MIH-OST7 (IgG) were mixed with the same volume of [¹³¹I]SIB-OST7 (IgG) prior to administration. [¹²⁵I]MIH-OST7 (IgG) was also mixed with the same volume of ¹¹¹In-cDTA-OST7 (IgG) or ¹¹¹In-EMCS-Bz-EDTA-OST7 (IgG) for *in vivo* studies. Biodistributions of radioactivity after intravenous administration of each mixture in 6-week-old male ddY mice (35) were monitored at 1, 3, 6, 24, 48, and 72 h postinjection. Groups of five mice, each receiving 20 μ g of radiolabeled OST7, were used for the experiments. Organs of interest were removed and weighed, and the radioactivity was determined with a well counter (ARC 2000). At 24 h postinjection of [¹²⁵I]MIH-OST7 (IgG), urine samples were collected and analyzed by TLC, RP-HPLC, and PC as described above. In RP-HPLC, free iodine, *m*-iodohippuric acid, and *m*-iodobenzoic acid had retention times of 2.9, 9.4, and 30.8 min, respectively.

Biodistributions of radioactivity after injection of a mixed solution of [¹³¹I]MIH-OST7 (Fab) and [¹²⁵I]SIB-OST7 (Fab) (10 μ g each) were determined at 10 and 30 min and 1, 3, 6, and 24 h postinjection according to the procedures described above.

Atymic mice bearing osteogenic sarcoma were also

treated intravenously with a mixture of [¹²⁵I]SIB-OST7 (IgG) and [¹³¹I]MIH-OST7 (IgG) (10 μ g each), and the animals were sacrificed at 24 and 48 h postinjection (five mice each group). Mean weights of mice and tumors used in this study were 20.30 ± 0.48 and 0.44 ± 0.13 g, respectively. Organs of interest were removed and weighed, and the radioactivity in each tissue was determined (ARC-2000).

Statistical Analysis. Data are expressed as means $\pm$ standard deviation where appropriate. Results were statistically analyzed using unpaired *t*-test for *in vitro* studies and paired *t*-test for *in vivo* studies. Differences were considered statistically significant when the *p* value was less than 0.05.

RESULTS

Preparation of Radiolabeled OST7s. Radioiodination of MIH was performed in 1% AcOH–MeOH in the presence of NCS as an oxidant (Scheme 1). After 45 min of reaction at room temperature, [^{125/131}I]MIH was obtained with a radiochemical yield of more than 83% as determined by TLC. A similar procedure was employed for the radioiodination of ATE, which produced [^{125/131}I]SIB with a radiochemical yield of over 80%. All of the radioiodinated compounds were treated with sodium bisulfite to quench any residual NCS, preventing the iodine species from attaching to tyrosine residues of the antibody molecule. Neutralization of NCS also prevented exposure of the protein to the oxidant.

Conjugation of [^{125/131}I]MIH with OST7s was performed by reactions of thiolated antibodies with the maleimide group of [^{125/131}I]MIH. 2-ME reduction of OST7 (IgG) and Fab exposed 6.4 and 2.3 molecules of the thiol groups per molecule of OST7 (IgG) and OST7 (Fab), respectively, and subsequent conjugation reactions with [^{125/131}I]MIH were performed at a maleimide to thiol molar ratio of 1:1. The radiochemical yields of [¹³¹I]MIH-labeled OST7 (IgG) and OST7 (Fab) were 50.2 and 63.7%, respectively, as determined by CAE. After purification by the centrifuged column procedure, [¹³¹I]MIH-OST7 (IgG) and [¹³¹I]MIH-OST7 (Fab) were obtained with respective radiochemical purities of 92.7 and 93.5% for subsequent studies. [¹³¹I]MIH-OST7 (IgG) and [¹³¹I]MIH-OST7 (Fab) showed specific activities of 55.5 MBq (1.5 mCi)/mg and 12.1 MBq (0.33 mCi)/mg, respectively. Size-exclusion HPLC analyses of [¹³¹I]MIH-labeled OST7 (IgG) and OST7 (Fab) indicated more than 96.2 and 95.5% radioactivities in fractions with retention times of 15.4

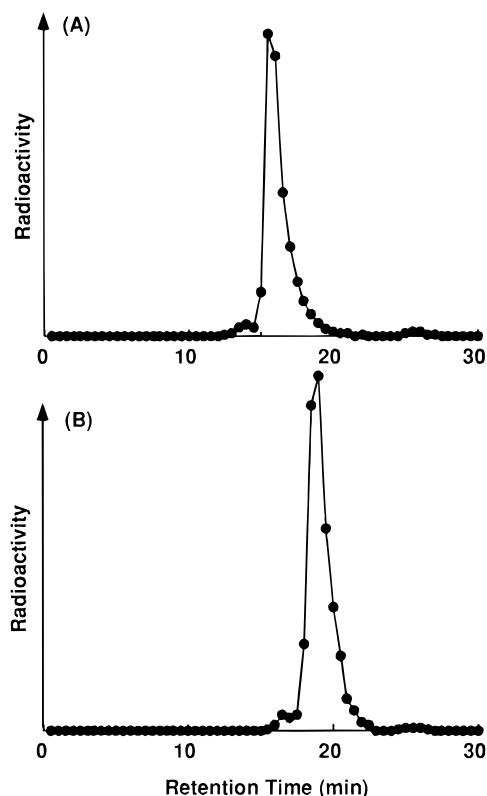


Figure 2. Size-exclusion HPLC profiles of the radioactivity of $[^{131}\text{I}]\text{MIH-OST7 (IgG)}$ (A) and $[^{131}\text{I}]\text{MIH-OST7 (Fab)}$ (B). More than 96% (A) and 95% (B) of the radioactivities were observed in fractions similar to those of unmodified OST7 (IgG) and OST7 (Fab), respectively. Under these conditions, thyroglobulin (670 000), OST7 (IgG), bovine serum albumin (68 000), OST7 (Fab), and cytochrome *c* (13 000) were eluted at retention times of 11.4, 15.4, 16.8, 18.8, and 21.0 min, respectively.

and 18.8 min, respectively, which were similar to those of the original (nontreated) OST7 (IgG) and OST7 (Fab), respectively (Figure 2). Under similar conditions, $[^{125}\text{I}]\text{MIH-OST7 (IgG)}$ was also prepared with radiochemical purity and specific activity of 98.1% and 10.36 MBq (0.28 mCi)/mg, respectively.

Conjugation of $[^{131}\text{I}]\text{SIB}$ with OST7 (IgG) and OST7 (Fab) was performed at active ester to protein molar ratios of 7:1 and 4:1, respectively, with radiochemical yields of 47.5 and 55.5%. The radiochemical purities and specific activities of the resulting $[^{131}\text{I}]\text{SIB-labeled mAbs}$ were 99.3% and 52 MBq (1.4 mCi)/mg for OST7 (IgG) and 93.5% and 11.8 MBq (0.32 mCi)/mg for OST7 (Fab), respectively. $[^{125}\text{I}]\text{SIB-OST7 (IgG)}$ was also prepared according to similar procedures with a radiochemical purity and a specific activity of 97.0% and 12.2 MBq (0.33 mCi)/mg, respectively. Direct radioiodination of OST7 (IgG) generated ^{125}I -labeled OST7 (IgG) with a radiochemical purity and a specific activity of 97.3% and 92.5 MBq (2.5 mCi)/mg, respectively.

OST7-cDTPA was prepared by reaction of acid anhydride with amine residues of the protein. ^{111}In radiolabeling of unpurified reaction solution and subsequent analysis by TLC estimated the number of cDTPA per molecule of OST7 to be 2.2. The radiochemical purity of $^{111}\text{In-cDTPA-OST7}$ was 99.4 and 68.6% when analyzed by TLC and CAE, respectively. Since further attempts to improve the radiochemical purity were unsuccessful (36), $^{111}\text{In-cDTPA-OST7 (IgG)}$ of this purity was used for subsequent *in vivo* study. EMCS-Bz-EDTA-OST7 (IgG) conjugate was prepared by maleimide-thiol chemistry using the maleimide group of EMCS-Bz-EDTA and the thiol groups of OST7 (IgG), generated by reducing

Table 1. Percent Radioactivity in Protein Fractions following Incubation of $[^{131}\text{I}]\text{MIH-OST7 (IgG)}$ and $[^{131}\text{I}]\text{MIH-OST7 (Fab)}$ in Human Serum, Murine Plasma, and Buffered Solution^a

incubation time (h)	$[^{131}\text{I}]\text{MIH-OST7 (IgG)}$			$[^{131}\text{I}]\text{MIH-OST7 (Fab)}$		
	serum	plasma	buffer	serum	plasma	buffer
1	97.15 (0.17)	98.68 (0.47)	99.80 (0.49)	94.33 (1.56)	97.69 (0.30)	99.26 (0.29)
3	90.66 (0.51)	96.12 (0.05)	97.12 (0.73)	81.68 (0.41)	91.92 (1.50)	97.01 (1.42)
6	79.81 (1.04)	89.10 (0.94)	95.89 (1.06)	66.80 (2.76)	81.43 (2.11)	96.47 (1.15)
24	43.44 (5.58)	66.40 (1.13)	82.50 (3.84)	27.22 (3.44)	54.46 (1.08)	83.78 (1.02)
half-life (days)	0.87 (0.09)	1.47 (0.05)	2.91 (0.57)	0.62 (0.02)	1.08 (0.03)	2.94 (0.29)

^a $[^{131}\text{I}]\text{MIH-OST7 (IgG)}$ or $[^{131}\text{I}]\text{MIH-OST7 (Fab)}$ (20 μL each) was added to 230 μL of human serum, murine plasma, or buffered solution (20 mM PBS, pH 7.4) at 37 $^{\circ}\text{C}$. Expressed as means (SD) from four experiments.

the disulfide bonds. The number of EMCS-Bz-EDTA molecules attached per molecule of OST7 was 2.2 by measuring the thiol groups before and after conjugation reaction. The radiochemical purity of $^{111}\text{In-EMCS-Bz-EDTA-OST7 (IgG)}$ after the centrifuged column procedure was 95.9% as determined by CAE.

Stability of the Ester Bond in $[^{131}\text{I}]\text{MIH-OST7s (IgG and Fab)}$. Stabilities of the ester bonds in both $[^{131}\text{I}]\text{MIH-OST7 (IgG)}$ and $[^{131}\text{I}]\text{MIH-OST7 (Fab)}$ were estimated in murine plasma, human serum, and buffered solution of neutral pH (pH 7.4) at 37 $^{\circ}\text{C}$. Table 1 shows the percentages of the protein-bound radioactivity of each $[^{131}\text{I}]\text{MIH-OST7}$ at 1, 3, 6, and 24 h postincubation as determined by TLC analysis. Both $[^{131}\text{I}]\text{MIH-OST7 (IgG)}$ and $[^{131}\text{I}]\text{MIH-OST7 (Fab)}$ released significantly more radioactivity in human serum than in murine plasma, with the lowest level of release in the buffered solution. In addition, while no significant differences were observed in the radioactivity release from the two $[^{131}\text{I}]\text{MIH-labeled OST7s}$ in the buffered solution, $[^{131}\text{I}]\text{MIH-OST7 (Fab)}$ liberated significantly more radioactivity than $[^{131}\text{I}]\text{MIH-OST7 (IgG)}$ in both human serum and murine plasma. The calculated half-lives of $[^{131}\text{I}]\text{MIH-OST7 (IgG)}$ and $[^{131}\text{I}]\text{MIH-OST7 (Fab)}$ in the respective media are also presented in Table 1. The radioactivity released from the two $[^{131}\text{I}]\text{MIH-labeled OST7s}$ showed R_f values on TLC and PC analyses similar to those of *m*-iodohippuric acid. Under similar experimental conditions, both $[^{125}\text{I}]\text{SIB-OST7 (IgG)}$ and $[^{125}\text{I}]\text{SIB-OST7 (Fab)}$ released <5% of the initial radioactivity in all incubation media up to 24 h postincubation.

Immunoreactivity. Figure 3 shows the binding of $[^{131}\text{I}]\text{MIH-OST7 (IgG)}$, $[^{125}\text{I}]\text{SIB-OST7 (IgG)}$, and $^{125}\text{I-OST7 (IgG)}$ to KT005 cells as a function of cell number. No significant differences were observed in the cell-bound radioactivity among the three radioiodinated OST7s.

Biodistribution Studies in Mice. Biodistributions of radioactivity after administration of a mixture of $[^{125}\text{I}]\text{MIH-OST7 (IgG)}$ and $[^{131}\text{I}]\text{SIB-OST7 (IgG)}$, $[^{125}\text{I}]\text{MIH-OST7 (IgG)}$ and $^{111}\text{In-cDTPA-OST7 (IgG)}$, or $[^{125}\text{I}]\text{MIH-OST7 (IgG)}$ and $^{111}\text{In-EMCS-Bz-EDTA-OST7 (IgG)}$ in ddY mice are shown in Figure 4. Since $[^{125}\text{I}]\text{MIH-OST7 (IgG)}$ showed similar radioactivity distributions in the three experiments, results from the experiments were combined and presented as $[^{125}\text{I}]\text{MIH-OST7 (IgG)}$ in Figure 4. $^{111}\text{In-cDTPA-OST7}$ indicated the fastest elimination rate of the radioactivity from the blood at an early postinjection time (up to 24 h). After this postinjection time, $^{111}\text{In-cDTPA-OST7}$ showed radioactivity clearance from the blood at a rate similar to those of $^{111}\text{In-EMCS-}$

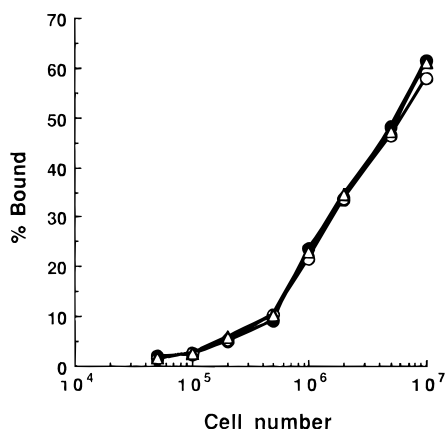


Figure 3. Reactivity of radioiodinated OST7s (IgG) with KT005 cells. Radioactivity bound to cells (%) is plotted against the cell number. Both [¹³¹I]MIH-OST7 (IgG) (●) and [¹³¹I]SIB-OST7 (IgG) (○) registered binding activities identical to that of [¹²⁵I]SIB-OST7 (IgG) (Δ).

Bz-EDTA-OST7 and [¹³¹I]SIB-OST7 (IgG). However, the highest and most persistent radioactivity levels in tissues such as the liver and kidney were observed with ¹¹¹In-cDTPA-OST7 (IgG). While the rest of three radio-labeled OST7s (IgG) registered similar radioactivity levels in the blood up to 6 h postinjection, [¹²⁵I]MIH-OST7 (IgG) had the fastest radioactivity clearance rate from the blood after this time. [¹³¹I]SIB-OST7 (IgG) showed the slowest radioactivity elimination from the blood. The elimination rates of the radioactivity from blood showed biexponential profiles with the half-lives of the elimination phase of 1.91, 5.59, and 4.92 (days) for [¹²⁵I]MIH-OST7 (IgG), [¹³¹I]SIB-OST7 (IgG), and ¹¹¹In-EMCS-Bz-EDTA-OST7 (IgG), respectively. [¹²⁵I]MIH-OST7 (IgG) demonstrated the lowest levels of radioactivity in all tissues examined at all time points. The radioactivity levels in the liver, kidney, and spleen were almost identical between [¹³¹I]SIB-OST7 (IgG) and ¹¹¹In-EMCS-Bz-EDTA-OST7 (IgG) at 48 and 72 h postinjection. No significant differences in the radioactivity levels in the stomach were observed between [¹³¹I]SIB-OST7 (IgG) and ¹¹¹In-EMCS-Bz-EDTA-OST7 (IgG) from 24 to 72 h postinjection (unpaired *t*-test), although the former showed slightly higher radioactivity levels at earlier postinjection time points. At 72 h postinjection, the amounts of radioactivity excreted in the urine and feces were 57.83 ± 2.93 and $1.38 \pm 0.30\%$ for [¹²⁵I]MIH-OST7 (IgG), 15.22 ± 2.01 and $1.64 \pm 0.34\%$ for [¹³¹I]SIB-OST7 (IgG), 17.88 ± 1.50 and $7.33 \pm 1.17\%$ for ¹¹¹In-EMCS-Bz-EDTA-OST7 (IgG), and 11.61 ± 2.74 and $7.52 \pm 1.82\%$ for ¹¹¹In-cDTPA-OST7 (IgG), respectively.

Figure 5 shows RP-HPLC and TLC radioactivity profiles of urine excreted 24 h after injection of [¹²⁵I]MIH-OST7 (IgG) in ddY mice. RP-HPLC analysis showed that 96.6% of the radioactivity was eluted at a retention time identical to that of *m*-iodohippuric acid. TLC analysis also indicated a single radioactivity peak (96.8%) at an *R_f* value (0.25) identical to that of *m*-iodohippuric acid. Similar results were obtained by the PC analysis of the urine samples.

Biodistributions of radioactivity after simultaneous administration of [¹³¹I]MIH-OST7 (Fab) and [¹²⁵I]SIB-OST7 (Fab) in ddY mice are illustrated in Figure 6. [¹³¹I]MIH-OST7 (Fab) showed much faster clearance of radioactivity from blood, liver, kidney, and intestine than [¹²⁵I]SIB-OST7 (Fab), even at an early postinjection time (30 min). At 6 h postinjection, 68.77 ± 2.17 and $62.48 \pm 4.86\%$ of the injected radioactivity was excreted in the

urine following administration of [¹³¹I]MIH-OST7 (Fab) and [¹²⁵I]SIB-OST7 (Fab), respectively.

Table 2 shows the biodistributions of radioactivity after simultaneous administration of both [¹³¹I]MIH-OST7 (IgG) and [¹²⁵I]SIB-OST7 (IgG) in athymic mice bearing osteogenic sarcoma. At 24 h postinjection, [¹³¹I]MIH-OST7 (IgG) manifested radioactivity levels in the target and nontarget tissues similar to and significantly lower than those of [¹²⁵I]SIB-OST7 (IgG), respectively. As a result, [¹³¹I]MIH-OST7 (IgG) provided significantly higher tumor-to-nontarget ratios of radioactivity than [¹²⁵I]SIB-OST7 (IgG) at this postinjection time (Figure 7). [¹³¹I]MIH-OST7 (IgG) also showed significantly higher tumor-to-nontarget ratios of radioactivity in the blood, liver, kidney, and intestine at 48 h postinjection (Figure 7). However, a small but significant decrease in the radioactivity level in the tumor was observed with [¹³¹I]MIH-OST7 (IgG) at this postinjection time when compared with [¹²⁵I]SIB-OST7 (IgG).

DISCUSSION

Ester bonds are representative chemical linkages that allow rapid and selective release of designed compounds with carboxylate from covalently conjugated protein. However, since low radioactivity levels in the target are observed in clinical studies following administration of radiolabeled mAbs without cleavable linkages (37, 38), the ester bond in MIH should be sufficiently stable in plasma to preserve target radioactivity levels delivered by mAbs, while facilitating the rapid release of *m*-iodohippuric acid in nontarget tissues. To achieve the discriminated cleavage of linkage in MIH-labeled OST7 (IgG and Fab), the ester bond was placed close and proximal to the antibody molecule using thiol groups generated by reducing their disulfide bonds to hinder access of esterase to the ester bond (8). As shown in Figure 2, such conjugation procedures preserved the molecular weight of IgG and Fab antibodies at near neutral pH. The lack of changes in immunoreactivity of OST7 (IgG) were also confirmed by cell binding studies (Figure 3). However, when incubated in murine plasma or human serum, [¹³¹I]MIH-OST7 (IgG) released significantly more *m*-iodohippuric acid than in the buffered solution (Table 1), indicating that the ester bond in [¹³¹I]MIH-OST7 (IgG) was cleaved by esterase-mediated hydrolysis even with the conjugation procedure. Greater release of *m*-iodohippuric acid from [¹³¹I]MIH-OST7 (IgG) was also observed in human serum than in murine plasma, presumably due to the higher esterase activity in primates than in rodents (39).

In biodistribution studies in mice, [¹²⁵I]MIH-OST7 (IgG) showed radioactivity clearance from the blood almost identical to [¹³¹I]SIB-OST7 (IgG) and ¹¹¹In-EMCS-Bz-EDTA-OST7 (IgG) at early postinjection intervals (up to 6 h), as shown in Figure 4. [¹²⁵I]MIH-OST7 (IgG) was eliminated from murine blood with a half-life of 1.91 days (elimination phase), which was longer than the *in vitro* half-life of the ester bond in murine plasma (1.47 days). In addition, ¹¹¹In-EMCS-Bz-EDTA-OST7 (IgG) was eliminated from the blood more quickly than [¹³¹I]SIB-OST7 (IgG), presumably due to partial dissociation of noncovalently bonded chains in plasma and subsequent elimination of the radiolabeled fragments, as observed previously (8). Since [¹²⁵I]MIH-OST7 (IgG) was prepared by conjugation procedures similar to those used for ¹¹¹In-EMCS-Bz-EDTA-OST7 (IgG), a similar acceleration effect of the noncovalently bonded chains in [¹²⁵I]MIH-OST7 (IgG) would also be involved in its elimination rate from the blood. These

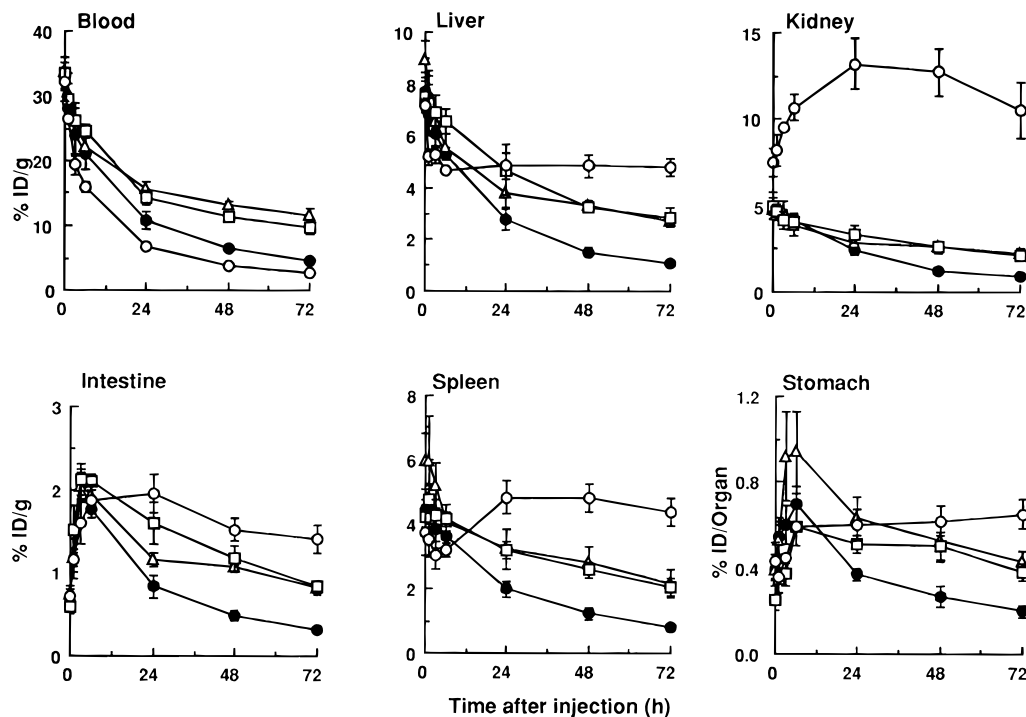


Figure 4. Biodistribution of radioactivity following administration of $[^{125}\text{I}]\text{MIH-OST7 (IgG)}$ (●), $[^{131}\text{I}]\text{SIB-OST7 (IgG)}$ (Δ), $[^{111}\text{In}]\text{-EMCS-Bz-EDTA-OST7 (IgG)}$ (□), and $[^{111}\text{In}]\text{CDTPA-OST7 (IgG)}$ (○) in normal mice.

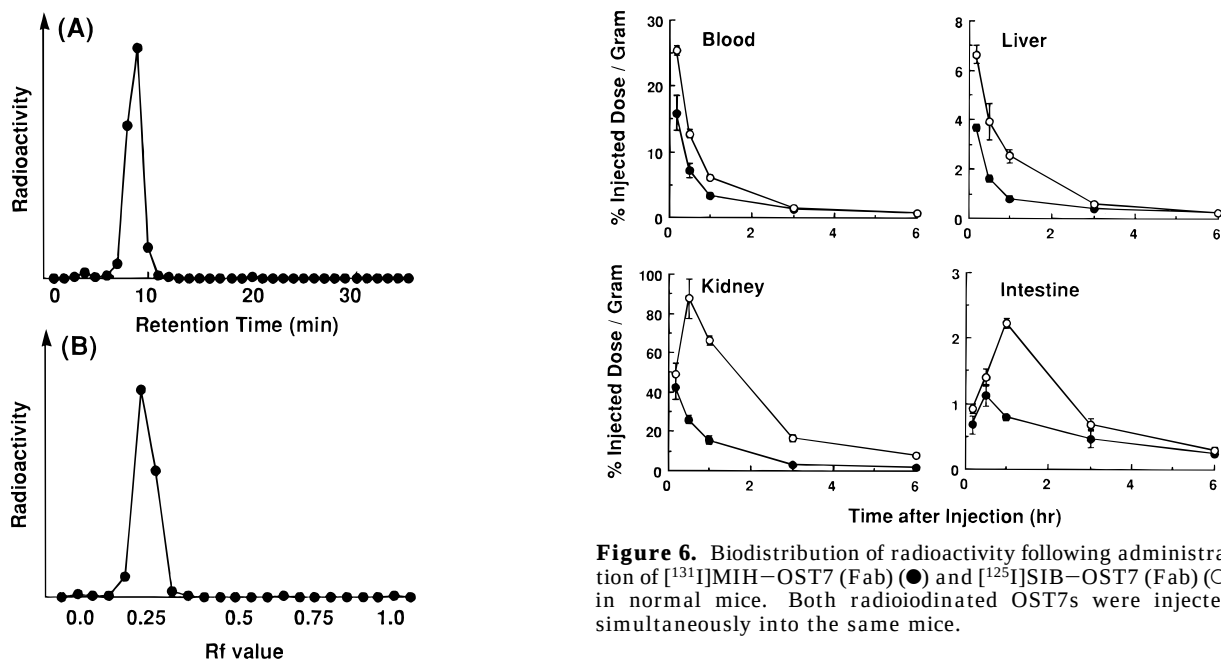


Figure 6. Biodistribution of radioactivity following administration of $[^{131}\text{I}]\text{MIH-OST7 (Fab)}$ (●) and $[^{125}\text{I}]\text{SIB-OST7 (Fab)}$ (○) in normal mice. Both radioiodinated OST7s were injected simultaneously into the same mice.

Figure 5. Radioactivity profiles of murine urine at 24 h postinjection of $[^{131}\text{I}]\text{MIH-OST7 (IgG)}$ analyzed by RP-HPLC (A) and TLC (B). The majority of the radioactivity (>96.6%) was detected at a retention time (A) or a R_f value (B) identical to those of *m*-iodohippuric acid by both analyses.

results indicated that the ester bond in $[^{125}\text{I}]\text{MIH-OST7 (IgG)}$ had higher stability against esterase-mediated hydrolysis *in vivo* than that observed *in vitro*. This was also supported by the similar radioactivity levels in the tumor at 24 h postinjection of $[^{131}\text{I}]\text{MIH-OST7 (IgG)}$ and $[^{125}\text{I}]\text{SIB-OST7 (IgG)}$ in athymic mice (Table 2). The differences in stability of the ester bond in $[^{125}\text{I}]\text{MIH-OST7 (IgG)}$ *in vitro* and *in vivo* may be attributable to the rapid blood stream *in vivo*, which may have hindered esterase access to its substrate. These results also suggested that the rapid hydrolysis of the ester bond in

$[^{125}\text{I}]\text{MIH-OST7 (IgG)}$ in human serum would not predict rapid cleavage of the ester bond when administered to humans.

$[^{125}\text{I}]\text{MIH-OST7 (IgG)}$ showed rapid systemic clearance of the radioactivity with over 96% of the excreted radioactivity being recovered in the urine. Analysis of urine confirmed selective release of *m*-iodohippuric acid (Figure 5). Low radioactivity levels in the stomach along with negligible amounts of free radioiodine in the urine following administration of $[^{125}\text{I}]\text{MIH-OST7 (IgG)}$ also indicated that *in vivo* deiodination was not involved in the rapid systemic clearance of radioactivity by the radioimmunoconjugate. Since radiolabeled mAbs are rapidly metabolized into smaller molecular weight radiometabolites in nontarget tissues (33, 40, 41), lower radioactivity levels in nontarget tissues with $[^{125}\text{I}]\text{MIH-OST7 (IgG)}$ would be attributable to faster elimination

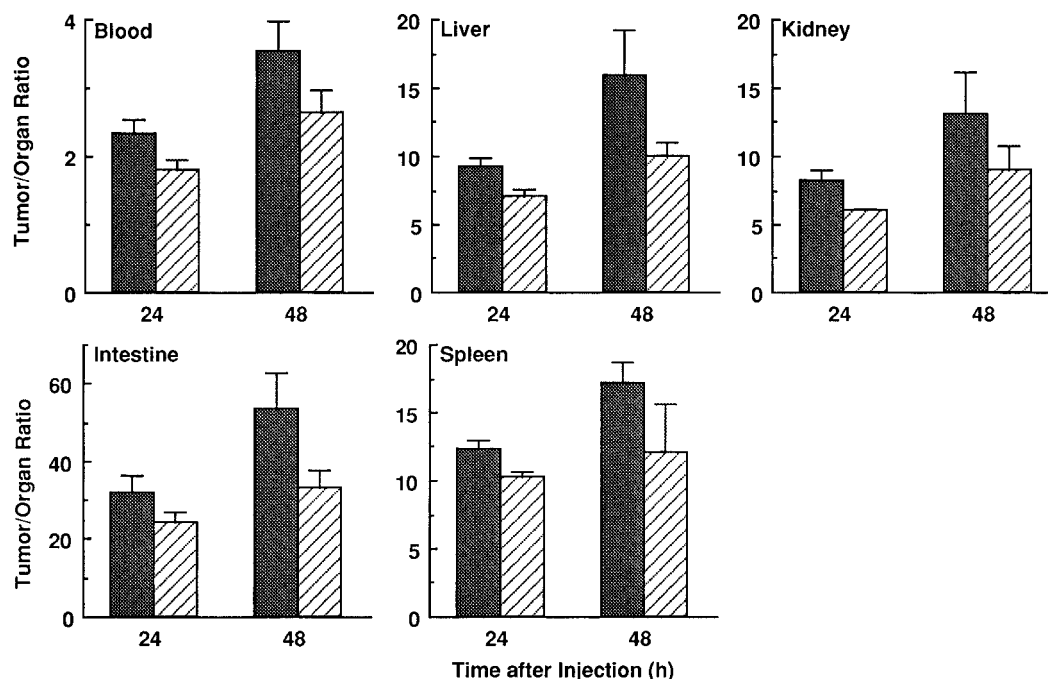


Figure 7. Tumor-to-organ ratios of the radioactivity at 24 and 48 h postinjection of [¹³¹I]MIH-OST7 (IgG) (solid column) and [¹²⁵I]SIB-OST7 (IgG) (hatched column) in athymic mice bearing osteogenic sarcoma.

Table 2. Biodistribution of Radioactivity after Simultaneous Administration of [¹³¹I]MIH-OST7 (IgG) and [¹²⁵I]SIB-OST7 (IgG) in Athymic Mice Bearing Osteogenic Sarcoma^{a,b}

tissue	[¹³¹ I]MIH-OST7 (IgG)		[¹²⁵ I]SIB-OST7 (IgG)	
	24 h	48 h	24 h	48 h
blood	8.74 (0.79)	4.66 (0.52)	12.51 (1.56)	8.98 (1.30)
liver	2.48 (0.14)	0.82 (0.16)	3.72 (0.32)	1.72 (0.33)
kidneys	2.43 (0.84)	1.23 (0.07)	3.70 (0.32)	2.78 (0.09)
intestine	0.68 (0.07)	0.32 (0.03)	1.09 (0.10)	0.68 (0.04)
spleen	1.69 (0.09)	0.79 (0.16)	2.32 (0.32)	1.87 (0.59)
lungs	3.58 (0.35)	1.78 (0.14)	5.19 (0.80)	3.46 (0.36)
stomach ^c	0.23 (0.06)	0.12 (0.05)	0.27 (0.06)	0.21 (0.05)
neck ^c	0.18 (0.04)	0.17 (0.03)	0.22 (0.08)	0.31 (0.07)
tumor	22.89 (3.39) ^d	16.60 (3.94)	23.47 (4.42) ^d	21.30 (5.42)

^a Mean weights of mice and tumors were 20.30 ± 0.48 g and 0.44 ± 0.13 g, respectively. ^b Expressed as percent of injected dose per gram of wet tissue. ^c Expressed as percent of injected dose per tissue. ^d Not significantly different by paired *t*-test.

rates of the radiometabolite derived from [¹²⁵I]MIH-OST7 (IgG), *m*-iodohippuric acid, compared with those from [¹³¹I]SIB-OST7 (IgG) and [¹¹¹In]-EMCS-Bz-EDTA-OST7 (IgG), although there would also be some contribution of faster blood clearance of [¹²⁵I]MIH-OST7 (IgG). This was more clearly demonstrated by the different radioactivity levels in the tissues such as the liver and kidney between [¹²⁵I]MIH-OST7 (IgG) and [¹¹¹In]-cDTPA-OST7 (IgG). While [¹²⁵I]MIH-OST7 (IgG) demonstrated higher radioactivity levels in the blood than [¹¹¹In]-cDTPA-OST7 (IgG) throughout the experimental period, the latter demonstrated significantly higher radioactivity levels in the liver and kidney after 24 h postinjection. Although nonchelated [¹¹¹In]-labeled species in [¹¹¹In]-cDTPA-OST7 (IgG) would be partially responsible for high radioactivity levels in these tissues, higher and persistent radioactivity levels in the liver, kidney, and spleen along with the lower radioactivity levels in the blood with [¹¹¹In]-cDTPA-OST7 (IgG) than with [¹²⁵I]MIH-OST7 (IgG) reinforced the important role played by the radiometabolites in the elimination of radioactivity from nontarget tissues, as documented previously (15, 17, 18, 23). Thus, these findings indicated that radiochemical design of mAbs that liberates radiometabolites

with rapid urinary excretion could improve the undesirable localization of radioactivity in nontarget tissues.

In biodistribution studies in athymic mice, [¹³¹I]MIH-OST7 (IgG) rendered radioactivity levels in the tumor and nontarget such as the liver and kidney similar to and significantly lower than those of simultaneously administered [¹²⁵I]SIB-OST7 (IgG), respectively (Table 2). These results also supported the preferential release of *m*-iodohippuric acid from [¹²⁵I]MIH-OST7 (IgG) in nontarget tissues. However, although [¹³¹I]MIH-OST7 (IgG) indicated target-to-nontarget ratios of radioactivity significantly higher than those of [¹²⁵I]SIB-OST7 (IgG) at both 24 and 48 h postinjection (Figure 7), the former showed a small but significant decrease in the target radioactivity level at 48 h postinjection when compared with the latter (Table 2). No significant decrease in the radioactivity level in the tumor was observed with [¹²⁵I]SIB-OST7 (IgG) between the postinjection intervals. In our previous study using NGA, although [¹³¹I]SIB-NGA was eliminated from murine liver more slowly than [¹³¹I]MIH-NGA, the former registered residual radioactivity levels in the liver similar to the latter at 24 h postinjection (19). In addition, radiolabeled OST7 (IgG) via direct radioiodination (26, 42), via [⁶⁷Ga]succinyldeferoxamine with an ester bond (8), and via [¹¹¹In]-cDTPA (43) indicated unchanged radioactivity levels in the same tumor model (KT005) during the same postinjection intervals. Therefore, it is likely that the decreased radioactivity level in the tumor at 48 h postinjection of [¹³¹I]MIH-OST7 (IgG) was caused by gradual cleavage of the ester bond even when the radioimmunoconjugate was bound to the antigen on the target cell surface, although cleavage of the ester bond in the blood may have also been involved. The slower elimination rate of radioactivity from the tumor than from blood, as reflected in the increased target-to-blood ratio with time (Figure 7), would be attributable to the formation of antigen-antibody complexes at the tumor site, which might have exerted additional steric interference against esterase access to its substrate. However, such steric interference on tumor cells would have little effect on chemical hydrolysis of the ester bond. This was supported by the

similar hydrolysis rates of [^{131}I]MIH-conjugated OST7 (IgG) and OST7 (Fab) in the buffered solution (Table 1). These findings suggested that while MIH may be applicable for diagnostic purposes in combination with ^{123}I , this reagent may not be suitable for therapeutic applications.

When MIH was applied to Fab fragment, the ester bond in [^{131}I]MIH-OST7 (Fab) was found to be much more labile against esterase-mediated hydrolysis than that in [^{131}I]MIH-OST7 (IgG) (Table 1). Such labile characteristics of the ester bond in [^{131}I]MIH-OST7 (Fab) would be primarily responsible for the rapid systemic clearance of its radioactivity (Figure 6), although partial dissociation of noncovalently bonded chains in blood would also be involved. These results suggested that the steric interference induced by the protein molecule plays a critical role in the stability of the ester bond in MIH-conjugated polypeptides and that MIH would be less applicable to conjugation of Fab or smaller molecular weight polypeptides.

In conclusion, the present study indicated that use of a metabolizable linkage between mAbs and radiolabeled compound with rapid urinary excretion characteristics constitutes an attractive strategy to decrease the radioactivity levels in nontarget tissues. Application of ^{123}I -labeled-MIH to IgG mAb is promising for tumor diagnosis at early postinjection times. However, this study also indicated problems associated with the use of an ester bond as the metabolizable linkage due to its labile characteristics and limited applicability to larger molecular weight proteins. These results call for development of a new class of metabolizable linkage that allows stable attachment of the radiolabel in plasma but liberates designed radiometabolites with carboxylate from covalently conjugated proteins following lysosomal proteolysis in nontarget tissues. Combination of such linkages with radiolabeled compounds with rapid urinary excretion characteristics would further enhance target-to-nontarget ratios of the radioactivity with various radiopharmaceuticals derived from not only mAbs of high molecular weight but also peptides of rather lower molecular weight.

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LITERATURE CITED

- Co, M. S., Yano, S., Hsu, R. K., Landolfi, N. F., Vasquez, M., Cole, M., Tso, J. T., Bringman, T., Laird, W., Hudson, D., Kawamura, K., Suzuki, K., Furuichi, K., Queen, C., and Masuho, Y. (1994) A Humanized Antibody Specific for the Platelet Integrin GpIIb/IIIa. *J. Immunol.* 152, 2968–2976.
- Kobayashi, H., Sakahara, H., Saga, T., Hosono, M., Shirato, M., Kanda, H., Ishibashi, K., Watanabe, T., Endo, K., Ishiwata, I., and Konishi, J. (1993) A Human/Mouse Chimeric Monoclonal Antibody Against CA-125 for Radioimmunotherapy of Ovarian Cancer. *Cancer Immunol. Immunother.* 37, 143–149.
- Yokota, T., Milenic, D. E., Whitlow, M., and Schlom, J. (1992) Rapid Tumor Penetration of a Single-Chain Fv and Comparison with Other Immunoglobulin Forms. *Cancer Res.* 52, 3402–3408.
- Brechbiel, M. W., Gansow, O. A., Atcher, R. W., Schlom, J., Esteban, J., Simpson, D. E., and Colcher, D. (1986) Synthesis of 1-(*p*-isothiocyanatobenzyl) Derivatives of DTPA and EDTA. Antibody Labeling and Tumor-Imaging Study. *Inorg. Chem.* 25, 2772–2781.
- Subramanian, R., Colony, J., Shaban, S., Sidrak, H., Haspel, M. V., Pomato, N., Hanna, M. G., and McCabe, R. P. (1992) New Chelating Agent for Attaching Indium-111 to Monoclonal Antibodies—Invitro and In vivo Evaluation. *Bioconjugate Chem.* 3, 248–255.
- Zalutsky, M. R., and Narula, A. S. (1987) A Method for the Radiohalogenation of Proteins Resulting in Decreased Thyroid Uptake of Radioiodine. *Appl. Radiat. Isot.* 38, 1051–1055.
- Pietersz, G. A., and Krauer, K. (1994) Antibody-Targeted Drugs for the Therapy of Cancer. *J. Drug Targeting* 2, 183–215.
- Arano, Y., Inoue, T., Mukai, T., Wakisaka, K., Sakahara, H., Konishi, J., and Yokoyama, A. (1994) Discriminated Release of a Hippurate-Like Radiometal Chelate in Nontarget Tissues for Target-Selective Radioactivity Localization Using pH-Dependent Dissociation of Reduced Antibody. *J. Nucl. Med.* 35, 326–333.
- Faivre-Chauvet, A., Gustin, J. F., Mease, R. C., Sai-Maurel, C., Thedrez, P., Slinkin, M., Meinken, G. E., Srivastava, S. C., and Chatal, J. F. (1993) Introduction of five Potentially Metabolizable Linking Groups Between In-111-Cyclohexyl EDTA Derivatives and F(ab')_2 Fragments of Anti-Carcinoembryonic Antigen Antibody. 2. Comparative Pharmacokinetics and Biodistribution in Human Colorectal Carcinoma-Bearing Nude Mice. *Nucl. Med. Biol.* 20, 763–771.
- Haseman, M. K., Goodwin, D. A., Meares, C. F., Kaminski, M. S., Wensel, T. G., McCall, M. J., and Levy, R. (1986) Metabolizable ^{111}In chelate conjugated anti-idiotypic monoclonal antibody for radioimmunodetection of lymphoma in mice. *Eur. J. Nucl. Med.* 12, 455–460.
- Meares, C. F., McCall, M. J., Deshpande, S. V., DeNardo, S., and Goodwin, D. A. (1988) Chelate Radiochemistry: Cleavable Linkers Lead to Altered Levels of Radioactivity in the Liver. *Int. J. Cancer, Suppl.* 2, 99–102.
- Paik, C. H., Yokoyama, K., Reymonds, J. C., Quadri, S. M., Min, C. Y., Shin, S. Y., Maloney, P. J., Larson, S. M., and Reba, R. C. (1989) Reduction of Background Activities by Introduction of a Diester Linkage Between Antibody and a Chelate in Radioimmunodetection of Tumor. *J. Nucl. Med.* 30, 1693–1701.
- Quadri, S. M., Vriesendorp, H. M., Leichner, P. K., and Williams, J. R. (1993) Evaluation of Indium-111-Labeled and Yttrium-90-Labeled Linker-Immunoconjugates in Nude Mice and Dogs. *J. Nucl. Med.* 34, 938–945.
- Weber, R. W., Boutin, R. H., Nedelman, M. A., Lister-Jones, J., and Dean, R. T. (1990) Enhanced Kidney Clearance with an Ester-Linked $^{99\text{m}}\text{Tc}$ -Radiolabeled Antibody Fab' -Chelator Conjugate. *Bioconjugate Chem.* 1, 431–437.
- Arano, Y., Mukai, T., Uezono, T., Wakisaka, K., Motonari, H., Akizawa, H., Taoka, Y., and Yokoyama, A. (1994) A biological method to evaluate bifunctional chelating agents to label antibodies with metallic radionuclides. *J. Nucl. Med.* 35, 890–898.
- Arano, Y., Mukai, T., Akizawa, H., Uezono, T., Motonari, H., Wakisaka, K., Kairiyama, C., and Yokoyama, A. (1995) Radiolabeled Metabolites of Proteins Play a Critical Role in Radioactivity Elimination from the Liver. *Nucl. Med. Biol.* 22, 555–564.
- Duncan, J. R., and Welch, M. J. (1993) Intracellular Metabolism of Indium-111-DTPA-Labeled Receptor Targeted Proteins. *J. Nucl. Med.* 34, 1728–1738.
- Franano, F. N., Edwards, W. B., Welch, M. J., and Duncan, J. R. (1994) Metabolism of Receptor Targeted ^{111}In -DTPA-Glycoproteins: Identification of ^{111}In -DTPA- ϵ -lysine as the Primary Metabolic and Excretory Product. *Nucl. Med. Biol.* 21, 1023–1034.
- Arano, Y., Wakisaka, K., Ohmomo, Y., Uezono, T., Mukai, T., Motonari, H., Shiono, H., Sakahara, H., Konishi, J., Tanaka, C., and Yokoyama, A. (1994) Maleimidoethyl 3-(tri-*n*-butylstannyl)hippurate: A useful radioiodination reagent for protein radiopharmaceuticals to enhance target selective radioactivity localization. *J. Med. Chem.* 37, 2609–2618.
- Boyle, C. C., Paine, A. J., and Mather, S. J. (1992) The Mechanism of Hepatic Uptake of a Radiolabelled Monoclonal Antibody. *Int. J. Cancer* 50, 912–917.

- (21) Sands, H., and Jones, P. L. (1987) Methods for the Study of the Metabolism of Radiolabeled monoclonal Antibodies by Liver and Tumor. *J. Nucl. Med.* 28, 390–398.
- (22) Deshpande, S. V., Subramanian, R., McCall, M. J., DeNardo, S. J., DeNardo, G. L., and Meares, C. F. (1990) Metabolism of Indium Chelates Attached to Monoclonal Antibody: Minimal Transchelation of Indium from Benzyl-EDTA chelate In Vivo. *J. Nucl. Med.* 31, 218–224.
- (23) Arano, Y., Mukai, T., Uezono, T., Motonari, H., Wakisaka, K., and Yokoyama, A. (1994) Biological Comparison of DTPA and SCN-Bz-EDTA as a Chelating Agent for Indium Labeling of Antibodies. *J. Labelled Compd. Radiopharm.* 35, 381–383.
- (24) Garg, P. K., Alston, K. L., and Zalutsky, M. R. (1995) Catabolism of radioiodinated murine monoclonal antibody F(ab')₂ fragment labeled using N-succinimidyl 3-iodobenzoate and iodogen methods. *Bioconjugate Chem.* 6, 493–501.
- (25) Koizumi, M., Endo, K., Kunimatsu, M., Sakahara, H., Nakashima, T., Kawamura, Y., Watanabe, Y., Saga, T., Konishi, J., Yamamuro, T., Hosoi, S., Toyama, S., Arano, Y., and Yokoyama, A. (1988) ⁶⁷Ga-labeled Antibodies for Immunoscintigraphy and Evaluation of Tumor Targeting of Drug-Antibody Conjugate in Mice. *Cancer Res.* 48, 1189–1194.
- (26) Sakahara, H., Endo, K., Koizumi, M., Nakashima, T., Kunimatsu, M., Watanabe, Y., Kawamura, Y., Nakamura, T., Ohta, H., Kotoura, Y., Yamamuro, T., Hosoi, S., Toyama, S., and Torizuka, K. (1988) Relationship Between In Vitro Binding Activity and In Vivo Tumor Accumulation of Radiolabeled Monoclonal Antibodies. *J. Nucl. Med.* 29, 235–240.
- (27) Hosoi, S., Nakamura, T., Higashi, S., Yamamuro, T., Toyama, S., Shinimiya, K., and Mikawa, H. (1982) Detection of human osteosarcoma-associated antigen(s) by monoclonal antibodies. *Cancer Res.* 42, 654–659.
- (28) Rousseaux, J., Rousseaux-Prevost, R., and Bazin, H. (1986) Optimal Conditions for the Preparation of Proteolytic Fragments from Monoclonal IgG of Different Rat IgG Subclasses. *Methods Enzymol.* 121, 663–669.
- (29) Meares, C. F., McCall, M. J., Reardan, D. T., Goodwin, D. A., Diamanti, C. I., and McTigue, M. (1984) Conjugation of Antibodies with Bifunctional Chelating Agents: Isothiocyanate and Bromoacetamide Reagents, Methods of Analysis, and Subsequent Addition of Metal Ions. *Anal. Biochem.* 142, 68–78.
- (30) Grasseti, D. R., and Murray, Jr., J. F. (1967) Determination of Sulfhydryl Groups with 2,2'- or 4,4'-Dithiodipyridine. *Arch. Biochem. Biophys.* 119, 41–49.
- (31) Wilbur, D. S., Hadley, S. W., Grant, L. M., and Hylarides, M. D. (1991) Radioiodinated Iodobenzoyl Conjugates of a Monoclonal Antibody Fab Fragment. In Vivo Comparisons with Chloramine-T-Labeled Fab. *Bioconjugate Chem.* 2, 111–116.
- (32) Paik, C. H., Hong, J. J., Ebbert, M. A., Heald, S. C., Reba, R. C., and Eckelman, W. C. (1985) Relative Reactivity of DTPA, Immunoreactive Antibody-DTPA Conjugates, and Nonimmunoreactive Antibody-DTPA Conjugates Toward Indium-111. *J. Nucl. Med.* 26, 482–487.
- (33) Paik, C. H., Sood, V. K., Le, N., Cioloca, L., Carrasquillo, J. A., Reynolds, J. C., Neumann, R. D., and Reba, R. C. (1992) Radiolabeled Products in Rat Liver and Serum After Administration of Antibody-Amide-DTPA-Indium-111. *Nucl. Med. Biol.* 19, 517–522.
- (34) Sakahara, H., Endo, K., Nakashima, T., Koizumi, M., Ohta, H., Furukawa, T., Ohmomo, Y., Yokoyama, A., Okada, K., Yoshida, O., and Nishi, S. (1985) Effect of DTPA Conjugation on the Antigen Binding Activity and Biodistribution of Monoclonal Antibodies Against alpha-Fetoprotein. *J. Nucl. Med.* 26, 750–755.
- (35) Imai, S., Morimoto, J., Tsubura, T., Esaki, K., Michalides, R., Holmes, R. S., Deimling, O., and Hilgers, J. (1986) Genetic marker patterns and endogenous mammary tumor virus genes in inbred mouse strains in Japan. *Exp. Anim.* 35, 263–273.
- (36) Arano, Y., Uezono, T., Akizawa, H., Ono, M., Wakisaka, K., Nakayama, M., Sakahara, H., Konishi, J., and Yokoyama, A. (1996) Reassessment of Diethylenetriaminepentaacetic Acid (DTPA) as a Chelating Agent for Indium-111 Labeling of Polypeptides Using a Newly Synthesized Monoreactive DTPA Derivative. *J. Med. Chem.* 39, 3451–3460.
- (37) Dykes, P. W., Bradwell, A. R., Chapman, C. E., and Vaughan, A. T. N. (1987) Radioimmunotherapy of cancer: clinical studies and limiting factors. *Cancer Treat. Rev.* 14, 87–106.
- (38) Gallinger, S., Reilly, R. M., Kirsh, J. C., Odze, R. D., Schmock, B. J., Hay, K., Polihronis, J., Damani, M. T., Shpitz, B., and Stern, H. S. (1993) Comparative Dual Label Study of First and Second Generation Antitumor-associated Glycoprotein-72 Monoclonal Antibodies in Colorectal Cancer Patients. *Cancer Res.* 53, 271–278.
- (39) Bormans, G., Nerom, C. V., Hoogmartens, M., Roo, M. D., and Verbruggen, A. (1989) Metabolism of Tc-99m ECD in Isolated Rat Organs. *J. Nucl. Med.* 30, 743 (Abstract).
- (40) Himmelsbach, M., and Wahl, R. L. (1989) Studies on the Metabolic Fate of ¹¹¹In-labeled Antibodies. *Nucl. Med. Biol.* 16, 839–845.
- (41) Motta-Hennessy, C., Sharkey, R. M., and Goldenberg, D. M. (1990) Metabolism of Indium-111-Labeled Murine Monoclonal Antibody in Tumor and Normal Tissue of the Athymic Mouse. *J. Nucl. Med.* 31, 1510–1519.
- (42) Kobayashi, H., Sakahara, H., Hosono, M., Yao, Z. S., Toyama, S., Endo, K., and Konishi, J. (1994) Improved clearance of radiolabeled biotinylated monoclonal antibody following the infusion of avidin as a "chase" without decreased accumulation in the target tumor. *J. Nucl. Med.* 35, 1677–1684.
- (43) Sakahara, H., Endo, K., Kunimatsu, M., Kawamura, Y., Ohta, H., Nakamura, T., Tanaka, H., Kotoura, Y., Yamamuro, T., Hosoi, S., Toyama, S., and Torizuka, K. (1987) Localization of Human Osteogenic Sarcoma Xenografts in Nude Mice by a Monoclonal Antibody Labeled with Radioiodine and Indium-111. *J. Nucl. Med.* 28, 342–348.

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PEG Thiazolidine-2-thione, a Novel Reagent for Facile Protein Modification: Conjugation of Bovine Hemoglobin

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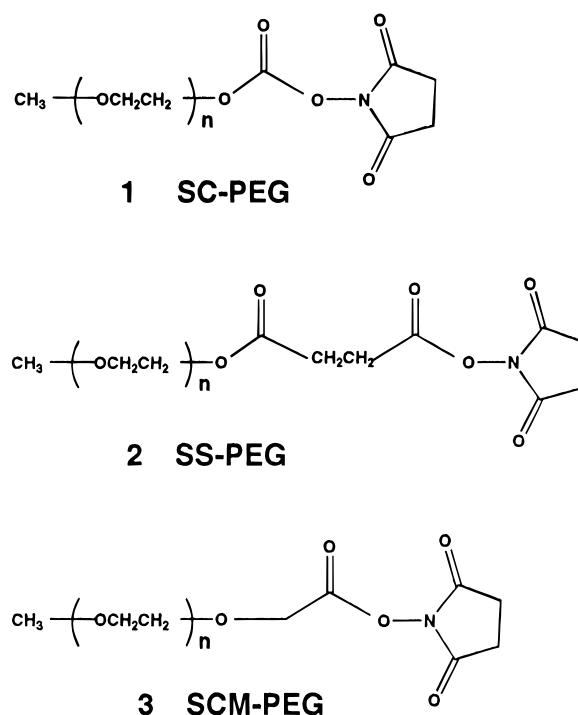
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A novel PEG linker that employs a thiazolidine-2-thione group has been synthesized. Kinetic studies done on this compound demonstrate a relatively long half-life compared to those of traditional succinimidyl linkers. This new PEG derivative reacts with proteins under mild conditions and was utilized to conjugate bovine hemoglobin (bHb) to provide a PEG amide-linked protein. The physical characteristics of this conjugate were compared with those of the known PEG carbamate-linked bHb.

Since the classic work, 25 years ago, of Abuchowski et al. (1) demonstrating the utility of poly(ethylene glycol) (PEG)–protein conjugates, the literature in this fascinating field has erupted with hundreds of contributions detailing the conjugation of PEG (2) with many different proteins, peptides, chemicals, surfaces, and the like. The popularity of PEGnology [a term coined by Katre (3)] is attested to by the numerous review papers (4) that have featured these conjugates and the various linker moieties synthesized to covalently attach PEG to other molecules. In the case of proteins, the ϵ -amino group of lysine amino acid residues is generally the focus of PEG conjugation strategies. These amino groups are more reactive at a pH greater than their pK_a (NH_2 unprotonated). The protein employed, and its stability, dictate the pH at which PEGylation is carried out. At higher pH the reaction proceeds more quickly, but hydroxide ion becomes a competitive nucleophile and the activated PEG hydrolyzes before aminolysis can occur, thus compromising the yield. Succinimidyl carbonate (SC)-activated PEG [1; SC-PEG (see Chart 1 for structure)], recently introduced by Zalipsky (5), has been a useful addition to the profusion of PEG linkers. The use of SC-PEG leads to the formation of carbamate bonds with the protein structure. These carbamate–protein conjugates have been claimed to be stable under physiological conditions, but no definitive comparison with amide-linked PEG has been done. Compared to *N*-hydroxysuccinimidyl (NHS) esters of PEG (2 and 3), carbonate 1 is thermally and hydrolytically more stable, thus enabling milder reaction conditions to be employed with proteins to achieve acceptable degrees of conjugation. Although 2 (SS-PEG) has been successfully conjugated to proteins (6), the PEG ester residue gamma to an amide or acid can undergo anchimeric assisted hydrolysis with cleavage of the PEG moiety under certain conditions (7, 8). In the case of the NHS ester 3, reaction with protein would form a stable amide, but $t_{1/2}$ hydrolysis has been reported (9) as <1 min at pH 8, thus minimizing its usefulness for protein modification in aqueous solution (Table 1). This enhanced reactivity is due to the electron-withdrawing PEG in the α position, which lowers the pK_a of the corresponding acid by over a full unit (10, 11).

We describe the synthesis of a novel PEG linker (6) that utilizes the thiazolidine-2-thione group and provides

Chart 1



$n = 113$ for mPEG(MW = 5000)

Table 1. Comparison of Half-Lives [$t_{1/2}$ Hydrolysis (min)] for Different Linkers

pH	T (°C)	T-PEG (6)	SC-PEG ^{5c} (1)	SS-PEG ^{5c} (2)	SCM-PEG ⁹ (3)	compd 7
7.4	22	240	129	65		
	37	50	6	18		
8	25	34	20.4 ^a	9.8	0.75	
	37	12	32 ^b	14		810

^a Reference 5c reports a 67 min half-life at pH 7.8 and 27 °C.

^b Reported for pH 7.8.

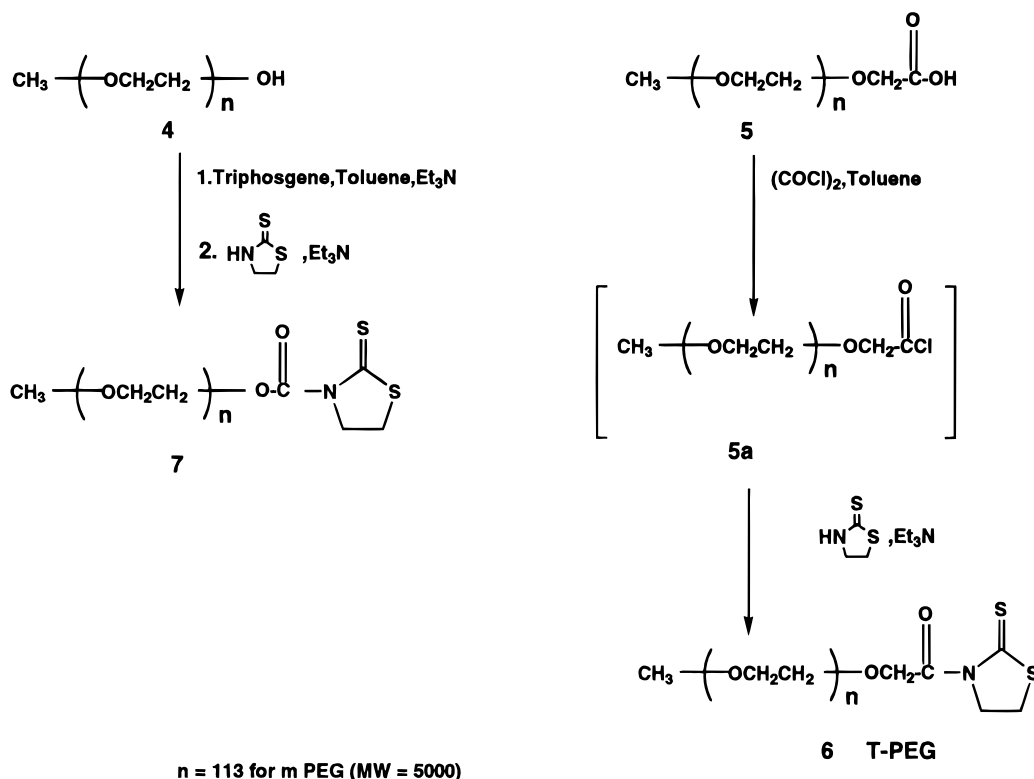
an exceptionally stable α -PEG derivative that can be used under mild conditions to form amide bonds with proteins. Additionally, utilization of this new linker to conjugate bovine hemoglobin (bHb) provided a PEG amide-linked protein having physical characteristics that can be compared with those of the known carbamate conjugate (5b). The preparation of the activated PEG derivative 6

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Scheme 1



was accomplished starting from the easily obtained acid **5** (Scheme 1).

Reaction of acid chloride **5a** with 2-mercaptothiazoline in the presence of base provided the PEG acylthiazolidine-2-thione linker, **6** (T-PEG) (**12**), in the same manner that simpler alkyl and aryl acylthiazolidine-2-thiones have been synthesized (**13**). It is known that the electron pair on N of the thiazolidine-2-thione heterocycle is involved in resonance with the thiocarbonyl group, thereby allowing the facile cleavage of the N–O acyl portion of the molecule by other N nucleophiles. Weaker nucleophilic reagents such as alcohols do not react, and so bifunctional reagents such as amino alcohols or amino acids like serine can be utilized without reaction occurring at O. Compound **6** was found to react in a similar fashion, providing this desirable selectivity to a functionalized PEG. Compound **7** was prepared by the reaction of PEG–OH **4** with triphosgene followed by condensation with 2-mercaptothiazoline in the presence of base as shown in Scheme 1. Kinetic experiments were done using **6** and **7** in various buffers at two different temperatures, and the data are summarized in Table 1.

Comparison of **6** (T-PEG) (**12**) with SC-PEG (**1**) shows that at physiological pH (7.4), T-PEG is a significantly more stable linker than the activated carbonate. Nonetheless, reaction of **6** with proteins occurred easily under mild reaction conditions and provided amide-linked bioconjugates in high yield. In fact, dissolution of **6** in water at room temperature prior to addition to protein is the preferred method of conjugation. On the other hand, the acylthiazolidine **7**, which is a carbamate derivative, is sufficiently stable to be completely unreactive toward proteins under normal conjugation conditions. At higher pH it can be seen that the stability of **6** declines to the level of SS-PEG, so that solid addition of T-PEG to protein under these conditions now becomes the preferred method.

EXPERIMENTAL PROCEDURES

Synthesis of Compound 6. A solution of m-PEG acid **5** (**14**) (5.0 g, 1.0 mmol, MW = 5000) in toluene (75 mL) was azeotroped with the removal of 25 mL of distillate. The reaction mixture was cooled to 30 °C, followed by the addition of oxalyl chloride (0.3 g, 2 mmol) and one drop of dimethylformamide. This mixture was stirred for 3 h at 30–40 °C, followed by the addition of 2-mercaptothiazoline (0.4 g, 3 mmol) and triethylamine (0.3 mL, 3 mmol). The reaction mixture was refluxed for 16 h, followed by filtration and removal of the solvent by distillation *in vacuo*. The crude residue was recrystallized from 2-propanol (100 mL) to yield **6** (4.3 g, 86%). The ¹³C NMR (CDCl₃) spectrum was consistent with the structure assigned: δ 200.5 (C=S), 170.9 (C=O), 54.8 (CH₂–N), 28.5 (CH₂–S), 58.0 (OCH₃). ¹H NMR (CDCl₃): δ 3.3–3.9 (br m, PEG), 3.91 (t, CH₂S), 4.62 (t, CH₂N).

Synthesis of Compound 7. A solution of m-PEG–OH **4** (100 g, 20 mmol, MW = 5000) in toluene (1 L) was azeotroped with the removal of 200 mL of distillate. The reaction mixture was cooled to 40 °C, followed by the addition of triphosgene (2.4 g, 8 mmol) and triethylamine (3.1 mL, 20 mmol). The resulting mixture was stirred for 4 h at 40 °C, followed by the addition of 2-mercaptothiazoline (3 g, 25 mmol) and triethylamine (3.5 mL, 25 mmol). The reaction mixture was stirred 16 h at 40 °C, followed by filtration and removal of the solvent by distillation *in vacuo*. The crude residue was recrystallized from 2-propanol (2 L) to yield **7** (90.3 g, 90%). The ¹³C NMR (CDCl₃) spectrum was consistent with the product structure: δ 198.7 (C=S), 149.8 (C=O), 54.8 (CH₂–N), 27.2 (CH₂–S), 57.8 (OCH₃). ¹H NMR (CDCl₃): δ 3.4–3.9 (br m, PEG), 3.91 (t, CH₂S), 4.61 (t, CH₂N).

Determination of the Half-Life of Hydrolysis of PEG Thiazolidine-2-thione Linkers 6 and 7. The

Table 2. Biochemical Characterization of bHb, PEG-Hb 8, and PEG-Hb 9

protein	purity (%)	P_{50}	PEG number	$t_{1/2}$ in rats (h)
bHb	>98	28		~4
PEG-Hb 8	>98	12.5	12	19.1 ± 0.8
PEG-Hb 9	>99	12.5	12.4	18.1 ± 1.5

rates of hydrolysis were obtained by employing a Hydro-pore-5-HIC column [Rainin, 4.6×100 mm, $5 \mu\text{m}$ particle size, 300 Å pore size spherical silica, chemically bonded to poly(ethylene glycol)], using a gradient mobile phase consisting of (a) hexane/2-propanol (80/20 v/v) and (b) acetonitrile/2-propanol (60/40 v/v). A flow rate of 1 mL/min was used, and chromatograms were monitored using a UV detector at 280 nm. Compound **6** or **7** was dissolved in acetonitrile at a concentration of 10.8 mg/mL (2 mM). From this stock solution 25 μL was transferred into a 1.5 mL polypropylene centrifuge tube and lyophilized. To the lyophilized powder was added 100 μL of 0.01 M, pH 7.4, PBS (or sodium phosphate buffer, 0.1 M, pH 8.0), and the mixture was incubated at 37 °C for various periods of time. Glacial acetic acid (40 μL) was added at the proper intervals to stop further hydrolysis, and the resulting solution was lyophilized to a powder. Acetonitrile (100 μL) was added to dissolve the solid, and 25 μL of this solution was injected into the HPLC. On the basis of the peak area, the amount of **6** or **7** was estimated, and the half-life of each compound in different media was calculated.

Modification of bHb with 6. Native bHb (supplied by Enzon Inc., Development Group) in 100 mM sodium phosphate (pH 8.4)/65 mM NaCl buffer was modified as follows. In a polypropylene container, 200 mL of **6** (40.9 g dissolved in 100 mM sodium phosphate/65 mM NaCl buffer at 4 °C) was added to 200 mL of bHb at 4 °C (22.2 g at 11 mg/mL) with gentle stirring. The pH of the reaction was monitored, and it was found that little if any fluctuation of pH occurred. The mixture was stirred for 1 h, the reaction was quenched by the addition of glycine, and stirring was continued for an additional 15 min. Cysteine (dissolved in 100 mM sodium phosphate/65 mM NaCl buffer at 4 °C, 30 mM final concentration) was added to reduce oxidized hemoglobin (met-Hb) formation, and the reaction mixture was stirred for 16 h at 4 °C. The PEG-Hb was diluted and diafiltered into formulation buffer (5 mM sodium bicarbonate, 4 mM Na_2HPO_4 , 1 mM NaH_2PO_4 , 150 mM NaCl, pH 7.4) to remove the unreacted PEG and/or PEG-glycine conjugate, followed by concentration to 60 mg/mL.

PEG-Hb Characterization. The purity of the PEG-Hb was determined by size exclusion HPLC (Superose 12; Pharmacia, Inc., Piscataway, NJ) and the percent modification. PEG number was measured by light scattering mass analysis (15). The P_{50} (ability to carry oxygen) was determined using a Hemox-Analyzer (TCS Medical Product Co., Huntington Valley, PA). Finally, the *in vivo* circulating life of the PEG-Hb was determined in female Sprague-Dawley rats as previously described (16). Plasma bHb/PEG-Hb levels were determined using Sigma Chemical Co. (St. Louis, MO) procedure 527 and reagents.

DISCUSSION

bHb was modified with activated **6** using a solution addition procedure. Compound **6** is relatively stable in water and buffer for extended periods of time (Table 1), thus allowing dissolution of T-PEG (**6**) without significant hydrolysis occurring and enabling liquid-liquid additions to be carried out. Another critical difference in the

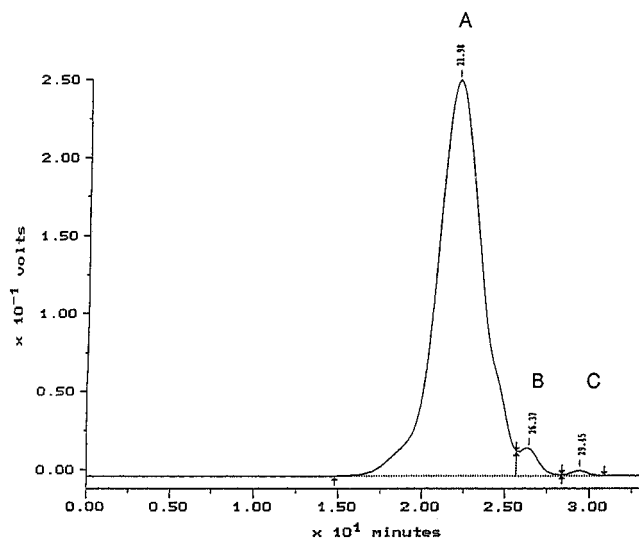


Figure 1. Size exclusion HPLC of **8** on a Superose 12 column. Ten micrograms of **8** was injected for each chromatograph. The Superose 12 flow rate was 0.5 mL/min using 100 mM sodium phosphate buffer, pH 7.0, containing 150 mM sodium chloride as the column buffer. Peaks A (21.98) and B (26.37) are PEG-modified Hbs. Peak C (29.45) is native Hb.

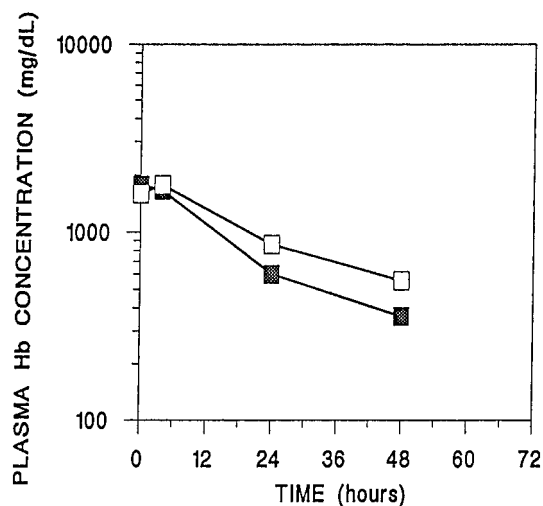


Figure 2. Plasma circulating half-lives in rats. Rats were given a top-loaded dose of 25 mg/kg ($\square$) **8** or ($\blacksquare$) **9**. Blood samples were drawn at the indicated times, and after centrifugation to remove red blood cells, the residual plasma hemoglobin levels were determined as described under Experimental Procedures.

modification of bHb with **6** versus other activated PEG derivatives such as **1** (SC-PEG) (**5**) or **2** (SS-PEG) (**5**) was that only minor fluctuations of pH during the modification process were observed. Addition of sodium hydroxide was not required to maintain the pH above 8.0, whereas modification of bHb with either **1** or **2** requires a significant addition of sodium hydroxide to maintain pH (**5**). Thus, the use of T-PEG eliminates the problem of protein denaturation that can occur with SC-PEG when it is necessary to add base (NaOH) to maintain the pH of the reaction. Biochemical characterization of the T-PEG-conjugated bHb (PEG-Hb, **9**) is summarized in Table 2. After 1 h, modification was essentially complete with 99% of the bHb modified (peaks A and B, Figure 1). The P_{50} of **9** is lower than that of bHb but identical to that of carbamate-linked PEG-Hb (**8**) (Table 2). The percent modification (PEG number/bHb tetramers) of amide-linked **9** was found to be equivalent to that of **8** (Table 2). The circulating half-life of **9** was determined using anesthetized rats, which were given a top-loaded

dose (25 mL/kg; 6.0 g%) of PEG-Hb. The results are summarized in Figure 2. The $t_{1/2}$ of **9** was 18.1 h, while the $t_{1/2}$ of **8** was 19.1 h. These half-life differences are not statistically different ($p = 0.48$; F -test; and Wilcoxon signed-rank test).

In summary, comparison of T-PEG **6** with SC-PEG **1**, by conjugating each with bHb, resulted in essentially equivalent products. However, T-PEG offers two important advantages over **1** and other current succinimidyl-activated linkers. First, T-PEG is relatively stable in aqueous solutions at room temperature, making possible liquid-liquid additions rather than the typical solid addition of activated PEG to protein solutions. Second, reaction occurs without concomitant change in pH, thus enabling pH sensitive proteins to be conjugated without difficulty. Thus, this novel linker should be a useful addition to the existing methodology for PEG conjugation.

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LITERATURE CITED

- (1) Abuchowski, A., Van Es, T., Palczuk, N. C., and Davis, F. F. (1977) Alteration of Immunological Properties of Bovine Serum Albumin by Covalent Attachment of Polyethylene Glycol. *J. Biol. Chem.* 252, 3578–3581.
- (2) Most poly(ethylene glycol) derivatives employed for protein conjugation are terminated on one end of the polymer, usually with a methyl group. The IUPAC nomenclature for this general polymer is α -hydroxy- ω -methyl-poly(oxy-1,2-ethanediyl). In this paper we refer to this material simply as PEG.
- (3) Katre, N. V. (1993) The Conjugation of Proteins with Polyethylene glycol and Other Polymers. *Adv. Drug Delivery Rev.* 10, 91–114.
- (4) For recent comprehensive reviews of the chemical and physical properties of poly(ethylene glycol)-modified compounds see: (a) Harris, J. M. (1992) In *Poly(Ethylene Glycol) Chemistry* (J. M. Harris, Ed.) Chapter 1, Plenum Press, New York. (b) Delgado, C., Francis, G. E., Fisher, D. (1992) *Crit. Rev. Ther. Drug Carrier Syst.* 9, 249–304. (c) Harris, J. M. (1985) *J. Macromol. Sci., Rev. Polym. Phys. Chem.* C25, 325–373. (d) Nucci, M. L., Shorr, R., Abuchowski, A. (1991) *Adv. Drug Delivery Rev.* 6, 133–151. (e) Zalipsky, S. (1995) Functionalized Poly(ethylene glycol) for Preparation of Biologically Relevant Conjugates. *Bioconjugate Chem.* 6, 150–165.
- (5) (a) Zalipsky, S. (1992) Active Carbonates of Polyalkylene Oxides for Modification of Polypeptides. U.S. Pat. 5,122,614. (b) Zalipsky, S., Seltzer, R., and Nho, K. (1991) Succinimidyl Carbonates of Polyethylene Glycol. In *Polymeric Drugs and Drug Delivery Systems* (R. L. Dunn and R. M. Ottenbrite, Eds.) Chapter 10, ACS Symposium Series 469, American Chemical Society, Washington, DC. (c) Zalipsky, S., Seltzer, R., and Menon-Rudolph, S. (1992) Evaluation of a New Reagent for Covalent Attachment of Polyethylene Glycols to Proteins. *Biotechnol. Appl. Biochem.* 15, 100–114.
- (6) Abuchowski, A., Kazo, G., Verhoest, C. R., Van Es, T., Kafkewitz, D., Nucci, M. L., Viau, A. T., and Davis, F. F. (1984) Cancer Chemotherapy with Chemically Modified Enzymes I. Antitumor Properties of Polyethylene glycol-Asparaginase Conjugates. *Cancer Biochem. Biophys.* 4, 175–186.
- (7) Tadayoni, B. M., Friden, B. M., Walus, L. R., and Musso, G. F. (1993) Synthesis, *In Vivo* Studies on Protein Conjugates of AZT: Evaluation of a Transport System to Increase Brain Delivery. *Bioconjugate Chem.* 4, 139–145.
- (8) Greenwald, R. B.; Pendri, A.; Bolikal, D. and Gilbert, C. (1994) Highly Water Soluble Taxol Derivatives: 2-Polyethylene Glycol Esters as Potential Prodrugs. *Bioorg. Med. Chem. Lett.* 4, 2465–2470.
- (9) Shearwater Polymers, Huntsville, AL, Jan 1996 catalog, p 46.
- (10) It is assumed that the electron-withdrawing effect of α -PEG is equivalent to that of α -methoxy. In this case the pK_a of acetic acid = 4.76, while that of methoxyacetic acid is 3.48 (11).
- (11) Fieser, L. F., and Fieser, M. (1961) *Advanced Organic Chemistry*, p 360, Reinhold Publishing, New York.
- (12) For short-hand notation we will refer to compound **6** as "T-PEG". Of passing interest is that T-PEG, when freshly prepared, has an odor reminiscent of the pudding, flan.
- (13) Fujita, L. A. (1981) New Efficient Aminolysis and its Application to Synthesis of Macrolactam Alkaloids. *Pure Appl. Chem.* 53, 1141–1154.
- (14) (a) Veronese, F. M., Caliceti, P., Pastorino, A., Schiavon, O., Satore, L., Scolaro, L. M. (1989) *J. Controlled Release* 10, 145–154. (b) Gehrhardt, H., and Mutter, M. (1987) *Polym. Bull.* 18, 487–493.
- (15) Lee, C., Sai, P., and McDonald, D. (Enzon Inc.) Manuscript in preparation.
- (16) Gilbert, C., Nho, K., Johnson, M., Linberg, R., and Shorr, R. (1994) Hemoglobinuria in rats: A sensitive test of renal filtering and absorption of PEG-hemoglobin, a red blood cell substitute. *Artif. Cells, Blood Substitutes, Immobilization Biotechnol.* 22, 535–541.

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CORRECTIONS

Volume 7, Number 6, November/December 1996.

Richard B. Greenwald,* Annapurna Pendri,
Anthony Martinez, Carl Gilbert, and Patricia
Bradley

PEG THIAZOLIDINE-2-THIONE, A NOVEL REA-
GENT FOR FACILE PROTEIN MODIFICATION:
CONJUGATION OF BOVINE HEMOGLOBIN

Page 638. The entry in the fourth column and second
row of Table 1 should be 36.

Page 640. In the legend for Figure 1, **9** should appear
in place of **8**, in both cases.

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Synthesis, Conformation, Biodistribution, and Hormone-Related *in Vitro* Antitumor Activity of a Gonadotropin-Releasing Hormone Antagonist–Branched Polypeptide Conjugate

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Since permanently high levels of GnRH analogues are necessary to exert direct and/or indirect antitumor effect on mammary tumors, much emphasis was put on the development of retarded-release devices (e.g. microcapsules) for GnRH derivatives. Alternatively, these compounds can be covalently coupled to high-molecular mass carrier molecules for the design of bioconjugates acting as (a) prodrugs producing prolonged release or (b) macromolecular therapeutics. In order to evaluate the feasibility of this approach, a prototype construct has been prepared with a potent GnRH antagonist Ac(D-Trp^{1,3}, D-Cpa², D-Lys⁶, D-Ala¹⁰)-GnRH (MI-1544). As a carrier, a representative of a new generation of synthetic, biodegradable branched poly[Lys(X_i-DL-Ala_m)] (XAK) type polypeptides with poly(L-lysine) backbone has been used in which X is an acetylated derivative of glutamic acid (AcEAK). This polyanionic polypeptide with free γ -carboxyl groups was conjugated to MI-1544, which has only a single amino group at position 6. In this paper, we describe (i) the synthesis and structure (primary structure, conformation) properties of the MI-1544–AcEAK conjugate with a 33% degree of substitution, (ii) the effect of the covalent attachment of MI-1544 to AcEAK on its blood clearance and tissue distribution, and (iii) the hormone-related indirect (ovulation inhibitory) or direct (antiproliferative) antitumor activity of the conjugate studied by *in vitro* assays. Data obtained with ¹¹¹In- and ¹²⁵I-labeled conjugates have demonstrated that in fact the body/blood survival of MI-1544 was prolonged by 1.5–3 times. The direct *in vitro* antitumor effect of MI-1544 was maintained or even enhanced in the MI-1544–AcEAK conjugate. Furthermore, we have shown that this conjugate was able to antagonize the effect of GnRH *in vitro* or to act as free MI-1544 both in short- and long-term inhibition of ovulation even after single subcutaneous injection. These data suggest that it is feasible to use a biodegradable polymeric polypeptide for development of a macromolecular therapeutic with GnRH antagonists.

INTRODUCTION

The native gonadotropin-releasing hormone (GnRH)¹ and its agonist analogues are capable of either stimulat-

ing or inhibiting secretion of the luteinizing hormone (LH) and the follicle-stimulating hormone (FSH), depending on the mode of their application (1). Knobil (2) published two different dose-dependent effects of GnRH agonists on the hypophysis. When GnRH agonists are given repeatedly, they desensitize the hypophysis, leading to decreases in gonadotropin and steroid release. In this way, they could inhibit the growth of hormone-dependent tumors (3). It has been reported that prolonged clinical treatment with high doses of agonists resulted in reversible inhibition of gonadal function.

There are differences between GnRH agonist and antagonist analogues in their mechanism of action. It has been demonstrated that a GnRH antagonist prevents GnRH action at the pituitary level by competitive GnRH receptor occupancy (4) rather than by down-regulation of GnRH binding sites. This GnRH antagonist did not affect the *de novo* synthesis of GnRH receptors *in vitro* (5). LH/FSH stimulation induced by native GnRH could be dose-dependently blocked by a GnRH antagonist, leading to inhibition of ovulation. Probably due to their different mode of action, GnRH antagonists may have stronger antitumor properties *in vitro* (6) than the agonists.

The GnRH analogues exert their antitumor action through chemical castration (7), a partial hypophysec-

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¹ Abbreviations: AcEAK, poly[Lys(Ac-Glu_{0.96}-DL-Ala_{3.1})]_n; AK, poly[Lys(DL-Ala_m)]_n; AUC, area under the curve; BOP, [(benzotriazol-1-yl)oxy]tris(dimethylamino)phosphonium hexafluorophosphate; CD, circular dichroism; DCM, dichloromethane; DIEA, *N,N*-diisopropylethylamine; DMF, dimethylformamide; DMSO, dimethyl sulfoxide; DTPA, diethylenetriaminepentaacetic acid; EAK, poly[Lys(Glu_{0.96}-DL-Ala_{3.1})]_n; FSH, follicle-stimulating hormone; GnRH, gonadotropin-releasing hormone; HOBt, 1-hydroxybenzotriazole; LH, luteinizing hormone; MI-1544, Ac(D-Trp^{1,3}, D-Cpa², D-Lys⁶, D-Ala¹⁰)-GnRH antagonist; PBS, phosphate-buffered saline; TEA, triethylamine; XAK, poly[Lys(X_i-DL-Ala_m)]_n; Z, benzyloxycarbonyl.

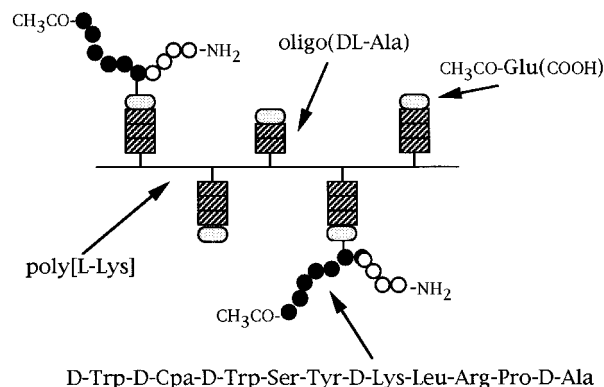


Figure 1. Schematic representation of MI-1544–AcEAK indicating the C- and the N-terminal portions of MI-1544 (○ and ●, respectively), starting from the grafting lysine residue.

tomy resulting in decreased levels of gonadotropin and prolactin (8), which consequently have a direct effect on mammary tumors (9–11). However, permanently high levels of GnRH agonist and antagonist analogues are necessary to reach direct and/or indirect antitumor effect (10). Therefore, much emphasis was put on the development of retarded-release devices for GnRH analogues and derivatives (12, 13). It has been found that the maintenance of a constant hormone analogue level can be efficiently achieved by formulation of microcapsules filled with GnRH agonists or antagonists (14–17).

Alternatively, GnRH agonists or antagonists can be covalently coupled to high-molecular mass carrier molecules for the design of bioconjugates acting as (a) potential prodrugs producing prolonged release or (b) macromolecular therapeutics with beneficially altered receptor binding or blood clearance. It should be noted that a similar strategy was successfully applied in passive tumor targeting of soluble macromolecules and drug conjugates (see reviews in refs 18–21). In order to evaluate the feasibility of this approach in relation to a GnRH antagonist, a prototype construct has been prepared with a potent GnRH antagonist Ac-D-Trp-D-Cpa-D-Trp-Ser-Tyr-D-Lys-Leu-Arg-Pro-D-Ala-NH₂ [Ac(D-Trp^{1,3},D-Cpa²,D-Lys⁶,D-Ala¹⁰)-GnRH, MI-1544] (22). As a carrier, a synthetic, biodegradable branched polypeptide with poly(L-lysine) backbone has been used (23–25). This polypeptide represents a new generation of the poly[Lys-(X_i-DL-Ala_m)] (XAK) type poly-α-amino acids developed in our laboratory (21, 23) in which X is an acetylated derivative of glutamic acid (AcEAK) (26, 27). This modification of the carrier structure resulted in a polyanionic polypeptide in which predominantly free γ-carboxyl groups are present. Consequently, this molecule seems to be ideal for conjugation to MI-1544, which has only a single amino group at position 6 (Figure 1).

In this paper, we describe (i) the synthesis and structural (primary structure, solution conformation) characterization of the MI-1544–AcEAK conjugate, (ii) the effect of the covalent attachment of MI-1544 to AcEAK on its blood clearance and tissue distribution, and (iii) the hormone-related indirect (ovulation inhibitory) or direct (antiproliferative) antitumor activity of the MI-1544–AcEAK conjugate studied by *in vitro* assays.

MATERIALS AND METHODS

DL-Alanine, glutamic acid, N^ε-Z-lysine, imidazole, and acetic anhydride were purchased from Reanal (Budapest, Hungary). N,N-Diisopropylethylamine (DIEA), 1-hydroxybenzotriazole (HOBt), and bis(trichloromethyl) car-

bonate were Fluka products (Buchs, Switzerland), while [(benzotriazol-1-yl)oxy]tris(dimethylamino)phosphonium hexafluorophosphate (BOP) was from Bachem (Bubendorf, Switzerland). Dimethylformamide (DMF) was stored over molecular sieves.

The human GnRH antagonist Ac(D-Trp^{1,3},D-Cpa²,D-Lys⁶,D-Ala¹⁰)-GnRH (MI-1544) was synthesized in our laboratory (MI) by the solid phase method on benzhydrylamine resin using N^α-tert-butyloxycarbonyl amino acids as described earlier (22).

The average degree of polymerization ($\overline{DP}_n$) of the polylysine used for the synthesis of AcEAK was estimated by sedimentation analysis and gel permeation chromatography (28). Sedimentation analysis was carried out in a MOM 3170 ultracentrifuge. The average relative molar mass of the branched polypeptides (AK, EAK, and AcEAK) was calculated from the $\overline{DP}_n$ of the poly(Lys) backbone and from the amino acid ratios Ala:Lys or Glu:Ala:Lys as described earlier (23, 28).

Coupling reactions between the GnRH antagonist and AcEAK were monitored by thin layer chromatography (TLC) on precoated silica gel plates purchased from Merck (Darmstadt, Germany) using ninhydrine, toluidine, and Ehrlich reagents.

Abbreviations for amino acids and their derivatives follow the revised recommendation of the IUPAC-IUB Committee on Biochemical Nomenclature, entitled Nomenclature and Symbolism for Amino Acids and Peptides (29). The nomenclature of branched polypeptides is used in accordance with the recommended nomenclature of graft polymers (30). For the sake of brevity, codes of branched polypeptides were constructed by us using the one-letter symbols of amino acids. All amino acids are of the L configuration unless otherwise stated. Visking tubes with a 12000–14000 Da cutoff (Fisons, Loughborough, U.K.) were used for the dialysis of polymer products.

Synthesis of Branched Polypeptides. (a) Poly[Lys-(Glu_i-DL-Ala_m)] (EAK), where $i = 0.96$ and $m = 3.1$, was synthesized as previously described (31) with minor modification using HOBt as a catalyst in the active ester coupling method for the attachment of the glutamic acid residue to the end of the side chains of the branched polypeptide poly[Lys(DL-Ala_{3.1})] (AK) (32). Briefly, poly(L-Lys) ($\overline{DP}_n = 155$) was prepared by the polymerization of N^α-carboxy-N^ε-(benzyloxycarbonyl)lysine anhydride. After cleavage of the protecting groups with HBr in glacial acetic acid, poly[Lys(DL-Ala_{3.1})] (AK) was formed by grafting of short oligomeric DL-alanine side chains onto the ε-amino groups of polylysine using N-carboxy-DL-alanine anhydride (31). Attachment of suitably protected and activated glutamic acid residues to AK was carried out by the active ester method using a 50% molar excess of Z-Glu(OBzl)-OPcp and HOBt over AK in a 1:5 water/DMF (v/v) solvent mixture in the presence of 4-methylmorpholine (at pH 7–8). The overnight reaction at room temperature was followed by removal of the solvent *in vacuo*, and the residue was triturated several times with ether containing 10% DCM. The protecting groups were cleaved with a HBr/acetic acid mixture afterward. Finally, EAK was purified by extensive dialysis using Visking tubing (molecular mass cutoff = 12000–14000 Da) followed by lyophilization. The removal of protecting groups was verified by UV spectroscopy (31). Amino acid analysis yielded Lys:Ala:Glu = 1:3.1:0.98.

(b) *Acetylation of Poly[Lys(Glu_{0.96}-DL-Ala_{3.1})]*. EAK (85 mg, 174 μmol) was dissolved in 2 mL of deionized water, and then the solution was diluted with 10 mL of DMF under cooling. TEA (24 μL, 174 μmol) was added to the

solution for the neutralization of the polymer. During this time, 90 μL (0.95 mmol) of acetic anhydride and 63 mg (0.93 mmol) of imidazole were mixed in 2 mL of DMF at 0 °C. The preactivation was continued for 10 min, and then the mixture was added to the solution of EAK. The acetylation was continued for 1 h at 0 °C and then overnight at room temperature. The polymer was purified by extensive dialysis against water using Visking tubing (molecular mass cutoff = 12000–14000 Da) and finally freeze-dried. No ninhydrine positive reaction was observed in the Kaiser test (33) (yield, 87 mg, 94%). Amino acid analysis yielded Lys:Ala:Glu = 1:3.0:0.96.

AcEAK which was to be labeled with ^{111}In was conjugated to diethylenetriaminepentaacetic acid (DTPA) anhydride first (26, 27) and then acetylated as described above.

Synthesis of Branched Polypeptide–Hormone Analogue Peptide Conjugates. AcEAK (49 mg, 95 μmol) was dissolved in 1 mL of deionized water, and its solution was diluted with 3 mL of DMF. Ac(D-Trp^{1,3}, D-Cpa², D-Lys⁶, D-Ala¹⁰)-GnRH (MI-1544) (60 mg, 47.5 μmol) (0.5 mol calculated for side chains of the polymer) dissolved in 3 mL of DMF was added to the solution of AcEAK. Following the addition of 42 mg (95 μmol) of BOP reagent in 1 mL of DMF, the pH of the reaction mixture was adjusted to pH $\sim$ 7.5 with *N,N*-diisopropylethylamine (DIEA) (65 μL , 382 μmol). The mixture was stirred overnight at room temperature. The solution was placed in Visking tubing (molecular mass cutoff = 12000–14000 Da) and dialyzed extensively against deionized water for 3 days afterward. Finally, the conjugate, MI-1544–AcEAK was isolated by lyophilization (yield, 66 mg). Amino acid analysis yielded Lys:Glu:Ala:Ser:Tyr:Leu:Arg:Pro:Trp = 1.7:0.98:3.35:0.35:0.29:0.35:0.33:0.34:0.69.

MI-1544 conjugate of AcEAK–DTPA for labeling with ^{111}In was prepared as described above using AcEAK–DTPA instead of AcEAK.

Amino Acid Analysis. The amino acid composition of branched polypeptides (AK, EAK, and AcEAK) and MI-1544–AcEAK conjugates was investigated by amino acid analysis using a Beckman (Fullerton, CA) model 6300 amino acid analyzer. Under these conditions, Cpa could be determined at the position of the Lys residue. Prior to analysis, samples were hydrolyzed in 6 M HCl in sealed and evacuated tubes at 110 °C for 24 h. Trp content was measured by UV spectroscopy at $\lambda = 280$ nm ($\epsilon = 10\,000$) in a solution of 0.1 M NaOH.

GPC–HPLC (Gel Permeation Chromatography–High-Performance Liquid Chromatography) Analysis of the MI-1544–AcEAK Conjugate. *Instrumentation.* The HPLC system consisted of a VARIAN 9021 solvent delivery system and of a Varian 9065 diode array detector (all from Varian, Zug, Switzerland) and a Model 7125 injector valve (Rheodyne, Cotati, CA). A Biosil TSK 125 type 30 cm $\times$ 7.5 mm column and a 5 cm $\times$ 7.5 mm guard column (Biorad, Richmond, CA) were used.

Conditions. The mobile phase was 0.25 M triethylammonium phosphate buffer (pH 2.25) prepared daily using high-purity (Analar) components and distilled, deionized water. The injection volume was 10 μL containing 10 μg of MI-1544–AcEAK, AcEAK, or MI-1544 dissolved in eluent immediately before application and filtered through 0.45 μm Spartan 13 (Schleicher and Schuell, Dassel, Germany) filters. Free MI-1544 or AcEAK samples were run as standards, and their retention times were determined. UV absorbance was monitored at wavelengths of 215 and 280 nm. All analyses were carried out at ambient temperature with a flow rate of 0.5 mL/min.

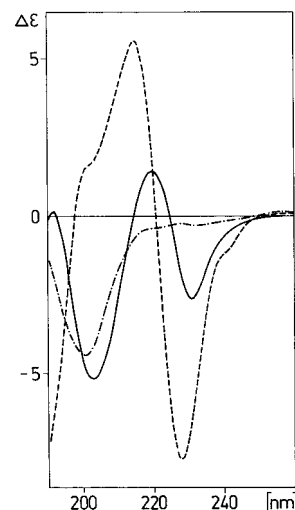


Figure 2. CD spectra of MI-1544 (—), AcEAK (---), and their conjugate MI-1544–AcEAK (—) at pH 7.3 in 0.02 M NaCl.

Circular Dichroism Spectroscopy. Circular dichroism (CD) was measured with a Roussel-Jouan model III dichrograph (Jobin-Yvon, Longjumeau, France). CD spectra from 195 to 260 nm were recorded in a quartz cell with an optical path of 0.02 cm at room temperature. The dichrograph was calibrated with D-(–)-pantoyllactone at 220 nm (34). CD spectra of free MI-1544, AcEAK, and their conjugate MI-1544–AcEAK were recorded in 0.02 M NaCl solution at pH 7.2, the concentrations of solutions being about 0.5 mg/mL (Figure 2). The data of CD in the figure are expressed in terms of $\Delta\epsilon$, calculated for one lysine residue in the polypeptide backbone carrying an average side chain. The interpretation of CD spectra was based on the well-characterized CD curves of polylysine (35), from which the branched polypeptides have been derived.

Labeling with ^{125}I . ^{125}I labeling of MI-1544 and MI-1544–AcEAK–DTPA was carried out with [^{125}I]sodium iodide [^{125}I] for protein iodination (IMS.30) in NaOH at pH 7–11, Amersham International plc, Amersham, U.K., by oxidative incorporation into the tyrosine of the MI-1544 using Iodogen (1,3,4,6-tetrachloro-3 α ,6 α -diphenylglycoluril) as the oxidizing agent. One hundred micrograms of Iodogen [Pierce and Warriner (U.K.) Ltd., Chester, U.K.] was coated onto the inner surface of polypropylene conical microfuge tubes by evaporation under nitrogen from solution in methylene chloride. Two hundred micrograms of MI-1544 or MI-1544–AcEAK–DTPA in 200 μL of PBS (pH 7.2) and $\sim$ 5 MBq of radioiodine solution were added to the tubes, and the solution was mixed with a Pasteur pipette. After incubation at room temperature for 10 min, the reaction was stopped by removal of the mixture from the reaction tube. Labeled MI-1544–AcEAK–DTPA was purified of unreacted [^{125}I]sodium iodide by passage through Sephadex G-25, elution being in PBS at pH 7.2, using prepacked PD-10 columns (Pharmacia, Milton Keynes, U.K.). Labeled MI-1544 was purified by dialysis against PBS (3 $\times$ 1000 volumes over 24 h at 4 °C) in 500 molecular mass cutoff dialysis tubing [Spectrum Spectra/por CE, molecular mass cutoff = 500 Da, Pierce and Warriner (U.K.) Ltd.]. Specific activities of the final products were $\sim$ 2 MBq/mg.

Labeling with ^{111}In . ^{111}In labeling by chelation to the DTPA of AcEAK–DTPA and MI-1544–AcEAK–DTPA was carried out by addition of 5 MBq of [^{111}In]InCl₃ (indium chloride in 0.04 M HCl, INS.1P, Amersham International) to solutions of 200 μg in 200 μL of pH 6.0

0.3 M sodium citrate/citric acid buffer. After 10 min at room temperature, the reaction mixtures were purified of unbound ^{111}In by passage through Sephadex G-25, elution being in PBS. Specific activities of the final products were $\sim 2\text{--}10\text{ MBq/mg}$.

Blood Clearance and Biodistribution Studies. All *in vivo* studies were carried out in adult ($\sim 20\text{ g}$) female Balb/c mice (Biomedical Services Unit, University of Nottingham) with appropriate licences from the U.K. Home Office and with due consideration for animal welfare.

Groups of mice ($n = 4$) were injected intravenously via a tail vein with $\sim 20\text{ }\mu\text{g}$ of labeled materials in 0.2 mL of PBS. Serial blood samples ($10\text{ }\mu\text{L}$) were taken from the tail tip at 1, 10, and 30 min and at 1, 2, and 3 h after injection directly into microcapillary pipettes (Drummond Microcaps, Drummond Scientific Co., Broomhall, PA). Blood clearance curves were constructed of the percent of injected count rates in the total blood volumes against time [the total intravascular blood volumes were calculated assuming the blood volume of the mice (in milliliters) to be 11.2% of the body weight (in grams)]. Areas under curves (AUC), as percent dose per hour, were calculated using the trapezoidal rule.

At 3 h after injection, mice were killed, and weighed samples of blood, visceral organs, and residual carcass were assayed for radioactivity. Results of the tissue distribution analysis were expressed as a percentage of the initially injected count rate recovered in the animal as a whole, and per gram of individual tissue.

In Vivo Ovation Inhibition Assay. Adult female rats (Wistar-R-Amsterdam), 200–250 g, were housed under controlled lighting (light period from 5:00 to 19:00) with free access to pelleted food and tap water.

MI-1544 or its AcEAK conjugate was dissolved in a 40–60% mixture of propylene glycol and of 0.9% sodium chloride solution. Aliquots of the solutions containing 10, 50, 75, and 100 μg of MI-1544–AcEAK and 1.5, 3, and 30 μg of MI-1544 were injected subcutaneously (sc) to female rats on the day of proestrus at noon. Only those rats that previously showed three consecutive regular 4-day cycles as tested from vaginal smears were used for these experiments. Animals were ovariectomized on the day of the first diestrus after the injection. Oviducts were prepared under a stereomicroscope and tested for ova as described earlier (22).

In Vitro Inhibition of LH Release. The GnRH antagonistic activity of MI-1544 and the MI-1544–AcEAK conjugate was assayed by using the superfused rat pituitary cell system (36, 37). Mixed cell populations of four regularly cycling female rats at random stages of the estrous cycle were used in each chamber of the superfusion apparatus. The cells were perfused overnight with Medium 199 (Sigma, St. Louis, MO) at a flow rate of 20 mL/h, and administration of the compounds was started the next morning. Pituitary cells were exposed to 9 min pulses of MI-1544 and the MI-1544–AcEAK conjugate in concentrations of 1, 5, 10, 50, and 100 nM prior to 1 nM GnRH. The peptide-containing solutions were prepared from the stock solutions immediately before use. At the beginning of each experiment, cells were challenged with 50 mM K^+ and two or three pulses of 1 nM GnRH for 3 min. At the end of the experiments, cells were rechallenged with K^+ to check the hormone receptor-independent LH secretion. One milliliter fractions per 3 min were collected, and the concentration of LH released in the system was determined by radioimmunoassay (RIA). Two independent experiments were performed for each concentration, and

the mean of data is summarized. The interassay variation was less than 10%.

NIAMD RIA kits (National Hormone and Pituitary Program) were used for the LH assay (LH-S-9 standard, LH-RP-2 reference preparation, and LH-1-6 hormone for iodination).

The superfusion data were analyzed by a special computer program (37). The amount of LH secreted was calculated from the peaks above the baseline after stimulation.

Clonogenic Assay. The human mammary carcinoma cell lines MCF-7 (38) and MDA-MB-231 (39) were used throughout. The cells were grown in Dulbecco's modified Eagle MEM (D-MEM) medium (GIBCO) supplemented with 5% estrogen-depleted (dextran-charcoal-treated) fetal calf serum (FCS-DCC). MCF-7 cells are estradiol (E_2) responsive, and MDA-MB-231 cells are E_2 unresponsive.

Three hundred cells were plated in 35 mm plastic petri dishes in 2.5 mL of medium. The next day, the cells were treated with doses of MI-1544, AcEAK, the MI-1544–AcEAK conjugate, and the MI-1544/AcEAK mixture. Compounds were dissolved in dimethyl sulfoxide (DMSO) and diluted with complete medium to reach 10, 30, and 50 μM final concentrations related to MI-1544 content. The cultures were incubated at 37 °C in a humidified CO_2 incubator (Heraeus) for 8–12 days. The cultures were then stained with crystal violet, and the colonies were counted under a stereomicroscope. Relative cloning efficiency of the treated cultures was expressed as a percentage of untreated controls (40).

RESULTS AND DISCUSSION

Preparation of the Hormone Analogue–Branched Polypeptide Conjugate. In order to prepare a structurally well-defined conjugate of the GnRH antagonist, Ac(D-Trp ^{1,3} , D-Cpa ^{2} , D-Lys ^{6} , D-Ala ^{10})-GnRH (MI-1544) with a single amino group at the lysine residue in position 6 and branched polypeptide AcEAK with free γ -carboxyl groups were produced from the amphoteric poly[Lys-(Glu $_{0.96}$ -DL-Ala $_{3.1}$)] (EAK) for coupling. Acetylation of EAK was performed in water/DMF (1:5 v/v) after neutralization of the α -amino groups of the polymer with imidazolyl acetate. The efficiency of the reaction was checked by ninhydrine after the isolation of the polymer product (AcEAK), and no detectable amount of free amino groups was observed. This procedure represents a new alternative to the previously described reaction scheme, where acetic anhydride was used as the acetylation agent (25, 26).

The coupling of MI-1544 to AcEAK was achieved by the BOP reagent-based activation method in which the γ -carboxyl group of glutamic acid of AcEAK was linked to the ϵ -amino group of lysine residue in the hormone analogue peptide to provide covalent isopeptide (γ,ϵ type amide) bonding. The carboxyl groups of the polymeric polypeptide were activated by equimolar BOP *in situ* using a tertiary amine (DIEA). It should be noted that no precipitate was observed during the synthesis of the conjugate under these conditions.

GPC–HPLC studies at two wavelengths were carried out to access free GnRH analogue in the MI-1544–AcEAK conjugate preparation. The retention time for MI-1544, absorbing at both 215 and 280 nm, was found to be 38.1 min. Free AcEAK was detected at 215 nm at the void volume, whereas the t_R value for MI-1544–AcEAK was 19.5 min at both 215 and 280 nm, indicating the presence of covalently bound MI-1544. Quantitative

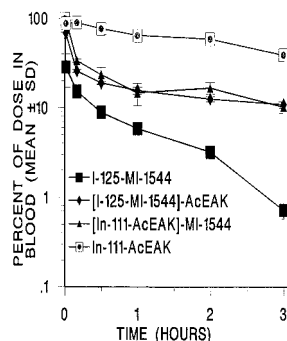


Figure 3. Blood clearance curves of ^{125}I -labeled MI-1544, its conjugate with AcEAK labeled with ^{125}I or ^{111}In , and ^{111}In -labeled AcEAK, with four mice per group. The areas under these time–activity curves are shown in Table 1.

analysis indicated less than 50 ppb of free MI-1544 in the conjugate samples. The amino acid composition of purified conjugates was determined by amino acid analysis. Considering the amino acid composition of the free carrier (AcEAK) and of the free peptide, the average degree of molar substitution (DS) was calculated, and it was found that $\text{DS} = \text{MI-1544:AcEAK} = 51:1$ mol/mol. This value indicates that 33% of the free carboxyl groups of AcEAK was modified by MI-1544. Consequently, the MI-1544–AcEAK conjugate still possesses polyanionic character.

Conformation of the MI-1544–AcEAK Conjugate.

The chiroptical properties of the conjugate in solution were studied by CD spectroscopy in the 190–250 nm wavelength region. CD spectra were recorded in water solution at physiological pH (pH 7.3) and 0.02 M NaCl ionic strength. The CD curves of free AcEAK, GnRH antagonist, and their conjugate are shown in Figure 2. In this wavelength region, the circular dichroic spectra indicate an essentially disordered conformation for the unsubstituted AcEAK. The CD signals in the 220–240 nm region of $\text{Ac(D-Trp}^{1,3}, \text{D-Cpa}^2, \text{D-Lys}^6, \text{D-Ala}^{10})\text{-GnRH}$ are indicative of the presence of aromatic amino acid residues.

In the case of the MI-1544–AcEAK conjugate, with this degree of substitution (33%), the shape of the CD spectrum in the 190–215 nm range was similar to the spectrum of AcEAK and corresponds to the unordered steric arrangement of the polypeptide backbone. Interestingly enough, a strong CD band was observed in the 220–240 nm wavelength region. This signal originated from the optical activity of the attached MI-1544 moiety. The red shift observed in the position of the CD bands ($\lambda = 202$ nm of AcEAK and $\lambda = 226$ nm of MI-1544) in the conjugate could verify the presence of MI-1544 peptide.

Previous studies provided evidence (41–44) that, besides the choice of carrier polypeptide, the number of hapten molecules attached to the carrier polypeptide influences markedly the spatial arrangement of the conjugate.

Biodistribution of Free and Conjugated MI-1544.

^{125}I labeling of GnRH and its derivatives into tyrosine by the chloramine T method is an established way of labeling the hormone-related peptides (44, 45). For our studies, radioiodinated MI-1544 and its AcEAK conjugate (MI-1544–AcEAK) were prepared with a similar strategy using Iodogen as the oxidizing agent. Similar to data reported on the pharmacokinetics of GnRH and its analogues after iv injection (47, 48), we found that ^{125}I -MI-1544 was cleared rapidly from circulation (Figure 3), with an AUC of only 17.8% dose/h (Table 1). The major sites of retention of ^{125}I at the 3 h dissection time were

Table 1. Biodistribution Parameters of MI-1544, Polypeptide AcEAK, and Their Conjugates^a

radiolabeled material	area under curve (% dose/h $\pm$ SD)	retention (% $\pm$ SD)
^{125}I -MI-1544	17.8 ± 1.1	38.8 ± 4.9
^{125}I -MI-1544–AcEAK	50.3 ± 1.6	60.2 ± 1.8
^{111}In -AcEAK–MI-1544	57.6 ± 9.6	80.3 ± 4.0
^{111}In -AcEAK	188.2 ± 0.8	41.0 ± 2.1

^a Mean of four mice per group to 3 h after injection.

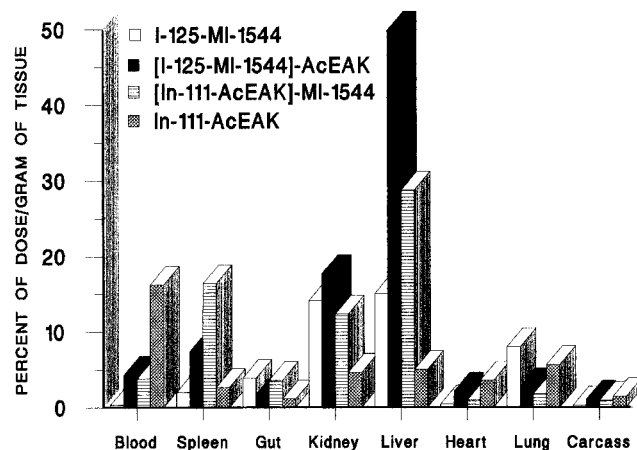


Figure 4. Biodistribution of ^{125}I -labeled MI-1544, its conjugate with AcEAK labeled with ^{125}I or ^{111}In , and ^{111}In -labeled AcEAK 3 h after injection, with four mice per group. The whole body retentions of the radiolabels are shown in Table 1.

kidney and liver, with about 15% of the injected dose/g in each of these organs, and the lung with about 7% (Figure 4). However, the whole body retention of ^{125}I was only 38.8% (Table 1), showing that much of the ^{125}I -MI-1544 had been excreted unchanged (via the kidneys) and/or ^{125}I had been removed from its site(s) of clearance following its metabolism and excreted as ^{125}I iodotyrosine or as free ^{125}I sodium iodide.

In contrast, ^{125}I -MI-1544 conjugated to AcEAK (^{125}I -MI-1544–AcEAK) showed longer blood survival (Figure 3), with an AUC of 50.3% dose/h (Table 1). There was also a different pattern of biodistribution, with reduced levels in the lung but higher levels in spleen and liver (Figure 4). The higher levels in these organs, and the greater blood survival, resulted in an increased whole body retention of ^{125}I , at about 60%.

The blood clearance curve and AUC of ^{111}In -AcEAK conjugated to MI-1544 (^{111}In -AcEAK–MI-1544) was virtually identical to that of the conjugate labeled in the MI-1544 with ^{125}I (Figure 3, Table 1). The main differences between their biodistributions were a reduced level in the spleen and a higher level in the liver with the ^{111}In -labeled conjugate (Figure 4), which was reflected in a greater whole body retention of about 80% (Table 1).

^{111}In -labeled AcEAK showed the greatest blood survival (Figure 3, Table 1), but with reduced levels in spleen, kidney, and liver compared with MI-1544–AcEAK labeled in either the MI-1544 or AcEAK parts of the conjugate.

The results of this comparative study suggest that the survival of free MI-1544 is prolonged and its tissue distribution is altered by conjugation to AcEAK. AcEAK in the conjugate elevated significantly the presence of MI-1544 in the circulation (AUC for the conjugate was 3 times higher than that for MI-1544) and in the whole body (1.5–2-fold increase for MI-1544–AcEAK compared to that of free MI-1544). Interestingly enough, the blood clearance profile for both ^{125}I - or ^{111}In -labeled conjugates was almost identical, indicating the lack of free labeled

Table 2. Short-Term and Long-Term Antioviulatory Effect of MI-1544 and the MI-1544–AcEAK Conjugate in Cycling Rats

inhibition at (h)	effect				
	1.5 μ g	3 μ g	30 μ g	50 μ g	75 μ g
MI-1544					
24	35% ^a (4/6)	100% (0/5)	100% (0/6)	nt	nt
48	nt ^b	nt	90% (1/6)	nt	nt
72	nt	nt	30% (4/6)	nt	nt
MI-1544–AcEAK					
24	nt	nt	nt	0% (4/4)	90% (1/10)
48	nt	nt	nt	nt	60% (3/7)
72	nt	nt	nt	nt	40% (5/8)

^a Antioviulatory activity % (number of rats ovulating/total number of rats). ^b nt = not tested. Compounds were administered subcutaneously on the day of proestrus at noon.

MI-1544 and free AcEAK and the stability of the γ,ϵ amide bond in the circulation. In contrast, the whole body retention and the spleen and kidney uptake were not the same for the two conjugates. For the interpretation of these findings, it should be borne in mind that the retention of ^{111}In tends to be different from that of ^{125}I at the site of clearance (27). This means that the tissue biodistribution results with ^{111}In -labeled and ^{125}I -labeled MI-1544–AcEAK are unlikely to be identical. Our recent comparative study with branched polypeptides indicated that generally kidney, spleen, and liver levels of radiometals (^{111}In or ^{51}Cr) were higher than those of radioiodine, while levels in the gastrointestinal tract were higher with radioiodine (49).

It is important to note that the covalent attachment of MI-1544 to branched polypeptide AcEAK resulted in some alterations (e.g. reduction in blood survival and increased liver, kidney, and spleen uptake) in the bio-distribution of the free carrier. This observation has to be considered for the design of future conjugates of MI-1544.

Inhibition of Ovulation. The inhibitory effect of the GnRH analogue, MI-1544, and the MI-1544–AcEAK conjugate is presented in Table 2. A marked difference was found in the short-term (24 h) antioviulatory potency of the analogue and the conjugate. The minimal antioviulatory dose (ED_{100}) of MI-1544 was found to be 3 μ g, while its AcEAK conjugate was only partially (90%) inhibitory even with a 75 μ g dose. Interestingly, in the long-term (72 h) antioviulatory study, there was 30% inhibition with free MI-1544 with a 30 μ g dose. A similar effect was observed with the MI-1544–AcEAK conjugate at a higher dose (40% inhibition at 75 μ g).

These data suggest that the MI-1544–AcEAK conjugate was able to act as free MI-1544 both in short- and long-term inhibition of ovulation even after single sc injection. The necessity for high-dose conjugate administration to achieve an antioviulatory effect comparable to that of free antagonist indicates differences in the bioavailability and/or pharmacokinetic properties (e.g. absorption and/or transport) of the free and conjugated MI-1544.

GnRH Antagonistic Effects. The *in vitro* GnRH inhibitory effect of MI-1544 and the MI-1544–AcEAK conjugate is presented in Table 3. MI-1544 prevented the LH-releasing effect of 1 nM GnRH even at 1 nM (95% inhibition), while MI-1544–AcEAK showed this effect (96% inhibition) only at a 50 nM concentration. At

Table 3. GnRH Inhibitory Effect of MI-1544 and Its Branched Polypeptide Conjugate (MI-1544–AcEAK) in an *in Vitro* Cell Perfusion System

GnRH:antagonist molar ratio (nmol/nmol)	MI-1544		MI-1544–AcEAK	
	NI/R ^a	inhibition (%)	NI/R ^a	inhibition (%)
1:0	1.00	—	1.00	—
1:1	0.05	95	1.00	0
1:5	0.02	98	0.89	11
1:10			0.57	43
1:50			0.04	96
1:100			0.02	98

^a Net integrated LH released by GnRH plus antagonist/net integrated LH released by GnRH alone (i.e. reference).

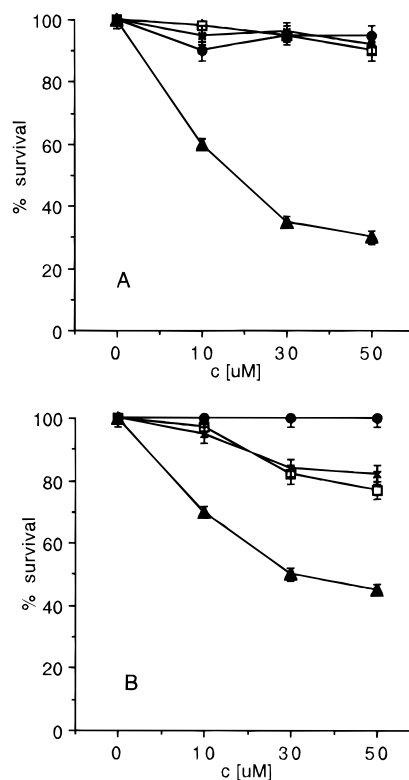


Figure 5. Effect of free MI-1544 ($\square$), its AcEAK conjugate ($\blacktriangle$), free AcEAK ($\bullet$), and the mixture of MI-1544 and AcEAK ($\times$) on the colony formation capacity of estradiol responsive (MCF-7) (A) and of estradiol unresponsive (MDA-231) (B) human mammary carcinoma cell lines. Relative cloning efficiencies of the treated cultures are expressed as a percentage of untreated controls.

concentrations of 1, 5, and 10 nM, it produced 0, 11, and 43% inhibition, respectively. These results show that MI-1544–AcEAK is capable of inhibiting LH release with high efficacy, but for this, the molar ratio of antagonist:GnRH must be 50–100. In contrast, the same level of inhibition can be achieved by antagonist:GnRH ratios of 1–5 with the free MI-1544. Consequently, the MI-1544–AcEAK conjugate is 20–50 times less inhibitory for GnRH than MI-1544 in the *in vitro* cell superfusion system. These findings might reflect the differences between the interaction of pituitary cells with free or covalently coupled MI-1544.

Clonogenic Assay. MCF-7 cells were essentially insensitive to the effect of the GnRH antagonist MI-1544. At a 50 μ M mg dose, the cell survival was reduced only by 10%. The cells were also resistant to free AcEAK (Figure 5A). MDA-MB-231 cells were somewhat more sensitive to the antagonist (Figure 5B), the relative plating efficiency being reduced to 78% compared to the controls. AcEAK did not reduce cell survival at all

(Figure 5B). The MI-1544–AcEAK conjugate, however, decreased cell survival by 70% (MCF-7) and 50% (MDA-231), respectively (Figure 5A,B). It should be noted that, in control experiments, when the mixture of free peptide and free carrier was applied, the survival profile in both cell lines was similar to that when free peptide was administered (Figure 5).

These data show that the survival of both cell lines could be only slightly reduced by the treatment with the antagonist MI-1544 in the concentration range studied. Conjugation but not mixing of this peptide with AcEAK however increased its effect significantly. The difference between the effect of the free peptide and the conjugate was especially considerable in the case of MCF-7 cells. The damaging effect of the MI-1544–AcEAK conjugate observed in the clonogenic assay is supposed to be the result of a direct antitumor effect, which can be explained by the presence of GnRH-binding sites on both MCF-7 and MDA-MB-231 mammary carcinoma cells (10). According to the literature, the GnRH antagonists inhibit the GnRH receptor-mediated signal transduction by blocking the GnRH receptors (50, 51). As the compounds were given to the cultures only once during the 8–12 days of incubation, it is thought that the moderate effect of the free peptide compared to that of the conjugate can be accounted for by its limited stability. This observation was supported by a comparative study in which the effects of these compounds were investigated on cell proliferation (52).

Conclusion. These studies have indicated that it is possible to prepare a synthetic branched polypeptide conjugate of a potent GnRH antagonist Ac(D-Trp^{1,3}, D-Cpa², D-Lys⁶, D-Ala¹⁰)-GnRH (MI-1544) with a relatively high molar substitution ratio. In this construct, a stable γ,ϵ type amide bonding was introduced between the carboxyl groups of the carrier AcEAK and the amino group at position 6 of MI-1544 to achieve prolonged body retention of the antagonist with maintained hormone-related antitumor activity. This conjugate, as revealed by CD spectroscopy, adopts a disordered conformation under physiological condition, indicating the sterically random arrangement of the carrier backbone and allowing potential accessibility of the GnRH antagonist to recognition by relevant receptors.

Data presented in this paper have demonstrated that in fact (a) the blood/body presence of MI-1544 can be significantly prolonged and (b) the direct *in vitro* antitumor effect of MI-1544 can be maintained or even enhanced by its coupling to an anionic branched polypeptide, AcEAK. On the other hand, in this study, we have shown that this prototype conjugate was able (a) to antagonize the effect of GnRH *in vitro* and (b) to prevent ovulation, but its efficiency was markedly decreased.

Although the precise mechanism of conjugate action is unclear, our *in vitro* data indicate that the presence of biodegradable polymeric carrier could provide new route(s) of cellular uptake and/or cell–antagonist interaction, which are not available for the free GnRH antagonist. This speculation is in accord with recently published *in vitro* data on MI-1544 conjugated to a nonbiodegradable polymer, poly(*N*-vinylpyrrolidone-maleic anhydride) (53).

For the reliable explanation of these results, more experimentation is needed to clarify potential aspects of polymer activity.

Taken together, these results can be considered as a proper basis for further development of the conjugate structure by e.g. selecting a different carrier, an attachment site, or a spacer for producing a functionally further improved bioconjugate.

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LITERATURE CITED

- (1) Hazum, E. (1988) Molecular mechanism of gonadotropin releasing hormone (GnRH) action. The GnRH receptor. *Endocr. Rev.* 9, 379–386.
- (2) Knobil, E. (1980) The neuroendocrine control of the menstrual cycle. *Recent Prog. Horm. Res.* 36, 53–88.
- (3) Lissoni, P., Barni, S., Crispino, S., Cattaeno, G., and Tancini, G. (1988) Endocrine and clinical effects of LHRH analogue in pretreated advanced breast cancer. *Tumori* 74, 303–308.
- (4) Folkers, K., Bowers, C. Y., Sieh, H. M., Yin-Zeng, L., Sao-Bo, X., Tang, P. F. L., and Ji-Yu, C. (1984) Antagonists of the luteinizing hormone releasing hormone (LHRH) with emphasis on the Trp7 of the salmon and chicken II LHRH's. *Biochem. Biophys. Commun.* 123, 1221–1226.
- (5) Braden, T. D., and Conn, P. M. (1990) Altered rate of synthesis of gonadotropin releasing hormone receptors: effects of homologous hormone appear independent of extracellular calcium. *Endocrinology* 126, 2577–2582.
- (6) Bajusz, S., Kovács, M., Gazdag, M., Bokser, L., Karashima, T., Csernus, V. J., Janáky, T., Guot, J., and Schally, A. V. (1988) Highly potent antagonists of LHRH free edematogenic effects. *Proc. Natl. Acad. Sci. U.S.A.* 85, 1637–1641.
- (7) Pedrosa, E., Vilchez-Martinez, J. A., Coy, D. H., Arimura, A., and Schally, A. V. (1980) Reduction of LH-RH pituitary and estradiol uterine binding sites by a superactive analogue of luteinizing hormone-releasing hormone. *Biochem. Biophys. Res. Commun.* 95, 1056–1062.
- (8) Lamberts, S. W. J., Vitterlinden, P., Zuiderwijk-Roest, J. M., Bons-van Evelingen, E. G., and De Jong, F. H. (1981) Effects of a luteinizing hormone-releasing hormone analog and tamoxifen in the growth of an estrogen-induced prolactin-secreting rat pituitary tumor and its influence on pituitary gonadotropins. *Endocrinology* 108, 1878–1884.
- (9) Miller, W. R., Scott, W. N., Morris, R., Frase, H. M., and Sharpe, R. M. (1985) Growth of human breast cancer cells inhibited by a luteinizing hormone-releasing hormone agonist. *Nature* 313, 231–233.
- (10) Vincze, B., Pályi, I., Daubner, D., Kremmer, T., Számel, I., Bodrogi, I., Sugár, J., Seprödi, J., Mező, I., Teplán, I., and Eckhardt, S. (1991) Influence of luteinizing hormone-releasing hormone agonists on human mammary carcinoma cell lines and their xenografts. *J. Steroid Biochem. Mol. Biol.* 38, 119–126.
- (11) Pályi, I., Vincze, B., Kálnay, A., Gaál, D., Mező, I., Seprödi, J., and Teplán, I. (1994) Antitumor effect of new chicken GnRH analogues in mammary cell lines. *Ann. Oncol.* 5 (Suppl. 5), 85.
- (12) Karten, M. J., and Rivier, J. E. (1986) Gonadotropin-releasing hormone analog design. Structure-function studies toward the development of agonists and antagonists: rationale and perspectives. *Endocr. Rev.* 7, 44–66.
- (13) Danforth, D. R., Williams, R. F., Gordon, K., Leal, J. A., and Hodgen, G. D. (1991) Inhibition of pituitary gonadotropin secretion by the gonadotropin-releasing hormone antagonist antide. *Endocrinology* 128, 2041–2044.

- (14) Redding, T. W., Schally, A. V., Tice, T. R., and Meyers, W. E. (1984) Long-acting delivery systems for peptides: inhibition of rat prostate tumors by controlled release of [D-Trp⁶]-luteinizing hormone-releasing hormone from injectable microcapsules. *Proc. Natl. Acad. Sci. U.S.A.* **81**, 5845–5848.
- (15) Gonzales-Barcena, D., Perez-Sanchez, P. L., Graef, A., Gomez, A. M., Berea, H., Comaru-Schally, A. M., and Schally, A. V. (1989) Inhibition of the pituitary-gonadal axis by a single intramuscular administration of D-Trp-6-LH-RH (Decapeptyl) in a sustained-release formulation in patients with prostatic carcinoma. *Prostate* **14**, 291–300.
- (16) Szende, B., Srkalovic, G., Goot, K., Lapis, K., and Schally, A. V. (1990) Growth inhibition of mouse MXT mammary tumor by the luteinizing hormone-releasing hormone antagonist SB-75. *J. Natl. Cancer Inst.* **82**, 513–517.
- (17) Yano, T., Korkut, E., Pinski, J., Szepesházi, K., Milovanovic, S., Groot, K., Clarke, R., Comaru-Schally, A. M., and Schally, A. V. (1992) Inhibition of growth of MCF-7 MIII human breast carcinoma in nude mice by treatment with agonists or antagonists of LH-RH. *Breast Cancer Res. Treat.* **21**, 35–45.
- (18) Duncan, R. (1992) Drug-polymer conjugates; potential for improved chemotherapy. *Anti-Cancer Drugs* **3**, 153–156.
- (19) Maeda, H., Seymour, L. W., and Miyamoto, Y. (1992) Conjugates of anticancer agents and polymers: advantages of macromolecular therapeutics in vivo. *Bioconjugate Chem.* **3**, 351–362.
- (20) Seymour, L. W. (1992) Passive tumor targeting of soluble macromolecules and drug conjugates. *Crit. Rev. Ther. Drug Carrier Syst.* **9**, 135–187.
- (21) Hudecz, F. (1995) Design of synthetic branched-chain polypeptides as carriers for bioactive molecules. *Anti-Cancer Drugs* **6**, 171–193.
- (22) Kovács, M., Mező, I., Flerkő, B., Teplán, I., and Nikolics, K. (1984) Long-term inhibition of ovulation by a GnRH-antagonist at low dose level. *Biochem. Biophys. Res. Commun.* **118**, 351–355.
- (23) Hudecz, F., Votavova, H., Gaál, D., Sponar, J., Kajtár, J., Blaha, K., and Szekerke, M. (1985) Branched polypeptides with a poly(L-lysine) backbone: synthesis, conformation and immunomodulation. In *Polymeric Materials in Medication* (Ch. G. Gebelein and Ch. E. Carraher, Eds.) pp 265–289, Plenum Press, New York.
- (24) Mező, G., Hudecz, F., Kajtár, J., Szókán, Gy., and Szekerke, M. (1989) The influence of the side chain sequence on the structure-activity correlations of immunomodulatory branched polypeptides. Synthesis and conformational analysis of new model polypeptides. *Biopolymers* **28**, 1801–1826.
- (25) Hudecz, F., Kutassi-Kovács, S., Mező, G., and Szekerke, M. (1989) Biodegradability of synthetic branched polypeptide with poly(L-lysine) backbone. *Biol. Chem. Hoppe-Seyler* **370**, 1019–1026.
- (26) Hudecz, F., Kojima, Y., Miyamoto, Y., Kajtár, J., and Maeda, H. (1994) The effect of charge on the biodistribution of synthetic branched polypeptides in tumour bearing mice. *J. Controlled Release* **28**, 301–302.
- (27) Pimm, M. V., Gribben, S. J., Bogdán, K., and Hudecz, F. (1995) The effect of charge on the biodistribution in mice of branched polypeptides with poly(L-lysine) backbone labelled with ¹²⁵I, ¹¹¹In or ⁵¹Cr. *J. Controlled Release* **37**, 161–172.
- (28) Hudecz, F., Kovács, P., Kutassi-Kovács, S., and Kajtár, J. (1984) GPC, CD and sedimentation analysis of poly-Lys and branched chain poly-Lys-poly-DL-Ala polypeptides. *Colloid Polym. Sci.* **262**, 208–212.
- (29) IUPAC-IUB Commission on Biochemical Nomenclature (1972) *Biochem. J.* **127**, 753–756.
- (30) IUPAC-IUB Commission on Biochemical Nomenclature (1984) *Eur. J. Biochem.* **138**, 9–37.
- (31) Hudecz, F., and Szekerke, M. (1984) Synthesis of new branched polypeptides with poly(lysine) backbone. *Collect. Czech. Chem. Commun.* **50**, 103–113.
- (32) Mező, G., Votavová, H., Hudecz, F., Kajtár, J., Sponar, J., and Szekerke, M. (1988) Conformation of branched polypeptides: the influence of relative position of leucine residues in the side chains. *Collect. Czech. Chem. Commun.* **53**, 2843–2853.
- (33) Kaiser, E., Colescott, R. L., Bossinger, C. D., and Cook, P. I. (1970) Color test for detection of free terminal amino groups in the solid-phase synthesis of peptides. *Anal. Biochem.* **34**, 595–598.
- (34) Schippers, P. H., and Dekkers, H. P. J. (1981) Direct determination of absolute circular dichroism data and calibration of commercial instruments. *Anal. Chem.* **53**, 778–782.
- (35) Greenfield, N., and Fasman, G. D. (1969) Computed circular dichroism spectra for the evaluation of protein conformation. *Biochemistry* **8**, 4108–4116.
- (36) Vigh, S., and Schally, A. V. (1984) Interaction between hypothalamic peptides in a superfused pituitary cell system. *Peptides* **5** (Suppl. 1), 214–247.
- (37) Csernus, V., and Schally, A. V. (1991) The dispersed cell superfusion system. In *Neuroendocrine Research Methods* (B. Greenstein, Ed.) pp 66–102, Harwood Academic Publishers, London.
- (38) Soule, H. D., Vasquez, J., Long, A., Albert, S., and Brennan, H. (1973) A human cell line from a pleural effusion derived from a breast carcinoma. *J. Natl. Cancer Inst.* **51**, 1409–1413.
- (39) Cailleau, R., Young, R., Olive, M., and Reeves, W. J. (1974) Breast tumor cell lines from pleural effusion. *J. Natl. Cancer Inst.* **53**, 661–674.
- (40) Pályi, I., Péter, I., Daubner, D., Vincze, B., and Lörincz, I. (1993) Establishment, characterization and drug sensitivity of a new anaplastic thyroid carcinoma cell line (BHT-101). *Virchows Arch. B* **63**, 263–269.
- (41) Hudecz, F., Kajtár, J., Kurucz, I., Kovács, A. L., and Szekerke, M. (1988) Investigation of the carrier function of polypeptides. Synthesis, conformation and cytotoxicity of oxazoline conjugates of poly(Lys) and poly(Lys-(DL-Ala)_m). *Makromol. Chem., Macromol. Symp.* **19**, 107–124.
- (42) Hudecz, F., and Price, M. R. (1992) Monoclonal antibody binding to peptide epitopes conjugated to synthetic branched polypeptide carriers. Influence of the carrier upon antibody recognition. *J. Immunol. Methods* **147**, 201–210.
- (43) Hudecz, F., Clegg, J. A., Kajtár, J., Embleton, M. J., Szekerke, M., and Baldwin, R. W. (1992) Synthesis, conformation, biodistribution, and in vitro cytotoxicity of daunomycin-branched polypeptide conjugates. *Bioconjugate Chem.* **3**, 49–57.
- (44) Hudecz, F., Clegg, J. A., Kajtár, J., Embleton, M. J., Pimm, M. V., Szekerke, M., and Baldwin, R. W. (1993) Influence of carrier on biodistribution and in vitro cytotoxicity of methotrexate-branched polypeptide conjugates. *Bioconjugate Chem.* **4**, 25–33.
- (45) Berger, H., Schäfer, H., Klauschenz, E., Albrecht, E., and Mehli, B. (1982) Rapid assay for in vitro degradation of luteinizing hormone releasing hormone. *Anal. Biochem.* **127**, 418–425.
- (46) Wynn, P. C., Suarez-Quian, C. A., Childs, G. V., and Catt, K. J. (1986) Pituitary binding and internalization of radioiodinated gonadotropin-releasing hormone agonist and antagonists ligands in vitro and in vivo. *Endocrinology* **119**, 1852–1853.
- (47) Berger, H., Heinrich, N., Schäfer, H., Baegar, I., and Mehli, B. (1988) Gonadotropin-releasing hormone (GnRH) pharmacokinetics: peptide hormone pharmacokinetics needs clarification. *Life Sci.* **42**, 985–991.
- (48) Handelsman, D. J., and Swerdloff, R. S. (1986) Pharmacokinetics of gonadotropin-releasing hormone and its analogs. *Endocr. Rev.* **7**, 95–105.
- (49) Pimm, M. V., Gribben, S. J., Mező, G., and Hudecz, F. (1995) Strategies for labelling branched polypeptides with a poly(L-lysine) backbone with radioiodines (¹²³I, ¹²⁵I, ¹³¹I) and radiometals (¹¹¹In, ⁵¹Cr) for biodistribution studies and radiopharmaceutical development. *J. Labelled Compd. Radiopharm.* **36**, 157–172.
- (50) Sharoni, Y., Bosin, E., Miinster, A., Levy, J., and Schally, A. V. (1989) Inhibition of growth of human mammary tumor cells by potent antagonists of luteinizing hormone-releasing hormone. *Proc. Natl. Acad. Sci. U.S.A.* **86**, 1648–1651.
- (51) Segal-Abramson, T., Giat, J., Levy, J., and Sharoni, Y. (1992) Guanine nucleotide modulation of high affinity gona-

- dotropin-releasing hormone receptors in rat mammary tumors. *Mol. Cell. Endocrinol.* **85**, 109–116.
- (52) Vincze, B., Pályi, I., Daubner, D., Kálnay, A., Mező, G., Hudecz, F., Szekerke, M., Teplán, I., and Mező, I. (1994) Antitumor effect of a GnRH antagonist and its conjugate on human breast cancer cells and their xenografts. *J. Cancer Res. Clin. Oncol.* **120**, 578–584.
- (53) Pályi, I., Vincze, B., Kálnay, A., Turi, G., Mező, I., Teplán, I., Seprődi, J., Pató, J., and Móra, M. (1996) Effect of gonadotropin-releasing hormone analogs and their conjugates on gonadotropin-releasing hormone receptor-positive human cancer cell lines. *Cancer Detect. Prev.* **20**, 146–152.

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Double-Lectin Site Ricin B Chain Mutants Expressed in Insect Cells Have Residual Galactose Binding: Evidence for More Than Two Lectin Sites on the Ricin Toxin B Chain

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Ricin toxin, the heterodimeric 65 kDa glycoprotein synthesized in castor bean seeds, contains a cell binding lectin subunit (RTB) disulfide linked to an RNA *N*-glycosidase protein synthesis-inactivating subunit (RTA). Investigations of the molecular nature of the lectin sites in RTB by X-ray crystallography, equilibrium dialysis, chemical modification, and mutational analysis have yielded conflicting results as to the number, location, and affinity of sugar-combining sites. An accurate assessment of the amino acid residues of RTB involved in galactose binding is needed both for correlating structure–function of a number of plant lectins and for the design and synthesis of targeted toxins for cancer and autoimmune disease therapy. We have performed oligonucleotide-directed mutagenesis on cDNA encoding RTB and expressed the mutant RTBs in insect cells. Partially purified recombinant proteins obtained from infected cell supernatants and cell extracts were characterized as to yields, immunoreactivities, asialofetuin binding, cell binding, ability to reassociate with RTA, and recombinant heterodimer cell cytotoxicity. Two single-site mutants (subdomain 1 α or 2 γ) and two double-site mutants (subdomains 1 α and 2 γ) were produced and studied. Yields varied by two logs with lower recoveries of double-site mutants. All the mutants showed immunoreactivity with a panel of anti-RTB monoclonal and polyclonal antibodies. Single-lectin site mutants displayed up to a 1 log decrease in asialofetuin binding avidity, while the double-site mutants showed close to a 2 log decrease in sugar binding. However, for each of the double-site mutants, residual sugar binding was demonstrated to both immobilized asialofetuin and cells, and this binding was specifically inhibitable with α -lactose. All mutants reassociated with RTA, and the mutant heterodimers were cytotoxic to mammalian cells with potencies 1000-fold or more times that of unreassociated wild-type RTA or RTB. These data support a model for three or more lectin binding subdomains in RTB.

INTRODUCTION

Lectins such as ricin toxin from the *Ricinus communis* plant mediate a wide range of biological effects due to binding cell surface carbohydrates. The B chain subunit of ricin (RTB)¹ binds to mammalian cell membranes by recognizing galactose-containing receptors, and this reaction is the first necessary step for intoxication of cells (1). RTB binds β -galactosides (2) with association constants from 10^3 to 3×10^4 M⁻¹ for simple sugars (3, 4) and 10^7 – 10^8 M⁻¹ for free and cell surface-bound glycoproteins (5, 6). These results lead to the hypothesis that multiple low-affinity sugar binding on ricin interact with complex oligosaccharides and cells to yield high-affinity binding. Clustering of target sugars in the proper

geometry to enhance lectin binding via interactions at multiple sites has been observed for the hepatic galactose *N*-acetylgalactosamine-binding receptor (7) and the macrophage mannose receptor (8).

The X-ray crystallographic structure of ricin provides a structural basis for this hypothesis. RTB has two domains each with three subdomains (9). The six subdomains have similar folding and primary amino acid sequences and resemble the primitive galactose binding fold in discoidin I from the slime mold *Dictyostelium discoideum*. Tripeptide kinks in the loops from subdomains 1 α , 1 β , 2 α , and 2 γ may interact with galactosides. Each of these subdomains has aromatic residues which can interact with the nonpolar face of galactose, and three of the four subdomain folds (1 α , 1 β , and 2 γ) have polar residues for hydrogen bond formation to the sugar hydroxyls. Cocrystallization of α -lactose at low concentrations (5 mM) with ricin permitted identification of bonds between lactose and amino acid residues of subdomains 1 α and 2 γ .

Biochemical modification studies have identified one to three sugar binding sites per RTB molecule. *N*-Bromosuccinimide modification of Trp-37 reduced sugar binding, demonstrating a sugar-binding site in the subdomain 1 α fold (10). *N*-Acetylimidazole *O*-acetylation of two tyrosines reduced sugar binding, implicating sites in subdomains 1 β pocket and 2 γ pocket (11). Further, three distinct sites on ricin were cross-linked by radio-labeled fetuin glycopeptide containing a dichlorotriazine-activated 6-(*N*-methylamino)-6-deoxy-D-galactose moiety, supporting the concept of three sugar-binding sites (12).

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¹ Abbreviations: RTB, ricin toxin B chain; RTA, ricin toxin A chain; PBS, phosphate-buffered saline; BSA, bovine serum albumin; ELISA, enzyme-linked immunoassay; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; cDNA, complementary DNA; IC₅₀, concentration of compound reducing cellular protein synthesis by 50%; moi, multiplicity of infection; EDTA, ethylenediaminetetraacetic acid; NTEAL, 50 mM NaCl/25 mM Tris (pH) 8/1 mM EDTA/0.01% sodium azide/25 mM lactose; NTEALT, 500 mM NaCl/25 mM Tris (pH) 9/1 mM EDTA/0.01% sodium azide/25 mM lactose/0.1% Tween 20.

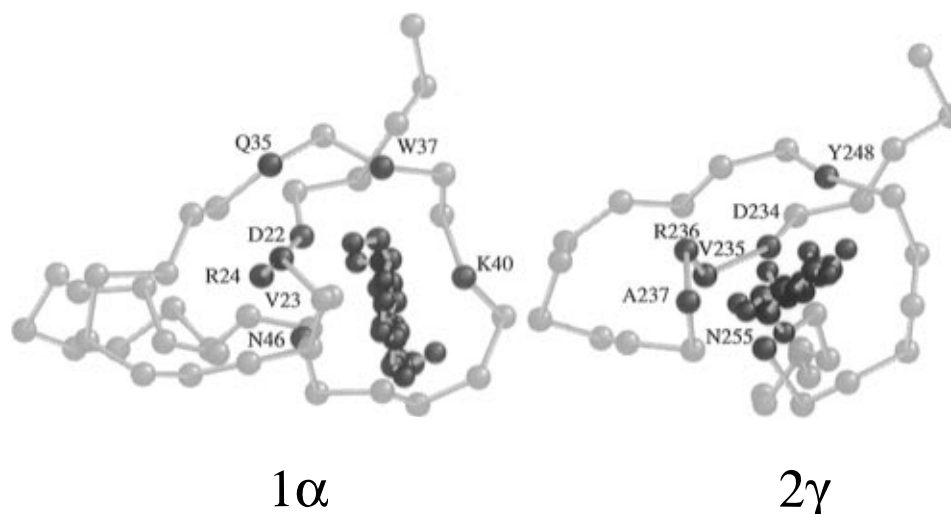


Figure 1. Model of subdomains 1 α and 2 γ of ricin B chain showing amino acid residues altered in this study. Coordinates derived from Rutember and Robertus (9). Ball and stick diagram display of Brookhaven coordinates on SYBYL software on Silicon Graphics Iris Indigo workstation with α carbon backbone displayed in gray and modified amino acid residues in black. Lactose molecules displayed in black also.

Mutational analysis of recombinant RTBs produced in Cos cells, bacteriophage, and *Xenopus laevis* oocytes has suggested either one active lectin site in subdomain 2 γ or two lectin sites in subdomains 1 α and 2 γ (13–15). RTB mutant N255A produced in Cos cells and RTB mutants K40M/N46G/N255G and D22Q/V23A/R24N/D234A/V235A/R236T synthesized in *Xenopus* oocytes lacked sugar binding (13, 14). However, very small amounts of proteins were made in each case, and purification and immunologic characterization of products were not done. In all three mutational studies, decreased sugar binding due to misfolding or aggregation of recombinant RTBs could lead to an overestimation of the effect of individual modifications.

To obtain more accurate quantitative information on the RTB lectin sites, our laboratory has expressed, partially purified, and characterized wild-type, single-site, and double-site RTB mutants in insect cells (16–19). We obtained microgram to milligram yields of recombinant proteins and were able to purify the lectins to 10–50% purity by Coomassie-stained SDS–PAGE. Wild-type recombinant RTB bound asialofetuin and cell surface oligosaccharides in a manner similar to that of plant RTB with half-maximal binding concentrations of about 5×10^{-9} M (16, 17). Single-site mutants of both the 1 α subdomain and the 2 γ subdomain had reduced binding affinity for asialofetuin and KB mammalian cells (18). However, the reduction in binding affinity was 1 log or less in each case. After reassociation with plant RTA, each single-site mutant retained HUT102 human leukemia cell cytotoxicity with ID_{50} 's within 1–1.5 logs of that of the wild-type heterodimer. The minor effect of genetic modification of lectin sites in subdomains 1 α and 2 γ suggested incomplete inactivation of lectin sites or additional sugar-combining sites on RTB. We then expressed, partially purified, and characterized three double-site mutant RTBs and one additional single-site mutant RTB (19). Two of the double-site mutants had unique properties suggesting either persistence of two main galactose binding sites operating at reduced levels or the theory of three ricin lectin sites. To resolve the issue of incomplete inactivation of the lectin sites in the single- and double-site mutants, we now report the biological properties of two additional single-site mutants and two additional double-site mutants. Modifications at each site were chosen to maximally alter each lectin

pocket. Our findings support the hypothesis of at least three independent sugar-combining sites on ricin.

EXPERIMENTAL PROCEDURES

Mutagenesis. pUC119-RTB plasmid containing a *Bam*HI-*Eco*RI DNA fragment coding for ADP–RTB was propagated in INV α F' *Escherichia coli* cells (InVitrogen, San Diego, CA) as previously described (16). Single-stranded DNA was produced by infection of transformants with M13K07 phage (Stratagene, La Jolla, CA) as previously described (20). Oligonucleotides were synthesized on an Applied Biosystems 380B DNA synthesizer and desalted with butan-1-ol. 39-mers were prepared with the modified codon flanked by 18 bases on each side matching RTB sequence and lacking an *Nci*I site. Site-specific mutagenesis was performed by the Eckstein method using the Sculptor *in vitro* mutagenesis system (Amersham, Arlington Heights, IL) and the manufacturer's instructions (21). Modifications were made at both the 1 α and 2 γ subdomains on the basis of the X-ray crystallographic model of the lectin binding sites (Figure 1) to either alter key polar residues which provide hydrogen bonds to sugar hydroxyls (D234E, N46G/K40M/N255G, and D22Q/V23A/R24N/D234A/V235A/R236T) or change aromatic ring residues which provide van der Waals interactions between the protein and sugar (Y248H). Sequences of mutant RTB DNAs were confirmed by double-stranded dideoxy sequencing by the Sanger method using the Sequenase kit (USB, Cleveland, OH) (22).

Construction of Transfer Vectors Encoding Mutant RTBs. Mutant RTB-encoding pUC119 DNAs were then restricted with *Bam*HI and *Eco*RI, and the RTB-encoding fragments were subcloned into pAcGP67A plasmid (PharMingen, San Diego, CA) and used to transform INV α F' *E. coli* cells. Transfer vectors with mutant RTBs were then purified by cesium chloride gradient centrifugation.

Isolation of Recombinant Baculoviruses. The Sf9 *Spodoptera frugiperda* ovarian cell line was maintained on TMNFH medium supplemented with 10% fetal calf serum and gentamicin sulfate (10 μ g/mL). pAcGP67A–mutant RTB DNAs (4 μ g) were cotransfected with 0.5 μ g of BaculoGold AcNPV DNA (PharMingen) into 2×10^6 Sf9 insect cells following the recommendations of the supplier. On day 7 post-transfection, media were cen-

trifuged and the supernatants tested in limiting dilution assays with Sf9 cells. Sf9 cells (2×10^4) were incubated with 10-fold dilutions of supernatants in 96-well plates. Seven days postinfection, supernatants were saved and cells in each assay well were lysed with NaOH and the lysates transferred to nitrocellulose. The nitrocellulose was then blocked with Blotto and reacted with random primer [^{32} P]-dCTP-labelled RTB DNA. After hybridization for 16 h at 67 °C, the dot blot membranes were washed with $0.1 \times$ (150 mM NaCl/15 mM sodium citrate)/1% SDS, dried, and exposed to X-ray film. Positive wells were identified and supernatants reassayed by limiting dilution until all wells up to 10^{-8} dilution were positive. Two rounds of selection were required for each mutant. Recombinant viruses in the supernatants were then amplified by infecting Sf9 cells at a multiplicity of infection (moi) of 0.1, followed by collection of day 7 supernatants.

Expression of Mutant B Chains in Sf9 Cells. Recombinant baculoviruses were used to infect 2×10^8 Sf9 cells at an moi of 5 in EX-CELL 400 media (JRH Scientific, Lexena, KS) with 50 mM α -lactose in spinner flasks. Media supernatants containing mutant RTBs were collected on day 6 postinfection.

Purification of Mutant RTBs. Media supernatants were adjusted to 0.01% sodium azide and maintained through all purification steps at 4 °C. The supernatants were concentrated 15-fold by vacuum dialysis, centrifuged at 3000g for 10 min to remove precipitate, dialyzed against 50 mM NaCl, 25 mM Tris (pH 8), 1 mM EDTA, 0.01% sodium azide, and 25 mM α -lactose (NTEAL), ultracentrifuged at 100000g for 1 h, and loaded onto a P2 monoclonal antibody-acrylamide column previously described (16). The affinity column was then washed sequentially with NTEAL and 500 mM NaCl, 25 mM Tris (pH 9), 1 mM EDTA, 0.01% sodium azide, 25 mM α -lactose, and 0.1% Tween 20 (NTEALT), and mutant RTBs were eluted with 0.1 M triethylamine (pH 11). The alkaline eluants were immediately neutralized with 1 M sodium phosphate (pH 4.8) and stored at -20 °C until they were assayed. Optical densities at 280 nm were determined and aliquots mixed with reducing 2 $\times$ SDS sample buffer, boiled for 4 min, submitted to a 15% SDS-PAGE, stained with Coomassie Blue R-250, and destained with acetic acid/methanol. Gels were scanned on an IBAS automatic image analysis system (Kontron, Germany) to estimate the fraction of the protein with a molecular mass of 32 kDa.

Several preparations of mutant RTBs were made from cell pellets by dissolving pellets in 10 volumes of 20 mM Tris (pH 8), 50 mM NaCl, 1% NP40, 1 mM PMSF, 2 μ g/mL aprotinin, 1.5 μ g/mL pepstatin, and 1.5 μ g/mL leupeptin. The extracts were frozen at -70 °C, thawed, and centrifuged at 22000g for 15 min at 4 °C. Extracts were then dialyzed against NTEAL and treated in the same manner as dialyzed concentrated cell supernatants.

Immunological Properties of Mutant RTBs. Aliquots of mutant RTBs, plant RTB (Inland Laboratories, Austin, TX), wild-type recombinant RTB, and prestained low-molecular mass standards (BioRad, Hercules, CA) were mixed with reducing 2 $\times$ SDS sample buffer, boiled for 4 min, submitted to a 15% SDS-PAGE, and electrophoresed for 90 min. Gels, Whatman 3M #1 paper, and nitrocellulose were equilibrated for 15 min in Towbin buffer (20 mM Tris/0.1 M glycine/20% methanol) and placed in a Semi-dry Trans-blot cell (BioRad). After electrophoresis at 15 V for 20 min, the nitrocellulose was blocked with 10% Carnation's nonfat dry milk/0.1% BSA/0.1% Tween 20/0.02% sodium azide. The blots were then washed with PBS plus 0.05% Tween 20 and PBS,

incubated with rabbit anti-ricin antibody (Sigma, St. Louis, MO) at 1:400 in PBS plus 0.5% BSA plus 0.01% sodium azide for 1 h, washed again, incubated with alkaline phosphatase-conjugated goat anti-(rabbit IgG) (Sigma) at 1:1000 in PBS plus 0.5% BSA plus 0.01% sodium azide for 1 h, washed again, and developed with the Vectastain alkaline phosphatase kit, following the manufacturer's recommendations (Vector Laboratories, Burlingame, CA). Blots were scanned as above to compare 32 kDa M_r band intensities.

Monoclonal antibody P2, P8, or P10 (gifts of Dr. Walter Blattler, ImmunoGen, Cambridge, MA) (100 μ L) at 5 μ g/mL in PBS was incubated in Costar EIA microtiter wells overnight at 4 °C. Samples of plant RTB, wild-type recombinant RTB, and mutant RTBs were treated for 20 min at room temperature with 5% β -mercaptoethanol and then dilutions made in EX-CELL 400. The antibody-coated microtiter wells were then washed with PBS plus 0.1% Tween 20, blocked with 3% BSA/PBS/0.01% sodium azide, rewashed and incubated with dilutions of the reduced RTB samples, rewashed and incubated with rabbit anti-ricin antibody at 1:400 in PBS plus 0.5% BSA plus 0.01% sodium azide, washed again, incubated with alkaline phosphatase-conjugated goat anti-(rabbit IgG) at 1:1000 in PBS plus 0.05% BSA plus 0.01% sodium azide, washed and developed with (*p*-nitrophenyl)phosphate at 1 mg/mL in 50 mM diethanolamine buffer (pH 9.8), and read on a BioRad 450 microplate reader at 405 nm. For each experiment, 12 different concentrations of plant RTB and recombinant RTBs were tested. A plot of absorbance versus dilution was made for plant RTB and recombinant proteins. Dilutions yielding half-maximal binding were used to calculate concentrations.

Lectin Activity of Mutant RTBs. Volumes (100 μ L) of 1 μ g/mL asialofetuin in PBS were added to wells of a Costar EIA plate and incubated overnight at 4 °C. Samples of plant RTB, wild-type recombinant RTB, or mutant RTBs in EX-CELL 400 were exposed to 5% β -mercaptoethanol for 20 min at room temperature to remove homodimers and dilutions made in EX-CELL 400 medium with or without 100 μ g/mL asialofetuin or 100 mM α -lactose. The asialofetuin-coated microtiter wells were then washed with PBS/0.1% Tween 20, blocked with 3% BSA/PBS/0.01% sodium azide, and rewashed. The dilutions of various reduced RTBs were added to wells for 1 h and then removed and the wells washed again. Rabbit anti-ricin antibody was added (1:400 dilution in 0.5% BSA/PBS/0.01% sodium azide) for 1 h, and the wells were washed again; alkaline phosphatase-conjugated goat anti-(rabbit IgG) (1:5000 in 0.5% BSA/PBS/0.01% sodium azide) was incubated in the wells, and finally, the wells were washed and reacted with 1 mg/mL (*p*-nitrophenyl)phosphate in 50 mM diethanolamine buffer (pH 9.6) and measured in a microtiter plate reader at 405 nm. In each experiment, 12 different concentrations of plant RTB and recombinant protein were tested. As in the antibody ELISA, relative reactivity to plant RTB was calculated from concentrations giving half-maximal binding. The effects of 100 μ g/mL asialofetuin or 100 mM α -lactose on half-maximal binding were calculated for plant RTB, wild-type recombinant RTB, and mutant RTBs.

KB cells were washed with PBS and attached to polylysine-coated tissue culture dishes and centrifuged at 2000g for 10 min. The cells were then incubated live at 4 °C. The cells were washed with 2 mg/mL BSA in PBS and incubated in PBS plus BSA with or without 100 μ g/mL asialofetuin and with 1 μ g/mL freshly reduced plant RTB, recombinant wild-type RTB, or mutant RTB. The incubation was done at 4 °C. The cells were then

washed with PBS and incubated with rabbit anti-ricin antibody at 1:400 in PBS plus BSA for 30 min at 4 °C. The cells were then washed with PBS and reacted with goat anti-(rabbit Ig) conjugated to rhodamine at 25 $\mu\text{g}/\text{mL}$ for 30 min at 4 °C. The cells were washed again in PBS, fixed in 3.7% formaldehyde in PBS, mounted under a #1 coverslip in glycerol–PBS (90:10), and examined under a Zeiss Axioplan epifluorescence microscope.

Reassociation of Mutant RTBs with Plant RTA To Form Heterodimers. Portions (30 μg) of plant RTB and wild-type recombinant RTB and 1–5 μg of mutant RTBs were mixed with a 4-fold molar excess of plant RTA (Inland Laboratories) in a total volume of 0.5 mL of 0.1 M triethylamine/sodium phosphate (pH 7) shaking overnight at room temperature. The reaction mixture was then analyzed by a modified ricin ELISA. Wells of an EIA plate were coated with 10 $\mu\text{g}/\text{mL}$ P2 monoclonal antibody to RTB diluted in PBS in a volume of 100 μL overnight at 4 °C. The wells were washed with PBS plus 0.1% Tween 20, blocked with 3% BSA in PBS plus 0.02% sodium azide, rewashed, and incubated with dilutions of ricin or reassociated heterodimers. The wells were again washed and incubated with biotin-conjugated αBR12 monoclonal antibody (αBR12 mouse monoclonal antibody reactive with RTA was a gift of Dr. Walter Blattler, ImmunoGen) at 5 $\mu\text{g}/\text{mL}$ in PBS/0.5% BSA/0.01% sodium azide. Biotinylation was performed using (*N*-hydroxysuccinimido)biotin (Sigma) following the manufacturer's instructions. Wells were washed and incubated with alkaline phosphatase-conjugated streptavidin (Sigma) at 1:1000 in PBS/0.5% BSA/0.01% sodium azide, washed again, and developed with (*p*-nitrophenyl)phosphate at 1 mg/mL in 50 mM diethanolamine (pH 9.8). Absorbance at 405 nm was read on a microtiter plate reader. Reassociated mixtures were also analyzed by nonreducing SDS–PAGE followed by immunoblots with αBR12 anti-RTA monoclonal antibody and P10 anti-RTB monoclonal antibody. Densitometric scanning using the IBAS 2000 automatic image analysis system was done to quantify shift of immunoreactive material from 30 to 60 kDa.

Cytotoxicity of Recombinant Mutant Heterodimers. HUT102 human T leukemia cells (1.5×10^4) in 100 μL were placed in 96-well flat-bottomed plates in leucine-poor RPMI1640 containing 10% dialyzed fetal bovine serum. Fifty microliters of ricin (Inland Laboratories), recombinant wild-type RTB–plant RTA heterodimer, and mutant RTB–plant RTA heterodimers at varying concentrations were added in the same medium and the cells incubated at 37 °C in 5% CO_2 for 24 h. [^3H]-Leucine, 0.5 μCi per well (120 mCi/mmol), in 50 μL of the same medium was added and incubated for 4 h. Cells were then harvested with a PhD cell harvester onto glass-fiber filter mats. The filters were dried, mixed with 3 mL of liquid scintillation fluid, and counted in an LKB-Wallac liquid scintillation counter gated for ^3H . Cells cultured with medium alone served as controls. All assays were performed in triplicate. The ID_{50} was the concentration of protein which inhibited protein synthesis by 50% compared with control.

RESULTS

Yields and Immunoreactivity of Mutant RTBs.

Two new single-site mutants were prepared (D22E and Y248H). Y248H was previously described as part of a single-domain RTB–gene 3 protein fusion on fd phage (23), but the properties of a full length RTB with the single mutation have not been reported. *R. communis* agglutinin B chain and ricin E B chain have histidines

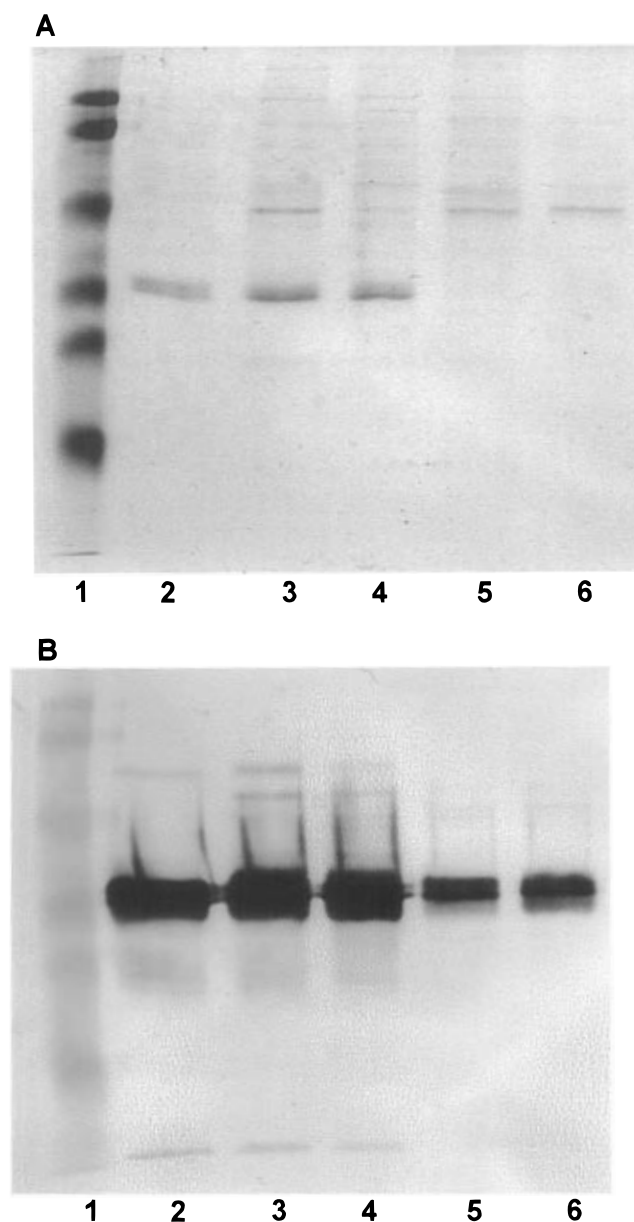


Figure 2. Insect-derived wild-type and mutant RTBs. (A) Coomassie-stained 15% reducing SDS–PAGE of mutant RTBs: lane 1, low-molecular mass prestained BioRad protein standards; lane 2, wild type; lane 3, D234E; lane 4, Y248H; lane 5, N46G/K40M/N255G; and lane 6, D22Q/V23A/R24N/D234A/V235A/R236T. (B) Immunoblot using rabbit anti-ricin antibody of 15% reducing SDS–PAGE of wild-type and mutant RTBs. Lanes are the same as in panel A.

at position 248 which may contribute to their lower galactose avidities (24). The two double-site mutants, N46G/K40M/N255G and D22Q/V23A/R24N/D234A/V235A/R236T, which were prepared had been previously expressed in *Xenopus* oocytes but have not been expressed and purified from insect cells (14).

The yields were estimated from the optical density at 280 nm of neutralized alkaline eluants with postaffinity chromatography (plant RTB OD = 1.44 for 1 mg/mL) and densitometry of Coomassie-stained reducing SDS–PAGE (10–30% of the protein migrated at 33 kDa, Figure 2A). These results were confirmed by densitometry of immunoblots with polyclonal rabbit anti-ricin antibody. As shown in Figure 2B, the single-site and double-site mutants were reactive with the polyclonal antibody. Finally, a monoclonal antibody anti-RTB ELISA was used to verify concentrations of each mutant. All three assays gave similar values. The yields of each of the four new

Table 1. Yields of Mutant RTBs Produced in Insect Cells^a

protein	type of mutant (subdomain)	amount of partially purified mutant per liter of culture (μ g)	reference
D234E	2 γ	1500	present study
Y248S	2 γ	640	18
Y248H	2 γ	420	present study
wild-type	—	400	16
W37S	1 α	264	18
D234E/A237R	2 γ	240	18
W37S/Y248H	1 α 2 γ	205	19
N255G	2 γ	160	18
D22E	1 α	150	19
Q35N	1 α	90	18, 19
N255A	2 γ	40	18
K40M/N46G	1 α	32	18
K40M	1 α	24	18
D22E/D234E	1 α , 2 γ	23	19
W37S/Y248S	1 α , 2 γ	22	19
N46G/K40M/N255G	1 α , 2 γ	10	present study
D22Q/V234A/R24N/D234A/V235A/R236T	1 α , 2 γ	2	present study

^a Quantity of RTB based on P2 ELISA and product of densitometry of Coomassie-stained gels and absorbance at 280 nm of partially purified protein. At least four preparations of each mutant made. Values given are mean. Agreement between densitometry and ELISA within 30% in each case.

Table 2. Binding of Monoclonal Anti-RTB Antibodies to Mutants^a

protein	type (subdomain)	% relative to monoclonal P2	
		P8	P10
D22E/D234E	1 α , 2 γ	60	100
wild-type	—	88	20
W37S/Y248H	1 α , 2 γ	90	710
W37S/Y248S	1 α , 2 γ	94	160
Q35N	1 α	96	250
D22E	1 α	100	100
D22Q/V23A/R24N/D234A/V235A/R236T	1 α , 2 γ	100	250
D234E	2 γ	100	100
D234E/A237R	2 γ	113	53
N46G/K40M	1 α	114	80
N255G	2 γ	117	80
W37S	1 α	119	55
N255A	2 γ	130	350
Y248H	2 γ	143	202
K40M	1 α	145	90
N46G/K40M/N255G	1 α , 2 γ	200	600
Y248S	2 γ	250	80

^a Measured by P2, P8, and P10 ELISA calibrated with known amounts of plant RTB. Each assay employed 5% β -mercaptoethanol to reduce homodimers. Average of two to four assays on each mutant.

mutants and 12 previously prepared mutants are shown in Table 1. The lower yields of 3/11 single-site mutants (N255A, K40M/N46G, and K40M) and 4/5 double-site mutants (D22E/D234E, W37S/Y248S, N46G/K40M/N255G, and D22Q/V23A/R24N/D234A/V235A/R236T) may be due to degradation of improperly folded proteins. Other investigators reported bioactivity of mutants without purification or characterization (13–15). Their assumption that modification of lectin site residues would not affect secondary structure may be inaccurate and overemphasize the role of individual residues and subdomains in sugar binding and cytotoxicity. Yields from cells extracts were similar to yields from supernatants in the four mutants tested (K40M, Q35N, N46G/K40M/N255G, and D22Q/V23A/R24N/D234A/V235A/R236T).

Reactivities of mutant RTBs with different monoclonal antibodies to RTB (P2, P8, and P10) were tested by substituting different monoclonal antibodies as capture reagents in the antibody ELISA. Equivalent results were observed for each antibody in most cases, suggesting similar folding of the mutants (Table 2).

Table 3. Binding of Mutant RTBs to Asialofetuin^a

protein	type of mutant	relative binding to asialofetuin (%)	reference
wild-type	—	83	16
N255A	2 γ	70	18
Q35N	1 α	50	18, 19
D234E	2 γ	33	this report
N255G	2 γ	33	18
Y248S	2 γ	32	18
D234E/A237R	2 γ	30	18
D22E	1 α	25	19
W37S	1 α	24	18
W37S/Y248S	1 α , 2 γ	20	19
Y248H	2 γ	17	this report
K40M	1 α	15	18
K40M/N46G	1 α	12	18
D22Q/V23A/R24N/D234A/V235A/R236T	1 α , 2 γ	10	this report
K40M/N46G/N255G	1 α , 2 γ	4	this report
W37S/Y248H	1 α , 2 γ	2	19
D22E/D234E	1 α , 2 γ	2	19

^a Quantity of RTB based on P2 ELISA and asialofetuin binding measured by asialofetuin ELISA. Both assays employed 5% β -mercaptoethanol to reduce homodimers. Average of three to four assays run on each mutant. Δ 32 nonsense mutant, DRA, and AcNPV antibody matrix eluants treated identically gave values of 0.01 μ g/mL for both the P2 and asialofetuin ELISA. Experimentals had P2 values of 0.2–150 μ g/mL and asialofetuin values of 0.02–58 μ g/mL. Q35N assays within 1 week of preparation.

Sugar Binding of Mutant RTBs. Binding of partially purified mutants to immobilized asialofetuin was quantitated by ELISA, and the results are shown in Table 3. The two new single-site mutants showed a less than 1 log drop in binding relative to recombinant or plant RTB. The two new double-site mutants showed a close to 2 log drop in sugar binding. The limit of detection of the assay was a 2.5–3 log decrease in sugar binding avidity.

An independent measure of mutant RTB binding to glycoproteins was made by detecting mutant RTB bound to cell surfaces. All mutants bound KB cells at 4 °C (Figure 3).

Competition Experiments. Binding of recombinant proteins to immobilized asialofetuin was performed in the presence of 100 mM α -lactose or 100 μ g/mL asialofetuin. In each case, the sugar binding of the RTB protein was inhibited by a competitor (Table 4). Lactose blocked wild-type and mutant RTB sugar binding between 2- and 81-

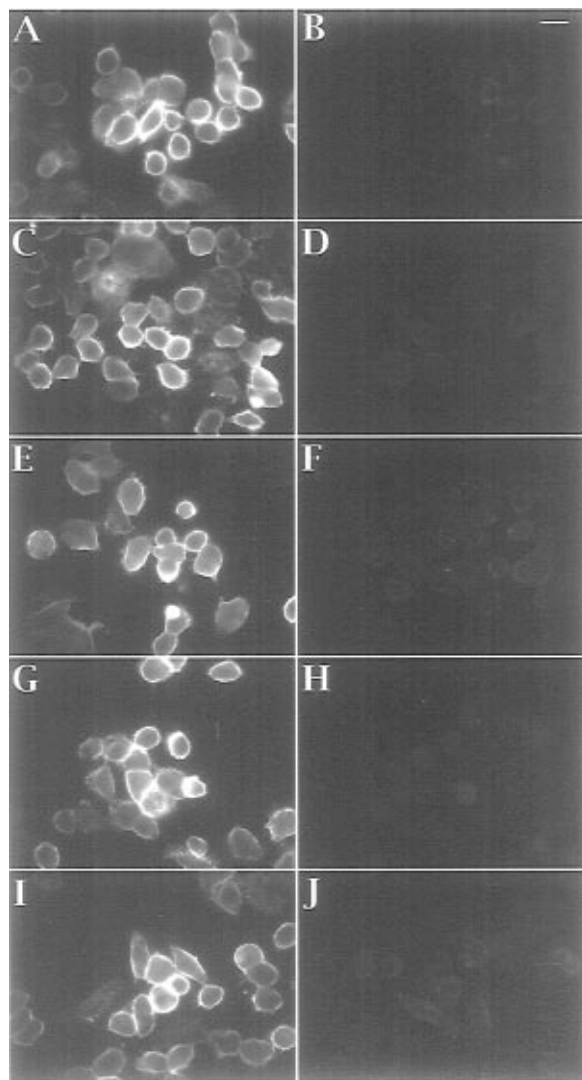


Figure 3. Binding of mutant RTBs to KB cells. Cells were attached to polylysine-coated tissue culture dishes, and all incubations were done at 4 °C. The cells were washed with 2 mg/mL BSA in PBS and the PBS plus BSA plus 1 μ g/mL purified mutant RTB or wild-type RTB, rewashed, incubated with 1:200 rabbit anti-ricin antibody (Sigma) in PBS plus BSA, rewashed, incubated with affinity-urified goat anti-(rabbit Ig) coupled to rhodamine (Jackson ImmunoResearch) at 25 μ g/mL, washed again, and fixed in 3.7% formaldehyde in PBS (magnification = 250 $\times$; bar = 20 μ m): (A, C, E, G, and I) without 100 μ g/mL asialofetuin and (B, D, F, H, and J) with asialofetuin. (A and B) wild-type RTB, (C and D) D234E, (E and F) Y248H, (G and H) N46G/K40M/N255G, and (I and J) D22Q/V23A/R24N/D234A/V235A/R236T.

fold. Asialofetuin blocked sugar binding 3–400-fold. The inhibition of soluble carbohydrates of double-site mutant binding was a lower estimate as the assay sensitivity was only 5–10-fold less than the observed binding to immobilized asialofetuin in the absence of added sugars for four of the double-site mutants. Each experiment was repeated three times, and 12 different concentrations of recombinant protein and plant RTB were used to compare half-maximal binding concentrations. Binding of mutant RTBs to KB cells was blocked by 100 μ g/mL asialofetuin (Figure 3).

Reassociation of Mutant RTBs with Plant RTA. Incubation of mutant RTBs at 3×10^{-8} to 3×10^{-7} M with plant RTA at a 4-fold molar excess overnight at room temperature led to 11–100% reassociation (Table 5 and Figure 4). Similar levels of reassociation were seen using plant RTB or recombinant wild-type RTB with plant RTA at the same concentrations. The heterodimer

Table 4. Competition of Mutant RTB Lectin Binding by Sugars^a

protein	type (subdomain)	fold inhibition	
		lactose	asialofetuin
Q35N	1 α	81	150
Y248H	2 γ	27	9
K40M/N46G	1 α	27	50
W37S	1 α	27	243
D234E	2 γ	27	100
N255A	2 γ	15	150
D22E	1 α	15	81
K40M	1 α	9	15
wild-type	—	9	400
W37S/Y248S	1 α , 2 γ	9	243
N255G	2 γ	9	243
D22Q/V23A/R24N/D234A/V235A/R236T	1 α , 2 γ	9	9
K40M/N46G/N255G	1 α , 2 γ	3	3
Y248S	2 γ	3	200
W37S/Y248H	1 α , 2 γ	3	5
D234E/A237R	2 γ	3	150
D22E/D234E	1 α , 2 γ	2	3

^a Asialofetuin ELISA performed in the presence or absence of 100 mM α -lactose or 100 μ g/mL asialofetuin. Twelve different concentrations of each protein tested and half-maximal binding concentrations compared to assess fold inhibition of binding.

Table 5. Reassociation of Mutant RTBs with RTA^a

protein	type (subdomain)	heterodimer formed (%)
W37S	1 α	11
N46G/K40M	1 α	22
W37S/Y248S	1 α , 2 γ	25
D22E	1 α	25
D234E	2 γ	27
N255A	2 γ	34
Y248S	2 γ	35
Y248H	2 γ	43
wild-type	—	45
D22E/D234E	1 α , 2 γ	50
D234E/A237R	2 γ	50
Q35N	1 α	65
W37S/Y248H	1 α , 2 γ	70
K40M	1 α	70
K40M/N46G/N255G	1 α , 2 γ	81
D22Q/V23A/R24N/D234A/V235A/R236T	1 α , 2 γ	90
N255G	2 γ	100

^a Assayed by modified ricin ELISA using P2 anti-RTB antibody capture and biotinylated α BR12 anti-RTA antibody detection reagent and confirmed by densitometry of immunoblots of nonreducing SDS–PAGE.

concentrations were quantitated by a modified ELISA which identified molecules with both RTA and RTB epitopes and by densitometry of 65 kDa bands of immunoblots with anti-RTB or anti-RTA antibodies. Both ELISA and immunoblots gave similar values and showed all mutants reassociated well with plant RTA and had minimal homodimer formation.

Cytotoxicity of Mutant Heterodimers. The ID₅₀ of ricin and recombinant mutant RTB–RTA heterodimers on HUT102 human leukemia cells is shown in Figure 5 and Table 6. The reduction in potency for single-site and double-site mutant RTB–RTA heterodimers compared to that for wild-type RTB–RTA and plant ricin was 1–2 log. Nevertheless, the potency for all the mutants was at least 3 log more than that for wild-type RTA or RTB alone.

DISCUSSION

The molecular mechanism of interaction between ricin and its saccharide receptors on the cell surface is the first

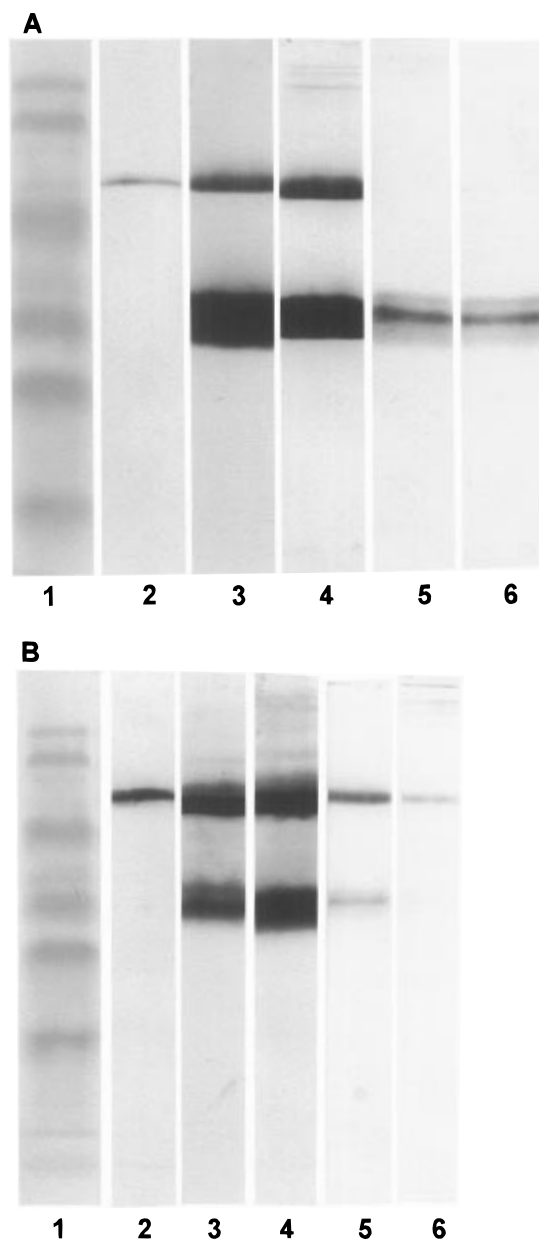


Figure 4. Reassociation of mutant RTBs with plant RTA. (A) Immunoblots of 15% nonreducing SDS-PAGE of reassociated mutant RTB-plant RTA. Low-molecular mass BioRad protein standards are 106, 80, 49.5, 32.5, 27.5, and 18.5 kDa. The heterodimer appears at 60 kDa, and subunits appear at 30 kDa. Immunoblot uses monoclonal antibody P2 and P10 anti-RTB. (B) Same as panel A except immunoblot uses monoclonal antibody α BR12 anti-RTA: lane 1, low-molecular mass prestained BioRad protein standards; lane 2, D234E-RTA; lane 3, Y248H-RTA; lane 4, N46G/K40M/N255G-RTA; lane 5, D22Q/V23A/R24N/D234A/V235A/R236T-RTA; and lane 6, ricin.

necessary step in intoxication of cells. Determination of the number of lectin sites on RTB and their affinities and participating amino acid residues are important both to define the structure-function relationships of this and other plant lectins, to interpret intracellular routing signals, and to design and synthesize genetically engineered cell-selective toxins.

While initial studies with ricin by equilibrium dialysis (25) and mutational analysis (13) suggested a single lectin site per molecule, subsequent equilibrium dialysis measurements (4, 26), X-ray diffraction analysis (9), chemical modification studies (10, 11), and mutational analyses (14, 15, 23) suggested two lectin sites in subdomains 1 α and 2 γ . However, for the mutational analy-

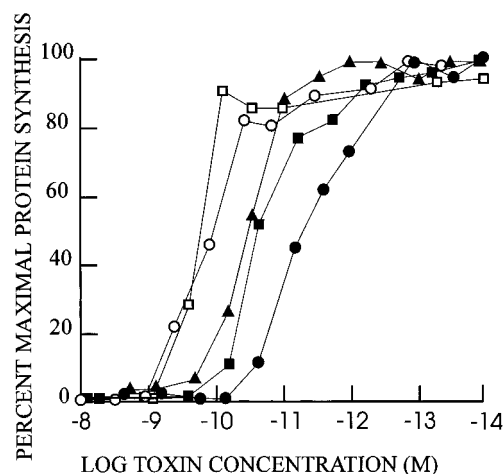


Figure 5. HUT102 cell cytotoxicity. Assay as described in text: (●) ricin, $IC_{50} = 4 \times 10^{-12}$ M; (○) D234E-RTA, $IC_{50} = 1 \times 10^{-10}$ M; (■) Y248H-RTA, $IC_{50} = 8 \times 10^{-11}$ M; (□) N46G/K40M/N255G-RTA, $IC_{50} = 2 \times 10^{-10}$ M; and (▲) D22Q/V23A/R24N/D234A/V235A/R236T-RTA, $IC_{50} = 5 \times 10^{-11}$ M. Yield of reassociated heterodimer tested by modified ELISA. Each experiment performed in triplicate.

Table 6. HUT102 Cell Sensitivity to Recombinant Heterodimers^a

protein	type (subdomain)	IC_{50} (M)
wild-type	—	5×10^{-12}
W37S/Y248S-RTA	1 α , 2 γ	1×10^{-11}
W37S-RTA	1 α	2×10^{-11}
Q35N-RTA	1 α	2×10^{-11}
Y248S-RTA	2 γ	5×10^{-11}
K40M/N46G-RTA	1 α	5×10^{-11}
D22Q/V23A/R24N/D234A/V235A/R236T-RTA	1 α , 2 γ	5×10^{-11}
Y248H-RTA	2 γ	8×10^{-11}
D234E/A237R-RTA	2 γ	8×10^{-11}
N255G-RTA	2 γ	8×10^{-11}
N255A-RTA	2 γ	8×10^{-11}
D22E/D234E-RTA	1 α , 2 γ	9×10^{-11}
K40M-RTA	1 α	1×10^{-10}
D234E-RTA	2 γ	1×10^{-10}
D22E-RTA	1 α	2×10^{-10}
K40M/N46G/N255G-RTA	1 α , 2 γ	2×10^{-10}
W37S/Y248H-RTA	1 α , 2 γ	2×10^{-10}

^a HUT102 cell cytotoxicity as described in text. Ricin IC_{50} was 4×10^{-12} M.

ses, no quantitation of soluble immunoreactive proteins was reported, and hence, the true specific activities of the recombinant proteins were unknown. If misfolding with aggregation or proteolytic degradation occurred, no residual lectin activity would be seen even if three or more lectin sites were present.

To more accurately measure sugar binding avidities of recombinant RTB proteins, we produced and purified wild-type and mutant RTBs from insect cells using the baculovirus system and monoclonal antibody affinity chromatography (16–19, present study). One log decreases in asialofetuin binding were observed with single-site mutants, and in 4/5 cases, 2 log decreases in asialofetuin binding were seen with double-site mutants. The persistent lactose and asialofetuin-inhibitable sugar and cell binding of a number of double-site (subdomain 1 α and 2 γ) mutants suggests either incomplete inactivation of the two sites in the mutants or the existence of a third lectin site. Since seven different residues in one subdomain (1 α) and seven different residues in the other domain (2 γ) were tested (Figure 1), we believe the first hypothesis is unlikely and that ricin has three or more lectin binding sites. Possible third lectin sites include

subdomain 1 β with aromatic ring residue Y78 and a lectin pocket kink and subdomain 2 α with aromatic residue W160, kink residues, and polar residues D153, E170, and Q171. Ongoing mutational analysis using the insect expression system should help address this question.

Other observations support the hypothesis of three lectin sites on ricin. Three distinct sites on ricin were cross-linked by radiolabeled fetuin glycopeptides containing a dichlorotriazine-activated 6-(*N*-methylamino)-6-deoxy-D-galactose moiety (12). When ricin molecules containing either two or three affinity cross-linkers were conjugated to monoclonal antibodies and tested for toxicity *in vitro* and *in vivo*, marked differences were observed (27). The residual nonspecific toxicity *in vitro* of the doubly blocked ricin conjugate could be blocked with excess lactose.

The finding of three or more independent sugar-combining sites on a lectin is not unique to ricin and has been reported for hepatic galactose *N*-acetylgalactosamine receptor and macrophage mannose receptor (7, 8). The results also have implications in the role of the residual sugar-combining site of blocked ricin conjugates in intracellular trafficking (28) and in the genetic engineering of tumor cell selective ricin fusion toxins (29).

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LITERATURE CITED

- (1) Olsnes, S., and Pihl, A. (1973) Different biological properties of the two constituent peptide chains of ricin, a toxic protein inhibiting protein synthesis. *Biochemistry* 12, 3121–3126.
- (2) Rivera-Sagredo, A., Solis, D., Diaz-Maurino, T., Jimenez-Barbero, J., and Martin-Lomas, M. (1991) Studies on the molecular recognition of synthetic methyl β -lactoside analogs by ricin, a cytotoxic plant lectin. *Eur. J. Biochem.* 197, 217–228.
- (3) Olsnes, S., Sandvig, K., Refsnes, K., and Pihl, A. (1976) Rates of different steps involved in the inhibition of protein synthesis by the toxic lectins abrin and ricin. *J. Biol. Chem.* 251, 3985–3992.
- (4) Zentz, C., Frenoy, J., and Bourrillon, R. (1978) Binding of galactose and lactose to ricin: equilibrium studies. *Biochim. Biophys. Acta* 536, 18–26.
- (5) Baenziger, J., and Fiete, D. (1979) Structural determinants of Ricinus communis agglutinin and toxin specificity for oligosaccharides. *J. Biol. Chem.* 254, 9795–9799.
- (6) Sandvig, K., Olsnes, S., and Pihl, A. (1976) Kinetics of binding of the toxic lectins abrin and ricin to surface receptors on human cells. *J. Biol. Chem.* 251, 3977–3984.
- (7) Lee, Y. (1992) Biochemistry of carbohydrate-protein interaction. *FASEB J.* 6, 3193–3200.
- (8) Drickamer, K. (1995) Multiplicity of lectin-carbohydrate interactions. *Nat. Struct. Biol.* 2, 437–439.
- (9) Rutenber, E., and Robertus, J. (1991) Structure of ricin B chain at 2.5 Å resolution. *Proteins* 10, 260–269.
- (10) Hatakeyama, T., Yamasaki, N., and Funatsu, G. (1986) Identification of the tryptophan residue located at the low-affinity saccharide binding site of ricin D. *J. Biochem.* 100, 781–788.
- (11) Youle, R., Murray, J., and Neville, D. (1981) Studies on the galactose-binding site of ricin and the hybrid toxin Man6P-ricin. *Cell* 23, 551–559.
- (12) Lambert, J., McIntyre, G., Gauthier, M., Zullo, D., Rao, V., Steeves, R., Goldmacher, V., and Blattler, W. (1991) The galactose-binding sites of the cytotoxic lectin ricin can be chemically blocked in high yield with reactive ligands prepared by chemical modification of glycopeptides containing triantennary N-linked oligosaccharides. *Biochemistry* 30, 3234–3247.
- (13) Vitetta, E., and Yen, N. (1990) Expression and functional properties of genetically engineered ricin B chain lacking galactose-binding activity. *Biochim. Biophys. Acta* 1049, 151–157.
- (14) Wales, R., Richardson, P., Roberts, L., Woodland, H., and Lord, J. (1991) Mutational analysis of the galactose binding ability of recombinant ricin B chain. *J. Biol. Chem.* 266, 19172–19179.
- (15) Swimmer, C., Lehar, S., McCafferty, J., Chiswell, D., Blattler, W., and Guild, B. (1992) Phage display of ricin B chain and its single binding domains: system for screening galactose-binding mutants. *Proc. Natl. Acad. Sci. U.S.A.* 89, 3756–3760.
- (16) Frankel, A., Roberts, H., Afrin, L., Vesely, J., and Willingham, M. (1994) Expression of ricin B chain in *Spodoptera frugiperda*. *Biochem. J.* 303, 787–794.
- (17) Afrin, L., Gulick, H., Vesely, J., Willingham, M., and Frankel, A. (1994) Expression of oligohistidine-tagged ricin B chain in *Spodoptera frugiperda*. *Bioconjugate Chem.* 5, 539–546.
- (18) Frankel, A., Tagge, E., Chandler, J., Burbage, C., Hancock, G., Vesely, J., and Willingham, M. (1996) Characterization of single-site ricin toxin B chain mutants. *Bioconjugate Chem.* 7, 30–37.
- (19) Frankel, A., Tagge, E., Chandler, J., Burbage, C., and Willingham, M. (1996) Double-site ricin B chain mutants retain galactose binding. *Protein Eng.* 9, 371–379.
- (20) Schlossman, D., Withers, D., Welsh, P., Alexander, A., Robertus, J., and Frankel, A. (1989) Expression and characterization of mutants of ricin toxin A chain in *E. coli*. *Mol. Cell Biol.* 9, 5012–5021.
- (21) Olson, D., and Eckstein, F. (1990) High-efficiency oligonucleotide-directed plasmid mutagenesis. *Proc. Natl. Acad. Sci. U.S.A.* 87, 1451–1455.
- (22) Sanger, F., Nicklen, S., and Coulson, A. (1977) DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. U.S.A.* 74, 5463–5467.
- (23) Lehar, S., Pedersen, J., Kamath, R., Swimmer, C., Goldmacher, V., Lambert, J., Blattler, W., and Guild, B. (1995) Mutational and structural analysis of the lectin activity in binding domain 2 of ricin B chain. *Protein Eng.* 7, 1261–1266.
- (24) Araki, T., and Funatsu, G. (1987) The complete amino acid sequence of ricin B-chain of ricin E isolated from small-grain castor bean seeds. Ricin E is a gene recombination product of ricin D and *Ricinus communis* agglutinin. *Biochim. Biophys. Acta* 911, 191–200.
- (25) Olsnes, S., Saltvedt, E., and Pihl, A. (1974) Isolation and comparison of galactose-binding lectins from *Abrus precatorius* and *Ricinus communis*. *J. Biol. Chem.* 249, 803–810.
- (26) Houston, L., and Dooley, T. (1982) Binding of two molecules of 4-methylumbelliferyl galactose or 4-methylumbelliferyl *N*-acetylgalactosamine to the B chains of ricin and Ricinus communis agglutinin and to purified ricin B chain. *J. Biol. Chem.* 257, 4147–4151.
- (27) Grossbard, M., Lambert, J., Goldmacher, V., Blattler, W., and Nadler, L. (1992) Correlation between *in vivo* toxicity and preclinical *in vitro* parameters for the immunotoxin anti-B4-blocked ricin. *Cancer Res.* 52, 4200–4207.
- (28) Goldmacher, V., Lambert, J., and Blattler, W. (1992) The specific cytotoxicity of immunoconjugate containing blocked ricin is dependent on the residual binding capacity of blocked ricin: evidence that the membrane binding and A-chain translocation activities of ricin cannot be separated. *Biochem. Biophys. Res. Commun.* 183, 758–766.
- (29) Frankel, A., Tagge, E., Chandler, J., Burbage, C., Hancock, G., Vesely, J., and Willingham, M. (1995) IL2-ricin fusion toxin is selectively cytotoxic *in vitro* to IL2 receptor-bearing tumor cells. *Bioconjugate Chem.* 6, 666–672.

Design, Synthesis, and Evaluation of Mitomycin-Tethered Phosphorothioate Oligodeoxynucleotides

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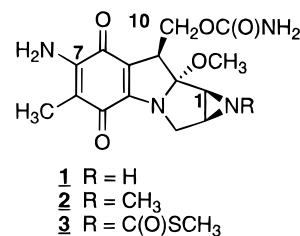
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Mitomycin C (**1**) is the prototypical bioreductive alkylating agent. Studies have shown that mitomycin C and its derivatives selectively alkylate guanine residues within di- and trinucleotide DNA sequences. This investigation sought to improve the selective DNA bonding properties of the mitomycins by coupling them with antisense oligodeoxynucleotides. Two procedures were developed that allowed the attachment of a phosphorothioate oligodeoxynucleotide containing a hexylamino spacer at the 5' terminus with a C(10)-activated mitomycin. In the first procedure, decarbamylation of **1** (NaOCH₃/benzene) afforded 10-decarbamoyletomycin C (**10**), which was treated with either dimethyl sulfate or methylthiochloroformate and base to yield 10-decarbamoyletomycin (**11**) and *N*(1a)-[(methylthio)carbonyl]-10-decarbamoyletomycin C (**12**), respectively. Activation of the C(10) site in **11** and **12** with 1,1'-carbonyldiimidazole or with 1,1'-thiocarbonyldiimidazole provided the *N*(1a)-substituted mitomycin 10-decarbamoyletomycin-10-*O*-carbonylimidazoles (**5**, **7**) and 10-decarbamoyletomycin-10-*O*-thiocarbonylimidazoles (**6**, **8**), respectively. Compounds **5**–**8** were reacted with glycine methyl ester hydrochloride (**17**) and base in both methylene chloride and aqueous buffered solutions to determine the ease and efficiency in which these C(10)-activated mitomycin derivatives coupled to amines. It was found that **5**–**8** all reacted with **17** in methylene chloride to give the coupled products **18**–**21** but that improved amine coupling yields in water were observed for the 10-decarbamoyletomycin-10-*O*-thiocarbonylimidazoles **6** and **8** as compared with the 10-decarbamoyletomycin-10-*O*-carbonylimidazoles **5** and **7**. This finding led to the coupling of the phosphorothioate oligodeoxynucleotide, H₂N(CH₂)₆–P(S)(OH)–GGCCCCGTG–GTGGCTCCAT (**22**) to **8**. Compound **22** complemented a 19-base sequence in the translation initiation region of the human *A-raf-1* gene. Use of excess **8** (28 equiv) with **22** gave only a 36% yield of the coupled product **23**, which proved difficult to separate from **22**. In the second procedure, phosphorothioate oligodeoxynucleotides that contained a hexylamino spacer at the 5' termini were coupled to 10-des(carbamoyloxy)-10-isothiocyanatoporphomycin (**9**). Compound **9** was prepared in four steps from **11**. Mesylation (methanesulfonyl chloride/pyridine) of **11** gave the C(10) mesylate **13**, which was then treated with NaN₃ (dimethylformamide, 90 °C) to give 10-des(carbamoyloxy)-10-azidoporphomycin (**14**). Catalytic reduction (PtO₂, H₂) of **14** in pyridine afforded C(10) amine **15**. Treatment of **15** with di-2-pyridyl thionocarbonate provided the desired 10-des(carbamoyloxy)-10-isothiocyanatoporphomycin (**9**). Compound **9** readily coupled with **17** and base in both methylene chloride and aqueous buffered solutions to give **25**. Use of the 5' hexylaminophosphorothioate oligodeoxynucleotides **32**–**35** in place of **17** gave the conjugated adducts **28**–**31**, respectively, in a 12% to near-quantitative yield. The products were purified by semipreparative HPLC. Antisense agents **28**–**31** were designed to target a 30-base-long region from the coding region of the human *FGFR1* gene. One adduct, **29**, reduced the number of FGFR1 receptors in human aortic smooth cells for bFGF on the cell surface, which suggested down-regulation of *FGFR1* gene expression. Further, **29** inhibited cultured human aortic smooth muscle cell proliferation and was less cytotoxic than porphomycin (**2**). The biological assay data suggest that the phosphorothioate oligodeoxynucleotide porphomycin conjugates may be more target selective and less toxic than either mitomycin or porphomycin and thus be promising therapeutic agents.

INTRODUCTION

In recent years we have witnessed technological advances that permit reagents to target specific sequences within single-stranded RNA and DNA (1–7) and double-helical DNA (8–15). One drug known to react with DNA is mitomycin C (**1**) (16). This compound is the prototypical bioreductive alkylating agent (17, 18). Drug function is expressed by quinone reduction permitting activation of the two DNA-bonding sites located at C(1) and C(10) (19). Alkylation occurs exclusively at guanine 2-amino residues located within the DNA minor groove (20). *In*

vitro reductive activation of mitomycin C (**1**) and por-



phomycin (**2**) leads to preferential alkylation of 5'CG* (G* = alkylation site)¹ sites (21–23), while *N*(1a)-[(methylthio)carbonyl]mitomycin C (**3**) selectively modifies 5'AG* and 5'TG* loci (24).

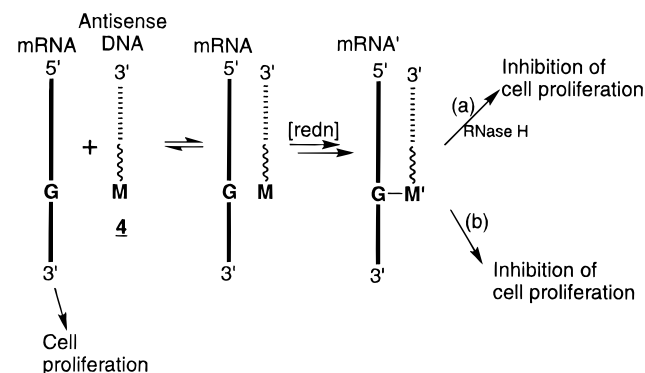
In this study, we sought to improve the selective DNA-bonding properties of the mitomycins by coupling them with antisense oligodeoxynucleotides. Recently, antisense oligodeoxynucleotides (25) have been developed to

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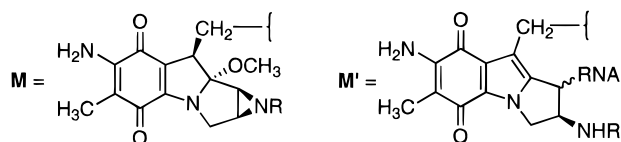
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Scheme 1. Proposed Pathway for Drug Function

where:

~~~~~ = linker



inhibit the expression of proteins, with emphasis given to their potential utility in antiviral (26) and anticancer therapy (27), and to inhibit restenosis and smooth muscle proliferation (28–33). The proposed mode of action of these agents involves complementary base pairing to a sense strand on mRNA leading to activation of RNase H activity, thereby removing a required RNA strand from the cellular machinery. We asked whether mitomycin–oligodeoxynucleotide conjugates **4** would provide an agent capable of destroying its cognate target mRNA strand by alkylating a specific guanine site upon reductive activation (Scheme 1, route b). This activity may potentiate the efficacy of these agents over previously reported antisense oligodeoxynucleotides that rely solely on RNase H-mediated destruction of their targets. We speculated that the tethered mitomycin (porfiromycin) **4** would exhibit reduced toxicity compared with the parent drug (16) since attachment of the mitomycin (porfiromycin) to the antisense oligodeoxynucleotide should diminish indiscriminate alkylation of DNA guanine residues. In this paper we report on the development of a novel strategy for the attaching of oligodeoxynucleotides to mitomycins and on their preliminary use to inhibit smooth muscle cell proliferation.

**EXPERIMENTAL PROCEDURES**

**Instrumentation, Materials, and Solvents.**  $^1\text{H}$  NMR and  $^{13}\text{C}$  NMR nuclear magnetic resonance spectra were recorded on either a Nicolet NT-300 or a General Electric QE-300 spectrometer. Chemical shifts are expressed in parts per million relative to the solvent employed, and coupling constants ( $J$  values) are given in hertz. Proton–proton interactions were confirmed in selective cases by the COSY spectrum. MS data for the nonoligonucleotide adducts were obtained on a Finnigan TSQ-70 triple quadrupole mass spectrometer under positive CI conditions by Dr. Mehdi Moini at the University of Texas at Austin. Mass spectrometric analyses of the phosphorothioate oligodeoxynucleotides and porfiromycin phosphorothioate oligodeoxynucleotide conjugates were conducted at UMIST (Manchester, U.K.) by Ms. Isabel

Riba and Dr. Simon J. Gaskell using ESI mass spectrometry on a Quattro tandem quadrupole mass spectrometer (upgraded to Quattro II specifications) supplied by VG Organic, Ltd. (Manchester, U.K.). Samples were prepared using the procedure of McCloskey (34). Samples were introduced to the electrospray interface in 2-propanol/water (2/1), and detection was in the negative ion mode. Each analyte gave a series of ions differing in the extent of deprotonation. The masses reported are those after transformation of the  $m/z$  data and correspond to the masses of the neutral phosphorothioate oligonucleotides. Melting points were determined with a Fisher-Johns melting point apparatus and are uncorrected. UV–vis absorption spectra were run on a Hitachi Model 100-80 spectrometer. FT-IR spectra were run on Mattson Galaxy Series FT-IR 5000 and Genesis infrared spectrophotometers, and absorption values are expressed in wavenumbers ( $\text{cm}^{-1}$ ). pH measurements were determined with either a Radiometer PHM 26 or a PHM 84 research meter equipped with a Radiometer GK 2320C combination glass electrode, which was standardized against aqueous buffer solutions.

Analytical HPLC analyses were conducted with the following Waters Associates units: 510 A pump, 510 B pump, Model 680 gradient controller, Model 490 multi-wavelength detector, U6K injector. The peak areas in the HPLC were determined with Waters Associates 740 and 745 and Hewlett-Packard 3392A integrators. The products were eluted from a  $\text{C}_{18}$   $\mu$ Bondapak (stainless steel) column ( $3.9 \times 300$  mm) using either of the two following linear gradient conditions: (a) program 1, 90% A (0.1 M triethylammonium acetate, pH 7.0) and 10% B (acetonitrile) isocratic for 5 min, then from 90% A and 10% B to 45% A and 55% B in 30 min; (b) program 2, 100% A (3 mM triethylammonium phosphate, pH 4.7) and 0% B (3 mM triethylamine in acetonitrile) to 50% A and 50% B in 25 min. In both cases, the column was fitted with a  $\mu$ Bondapak Guardpak precolumn and a flow rate of 1 mL/min was used. The eluent was monitored at 260, 313, and 365 nm. The HPLC solvents were filtered (aqueous solution with Millipore HA, 0.45  $\mu\text{m}$ ; acetonitrile with Millipore FH, 0.5  $\mu\text{m}$ ) and degassed before utilization. For the purification of mitomycin-conjugated phosphorothioate oligodeoxynucleotides a Rainin HPLC system (flow rate 2.0 mL/min) connected to a  $\text{C}_8$  reversed-phase semipreparative column (5  $\mu\text{m}$ , 25 cm, 10 mm ID) was used employing program 1 during which the aqueous triethylammonium acetate solution was maintained at 0.025 M.

All water used for the reactions was of HPLC grade. Mitomycin C was purchased from Janssen Chimica (Spectrum Chemical Manufacturing Corp., New Brunswick, NJ). HPLC-purified phosphorothioate oligodeoxynucleotides **32–35** (>90% purity) were purchased from Oligos Etc. (Wilsonville, OR). Tetrahydrofuran and benzene were both distilled from Na metal and benzophenone, methylene chloride was distilled from  $\text{P}_2\text{O}_5$ , and ethylene glycol dimethyl ether was distilled from  $\text{LiAlH}_4$ . All other solvents were of the best commercial grade available and used without further purification. Thin-layer chromatography and thick-layer chromatography were run on precoated silica gel GHLF microscope slides (2.5  $\times$  10 cm; Analtech No. 21521) and silica gel GF preparative uniplates (20  $\times$  20 cm; Analtech No. 02013).

**Preparation of *N*(1a)-[(Methylthio)carbonyl]-10-decarbamoylemitomycin C (12).** To an anhydrous ethylene glycol dimethyl ether solution (3 mL) containing **10** (35, 36) (9.0 mg, 0.03 mmol) and triethylamine (5.2  $\mu\text{L}$ , 0.04 mmol) was added methyl chlorothioformate (4.1 mg, 3.2  $\mu\text{L}$ , 0.04 mmol) under Ar. The reaction mixture

<sup>1</sup> Abbreviations: C, cytosine; G, guanine; A, adenine; T, thymine; CI, chemical ionization; ESI, electrospray ionization; PBS, phosphate-buffered saline; HASMC, human aortic smooth muscle cells.

was stirred at room temperature (1 h), and the solvent was removed under reduced pressure. The reaction mixture was purified by TLC (10% methanol/chloroform) to afford **12** (10.0 mg, 89%) as a purple solid: mp 82–83 °C; HPLC  $t_R$  (program 2) 24.1 min, (program 1) 24.5 min;  $R_f$  0.76 (10% CH<sub>3</sub>OH/CHCl<sub>3</sub>); UV-vis (CH<sub>3</sub>OH)  $\lambda_{max}$  360 nm; <sup>1</sup>H NMR (CDCl<sub>3</sub>)  $\delta$  1.78 [s, C(6)CH<sub>3</sub>], 2.32 [s, N(1a)C(O)SCH<sub>3</sub>], 3.21 [s, C(9a)OCH<sub>3</sub>], 3.36 [dd,  $J$  = 1.8, 4.7 Hz, C(2)H], 3.48–3.50 [m, C(9)H, overlapped], 3.51 [m, C(3)-H <sub>$\alpha$</sub> , overlapped], 3.54–3.56 [m, C(1)H, overlapped], 3.93–3.96 [m, C(10)H H'], 4.12 [dd,  $J$  = 8.4, 10.7 Hz, C(10)HH'], 4.46 [d,  $J$  = 13.1 Hz, C(3)H <sub>$\beta$</sub> ], 5.24 [br, C(7)NH<sub>2</sub>]; <sup>13</sup>C NMR (CDCl<sub>3</sub>) 7.9, 12.9, 42.1, 43.8, 45.5, 48.8, 49.7, 61.8, 105.0, 105.4, 113.8, 147.2, 154.4, 176.8, 178.3, 180.3 ppm; MS (+CI),  $m/e$  (rel intensity) 366 [M + 1, 100]<sup>+</sup>, 348 (223), 304 (9), 154 (55);  $M_r$  (+CI) 366.112 25 [M + 1]<sup>+</sup> (calcd for C<sub>16</sub>H<sub>20</sub>N<sub>3</sub>O<sub>5</sub>S, 366.112 36).

**Preparation of N-[10-Decarbamoyl-10-O-carbonylporfiromycin]imidazole (5).** To a stirred methylene chloride solution (1 mL) of **11** (35, 37) (6.8 mg, 0.02 mmol) and 4-(dimethylamino)pyridine (1.3 mg, 0.01 mmol) was added 1,1'-carbonyldiimidazole (33 mg, 0.20 mmol). The reaction mixture was stirred at room temperature (19 h), and then the solvent was removed under reduced pressure to give a crude purple solid. Purification by TLC (10% methanol/chloroform) afforded **5** (4.0 mg, 45%) as a purple solid: mp 94–95 °C; HPLC  $t_R$  (program 2) 30.3 min;  $R_f$  0.47 (10% CH<sub>3</sub>OH/CHCl<sub>3</sub>); UV-vis (CH<sub>3</sub>OH)  $\lambda_{max}$  360 nm; <sup>1</sup>H NMR (CDCl<sub>3</sub>)  $\delta$  1.77 [s, C(6)CH<sub>3</sub>], 2.19–2.21 [m, C(2)H, overlapped], 2.24 [s, N(1a)CH<sub>3</sub>], 2.29–2.31 [m, C(1)H], 3.20 [s, C(9a)OCH<sub>3</sub>], 3.49 [dd,  $J$  = 2.0, 13.0 Hz, C(3)H <sub>$\alpha$</sub> ], 3.72 [dd,  $J$  = 5.0, 10.4 Hz, C(9)H], 4.23 [d,  $J$  = 13.0 Hz, C(3)H <sub>$\beta$</sub> ], 4.69 [t,  $J$  = 10.4 Hz, C(10)H H'], 5.04 [dd,  $J$  = 5.0, 10.4 Hz, C(10)HH'], 5.20 [br, C(7)NH<sub>2</sub>], 7.10 [s, imidazole C(4)H], 7.50 [s, imidazole C(5)H], 8.22 [s, imidazole C(2)H]; <sup>13</sup>C NMR (CDCl<sub>3</sub>) 7.9, 42.7, 42.9, 43.2, 45.8, 49.7, 49.8, 65.5, 105.2, 105.7, 109.5, 117.2, 130.8, 137.2, 147.3, 148.3, 154.8, 175.6, 178.4 ppm; MS (+CI),  $m/e$  (rel intensity) 218 (52), 186 (12), 129 (100), 115 (30), 111 (42), 106 (12);  $M_r$  (+CI) 400.160 79 [M + 1]<sup>+</sup> (calcd for C<sub>19</sub>H<sub>22</sub>N<sub>5</sub>O<sub>5</sub>, 400.162 09).

**Preparation of N-[10-Decarbamoyl-10-O-thiocarbonylporfiromycin]imidazole (6).** Using the preceding procedure and **11** (35, 37) (14 mg, 0.05 mmol), 4-(dimethylamino)pyridine (2.8 mg, 0.02 mmol), and 1,1'-thiocarbonyldiimidazole (40.9 mg, 0.23 mmol) afforded **6** (11.7 mg, 61%) as a purple solid: mp 89–90 °C; HPLC  $t_R$  (program 1) 35.1 min;  $R_f$  0.51 (10% CH<sub>3</sub>OH/CHCl<sub>3</sub>); UV-vis (CH<sub>3</sub>OH)  $\lambda_{max}$  360 nm; <sup>1</sup>H NMR (CDCl<sub>3</sub>)  $\delta$  1.77 [s, C(6)CH<sub>3</sub>], 2.16–2.17 [m, C(2)H, overlapped], 2.20 [s, N(1a)CH<sub>3</sub>], 2.28–2.30 [m, C(1)H], 3.22 [s, C(9a)OCH<sub>3</sub>], 3.49 [dd,  $J$  = 1.6, 13.1 Hz, C(3)H <sub>$\alpha$</sub> ], 3.82 [dd,  $J$  = 4.9, 10.4 Hz, C(9)H], 4.24 [d,  $J$  = 13.1 Hz, C(3)H <sub>$\beta$</sub> ], 4.91 [t,  $J$  = 10.4 Hz, C(10)H H'], 5.18 [dd,  $J$  = 4.9, 10.4 Hz, C(10)-HH'], 5.24 [br, C(7)NH<sub>2</sub>], 7.07 [s, imidazole C(4)H], 7.73 [s, imidazole C(5)H], 8.46 [s, imidazole C(2)H]; <sup>13</sup>C NMR (CDCl<sub>3</sub>) 7.9, 42.4, 43.0, 43.3, 45.8, 49.8, 49.9, 70.7, 105.3, 105.8, 109.6, 117.9, 131.0, 136.9, 147.2, 155.0, 175.7, 178.4, 183.5 ppm; MS (+CI),  $m/e$  (rel intensity) 380 (6), 306 (6), 288 [M<sup>+</sup> – OC(S)imidazole, 100];  $M_r$  (+CI) 416.138 46 [M + 1]<sup>+</sup> (calcd for C<sub>19</sub>H<sub>22</sub>N<sub>5</sub>O<sub>4</sub>S, 416.139 25).

**Preparation of N-[10-Decarbamoyl-10-O-carbonyl-[N(1a)-(methylthio)carbonyl]mitomycin]imidazole (7).** Utilizing the procedure for **5** and anhydrous ethylene glycol dimethyl ether (2 mL) as the solvent, **12** (14.8 mg, 0.04 mmol) and 4-(dimethylamino)pyridine (2.5 mg, 0.02 mmol) were treated with 1,1'-carbonyldiimidazole (33.0 mg, 0.20 mmol) to give **7** (9.0 mg, 82%) as a purple solid: mp 94–95 °C; HPLC  $t_R$  (program 2) 29.8 min;  $R_f$  0.42 (10% CH<sub>3</sub>OH/CHCl<sub>3</sub>); UV-vis (CH<sub>3</sub>OH)  $\lambda_{max}$

360 nm; <sup>1</sup>H NMR (CDCl<sub>3</sub>)  $\delta$  1.79 [s, C(6)CH<sub>3</sub>], 2.31 [s, N(1a)C(O)SCH<sub>3</sub>], 3.21 [s, C(9a)OCH<sub>3</sub>], 3.37 [dd,  $J$  = 1.5, 4.5 Hz, C(2)H], 3.48 [d,  $J$  = 4.5 Hz, C(1)H], 3.57 [dd,  $J$  = 1.5, 13.4 Hz, C(3)H <sub>$\alpha$</sub> ], 3.82 [dd,  $J$  = 5.4, 10.9 Hz, C(9)H], 4.48 [d,  $J$  = 13.4 Hz, C(3)H <sub>$\beta$</sub> ], 4.56 [t,  $J$  = 10.9 Hz, C(10)-HH'], 5.15 [dd,  $J$  = 5.4, 10.9 Hz, C(10)HH'], 5.15 [br, C(7)-NH<sub>2</sub>], 7.09 [s, imidazole C(4)H], 7.52 [s, imidazole C(5)H], 8.24 [s, imidazole C(2)H]; <sup>13</sup>C NMR (CDCl<sub>3</sub>) 7.9, 12.9, 42.1, 42.2, 43.8, 48.9, 49.9, 64.8, 105.1, 105.4, 109.8, 117.3, 130.6, 137.3, 147.0, 148.2, 154.2, 175.9, 178.2, 179.6 ppm; MS (+CI),  $m/e$  (rel intensity) 460 [M + 1, 100]<sup>+</sup>, 366 (28), 348 (29), 154 (18);  $M_r$  (+CI) 460.128 93 [M + 1]<sup>+</sup> (calcd for C<sub>20</sub>H<sub>22</sub>N<sub>5</sub>O<sub>6</sub>S, 460.129 08).

**Preparation of N-[10-Decarbamoyl-10-O-thiocarbonyl-[N(1a)-(methylthio)carbonyl]mitomycin]imidazole (8).** Utilizing the procedure for **5** and anhydrous ethylene glycol dimethyl ether (2 mL) as the solvent, **12** (24.0 mg, 0.07 mmol) and 4-(dimethylamino)pyridine (4.0 mg, 0.03 mmol) were treated with 1,1'-thiocarbonyldiimidazole (58.5 mg, 0.33 mmol) to give **8** (25 mg, 80%) as a purple solid: mp 92–93 °C; HPLC  $t_R$  (program 1) 38.2 min;  $R_f$  0.56 (10% CH<sub>3</sub>OH/CHCl<sub>3</sub>); UV-vis (CH<sub>3</sub>OH)  $\lambda_{max}$  360 nm; <sup>1</sup>H NMR (CDCl<sub>3</sub>)  $\delta$  1.79 [s, C(6)CH<sub>3</sub>], 2.26 [s, N(1a)C(O)SCH<sub>3</sub>], 3.22 [s, C(9a)OCH<sub>3</sub>], 3.37 [dd,  $J$  = 1.5, 4.6 Hz, C(2)H], 3.47 [d,  $J$  = 4.6 Hz, C(1)H], 3.56 [dd,  $J$  = 1.5, 13.5 Hz, C(3)H <sub>$\alpha$</sub> ], 3.93 [dd,  $J$  = 4.8, 10.9 Hz, C(9)H], 4.48 [d,  $J$  = 13.5 Hz, C(3)H <sub>$\beta$</sub> ], 4.78 [t,  $J$  = 10.9 Hz, C(10)HH'], 5.23 [br, C(7)NH<sub>2</sub>], 5.49 [dd,  $J$  = 4.8, 10.9 Hz, C(10)HH'], 7.06 [s, imidazole C(4)H], 7.75 [s, imidazole C(5)H], 8.47 [s, imidazole C(2)H]; <sup>13</sup>C NMR (CDCl<sub>3</sub>) 7.9, 13.0, 42.2, 42.6, 43.8, 48.9, 49.9, 69.4, 105.4, 105.5, 109.8, 118.1, 130.8, 137.0, 146.9, 154.4, 176.0, 178.2, 179.6, 183.4 ppm; MS (+CI),  $m/e$  (rel intensity) 348 [M<sup>+</sup> – OC(S)imidazole];  $M_r$  (+CI) 476.105 64 [M + 1]<sup>+</sup> (calcd for C<sub>20</sub>H<sub>22</sub>N<sub>5</sub>O<sub>5</sub>S<sub>2</sub>, 476.106 24).

**Preparation of 10-Des(carbamoyloxy)-10-azidoporfiromycin (14).** A dimethylformamide (1 mL) solution of **13** (35, 38–40) (6.6 mg, 0.017 mmol) and NaN<sub>3</sub> (9.1 mg, 0.14 mmol) was heated (~90 °C) under Ar (1 h), and then the solution was diluted with chloroform (~10 mL) and filtered through a fritted funnel. The insoluble materials were washed with chloroform and partially concentrated *in vacuo*. The residue was purified by TLC (diethyl ether) to give **14** as a dark brown solid (2.6 mg, 45%); HPLC  $t_R$  28.1 min (program 1);  $R_f$  0.43 (ether); IR (KBr) 3437, 3329, 2100, 1601, 1555 cm<sup>-1</sup>; UV-vis (CH<sub>3</sub>OH)  $\lambda_{max}$  358 nm; <sup>1</sup>H NMR (CDCl<sub>3</sub>)  $\delta$  1.27 [s, C(6)CH<sub>3</sub>], 2.15 [overlapped dd, C(2)H], 2.27 [s, N(1a)CH<sub>3</sub>], 2.42 [d,  $J$  = 4.7 Hz, C(1)H], 3.17 [s, C(9a)OCH<sub>3</sub>], 3.46 [dd,  $J$  = 2.2, 12.9 Hz, C(3)H <sub>$\alpha$</sub> ], 3.40–3.54 [m, C(9)H and C(10)-HH'], 4.16 [d,  $J$  = 11.5 Hz, C(10)HH'], 4.17 [d,  $J$  = 12.9 Hz, C(3)H <sub>$\beta$</sub> ], 5.23 [br, C(7)NH<sub>2</sub>]; <sup>13</sup>C NMR (CDCl<sub>3</sub>) 7.9, 42.7, 43.3, 43.4, 46.0, 48.9, 49.7, 49.8, 105.2, 105.9, 110.7, 147.2, 154.5, 175.6, 178.5 ppm; MS (+CI),  $m/e$  (rel intensity) 331 [M + 1, 93]<sup>+</sup>, 330 [M, 100]<sup>+</sup>;  $M_r$  (+CI) 331.150 96 [M + 1]<sup>+</sup> (calcd for C<sub>15</sub>H<sub>19</sub>N<sub>6</sub>O<sub>3</sub>, 331.151 86), 330.143 11 [M]<sup>+</sup> (calcd for C<sub>15</sub>H<sub>18</sub>N<sub>6</sub>O<sub>3</sub>, 330.144 04).

**Preparation of 10-Des(carbamoyloxy)-10-amino-porfiromycin (15).** A pyridine solution (5 mL) containing **14** (6.5 mg, 0.016 mmol) and a catalytic amount of PtO<sub>2</sub> was stirred for 2 h under an atmosphere of H<sub>2</sub> at room temperature and then for 10 min in the air. Methanol (2–3 drops) was added to the mixture and stirred (3 min), and then the mixture was filtered through a Celite pad and the pad washed with a minimum amount of warm methanol. The filtrate was concentrated *in vacuo*, and the residue was purified by repetitive TLC (a, 10% methanol/chloroform; b, 20% methanol/chloroform) to obtain **15** as a dark brown solid (3.3 mg, 55%); HPLC  $t_R$  10.6 min (program 1);  $R_f$  0.24 (20% CH<sub>3</sub>OH/CHCl<sub>3</sub>); UV-vis (CH<sub>3</sub>OH)  $\lambda_{max}$  362 nm; <sup>1</sup>H NMR (pyri-

dine- $d_5$ )  $\delta$  2.02 [s, C(6)CH<sub>3</sub>], 2.15 [dd,  $J$  = 2.1, 4.8 Hz, C(2)H], 2.15 [s, N(1a)CH<sub>3</sub>], 2.59 [d,  $J$  = 4.8 Hz, C(1)H], 3.16 [s, C(9a)OCH<sub>3</sub>], 3.37 [t,  $J$  = 5.8 Hz, C(9)H], 3.50 [dd,  $J$  = 2.2, 12.9 Hz, C(3)H <sub>$\alpha$</sub> ], 3.47–3.60 [m, C(10)HH'], 3.71 [dd,  $J$  = 5.8, 12.3 Hz, C(10)HH'], 4.51 [d,  $J$  = 12.9 Hz, C(3)H <sub>$\beta$</sub> ], the C(7)NH<sub>2</sub> and C(10)NH<sub>2</sub> signals were not detected and are believed to be beneath the water peak; <sup>13</sup>C NMR (pyridine- $d_5$ ) 8.8, 42.7, 42.9, 43.1, 46.0, 49.4, 50.7, 104.1, 107.9, 114.2, 155.5, 177.4, 178.6 ppm (two carbons were not detected); MS (+CI),  $m/e$  (rel intensity) 306 [M + 1, 94]<sup>+</sup>, 305 [M, 10]<sup>+</sup>;  $M_r$  (+CI) 305.160 82 [M + 1]<sup>+</sup> (calcd for C<sub>15</sub>H<sub>20</sub>N<sub>4</sub>O<sub>3</sub>, 305.161 37).

**Preparation of 10-Des(carbamoyloxy)-10-acetamidoporfiromycin (16).** Amine **15** (1.0 mg, 3.40  $\mu$ mol) was stirred in pyridine- $d_5$  (0.5 mL) under Ar in an ice bath, and then acetic anhydride (4  $\mu$ L, 0.04 mmol) was injected and the ice bath removed. The reaction was stirred (30 min), and then the reaction was concentrated by an Ar stream and the residue purified by TLC (5% methanol/chloroform) to yield **16** as a brown solid (0.9 mg, 79%): HPLC  $t_R$  21.1 min (program 1);  $R_f$  0.32 (5% CH<sub>3</sub>OH/CHCl<sub>3</sub>); <sup>1</sup>H NMR (pyridine- $d_5$ )  $\delta$  2.02 [s, C(6)-CH<sub>3</sub>], 2.10 [s, COCH<sub>3</sub>], 2.16 [dd,  $J$  = 1.8, 4.8 Hz, C(2)H], 2.22 [s, N(1a)CH<sub>3</sub>], 2.48 [d,  $J$  = 4.8 Hz, C(1)H], 3.10 [s, C(9a)OCH<sub>3</sub>], 3.48 [dd,  $J$  = 1.8, 12.9 Hz, C(3)H <sub>$\alpha$</sub> ], 3.69 [t,  $J$  = 7.5 Hz, C(9)H], 3.93 [ddd,  $J$  = 2.1, 7.5, 13.1 Hz, C(10)-HH'], 4.43–4.53 [m, C(10)HH'], 4.56 [d,  $J$  = 12.9 Hz, C(3)-H <sub>$\beta$</sub> ], 8.56 [br t,  $J$  = 2.1 Hz, NHCO], the C(7)NH<sub>2</sub> was not detected and is believed to be beneath the water peak; <sup>13</sup>C NMR (pyridine- $d_5$ ) 8.8, 23.4, 40.3, 42.9, 43.3, 44.0, 45.7, 49.5, 50.7, 104.3, 107.6, 113.8, 148.8, 156.0, 169.8, 177.6, 178.4 ppm; MS (+CI),  $m/e$  (rel intensity) 347 [M + 1, 100]<sup>+</sup>, 314 (M – 32, 60)<sup>+</sup>;  $M_r$  (+CI) 347.172 13 [M + 1]<sup>+</sup> (calcd for C<sub>17</sub>H<sub>23</sub>N<sub>4</sub>O<sub>4</sub>, 347.171 93).

**Preparation of 10-Des(carbamoyloxy)-10-isothiocyanatoporfiromycin (9).** To a methylene chloride solution (2 mL) of di-2-pyridyl thionocarbonate (4.6 mg, 0.02 mmol) was added dropwise a methylene chloride solution (0.5 mL) of **15** (1.5 mg, 0.004 mmol), and then the reaction was stirred under Ar (2 h). The volatile solvent was removed by a stream of Ar, and the concentrated solution was purified by TLC (diethyl ether) to afford **9** as a dark brown solid (1.4 mg, 84%): HPLC  $t_R$  = 33.9 min (program 1);  $R_f$  0.38 (25% hexane/ether); UV-vis (CH<sub>3</sub>OH)  $\lambda_{max}$  362 nm; <sup>1</sup>H NMR (pyridine- $d_5$ )  $\delta$  2.02 [s, C(6)CH<sub>3</sub>], 2.21 [s, N(1a)CH<sub>3</sub>], 2.24 [dd,  $J$  = 2.1, 4.7 Hz, C(2)H], 2.57 [d,  $J$  = 4.7 Hz, C(1)H], 3.16 [s, C(9a)OCH<sub>3</sub>], 3.54 [dd,  $J$  = 2.1, 12.9 Hz, C(3)H <sub>$\alpha$</sub> ], 3.75 [dd,  $J$  = 4.0, 11.1 Hz, C(9)H], 4.06 [dd,  $J$  = 11.1, 14.3 Hz, C(10)-HH'], 4.39 [d,  $J$  = 12.9 Hz, C(3)H <sub>$\beta$</sub> ], 4.43 [dd,  $J$  = 4.0, 14.3 Hz, C(10)HH'], the C(7)NH<sub>2</sub> was not detected and is believed to be beneath the water peak; <sup>13</sup>C NMR (pyridine- $d_5$ ) 8.8, 42.9, 43.4, 43.5, 45.6, 45.9, 49.9, 50.5, 103.2, 106.3, 112.2, 128.7, 146.6, 155.3, 178.0, 181.2 ppm; MS (+CI),  $m/e$  (rel intensity) 347 [M + 1, 100]<sup>+</sup>, 346 [M, 53]<sup>+</sup>;  $M_r$  (+CI) 347.117 84 [M + 1]<sup>+</sup> (calcd for C<sub>16</sub>H<sub>19</sub>N<sub>4</sub>O<sub>3</sub>S, 347.117 79).

**Preparation of N-[10-Decarbamoyl-10-O-carbonylporfiromycin]glycine Methyl Ester (18).** To a stirred methylene chloride solution (1 mL) of **5** (1 mg, 0.003 mmol) and 4-(dimethylamino)pyridine (1.8 mg, 0.015 mmol) was added **17** (1.6 mg, 0.01 mmol). The reaction mixture was stirred at room temperature (25 h), and then the solvent was removed under reduced pressure to give a crude purple solid. Purification by TLC (10% methanol/chloroform) afforded **18** (0.6 mg, 57%) as a purple solid: mp 89–90 °C; HPLC  $t_R$  (program 2) 22.8 min;  $R_f$  0.42 (10% CH<sub>3</sub>OH/CHCl<sub>3</sub>); UV-vis (CH<sub>3</sub>OH)  $\lambda_{max}$  360 nm; <sup>1</sup>H NMR (CDCl<sub>3</sub>)  $\delta$  1.75 [s, C(6)CH<sub>3</sub>], 2.25 [s, N(1a)CH<sub>3</sub>], 2.24–2.28 [m, C(2)H, overlapped], 2.32 [d,  $J$

= 4.5 Hz, C(1)H], 3.18 [s, C(9a)OCH<sub>3</sub>], 3.44–3.50 [m, NHCH<sub>2</sub>CO<sub>2</sub>CH<sub>3</sub>], 3.59 [dd,  $J$  = 4.3, 10.8 Hz, C(9)H], 3.76 [s, CO<sub>2</sub>CH<sub>3</sub>], 3.99 [dd,  $J$  = 6.0, 14.0 Hz, C(3)H <sub>$\alpha$</sub> ], 4.20 [d,  $J$  = 14.0 Hz, C(3)H <sub>$\beta$</sub> ], 4.41 [t,  $J$  = 10.8 Hz, C(10)HH'], 4.71 [dd,  $J$  = 4.3, 10.8 Hz, C(10)HH'], 5.20 [br, C(7)NH<sub>2</sub>], 5.28 [br, NHCH<sub>2</sub>CO<sub>2</sub>CH<sub>3</sub>], the assignment was further confirmed by the COSY spectrum; <sup>13</sup>C NMR (CDCl<sub>3</sub>) 7.9, 42.6, 42.8, 43.2, 46.0, 49.7 (2 C), 52.3, 62.7, 105.1, 105.6, 110.1, 147.3, 154.6, 156.0, 170.5, 175.6, 178.6 ppm, the remaining one-carbon signal was not detected; MS (+CI),  $m/e$  (rel intensity) 421 [M + 1, 100]<sup>+</sup>, 288 (8), 256 (15), 154 (5);  $M_r$  (+CI) 421.173 29 [M + 1]<sup>+</sup> (calcd for C<sub>19</sub>H<sub>25</sub>N<sub>4</sub>O<sub>7</sub>, 421.172 32).

**Preparation of N-[10-Decarbamoyl-10-O-thiocarbonylporfiromycin]glycine Methyl Ester (19).** Utilizing the procedure for **18** and using **6** (9.5 mg, 0.02 mmol), 4-(dimethylamino)pyridine (16.8 mg, 0.14 mmol), and **17** (14.4 mg, 0.11 mmol) afforded **19** (8.4 mg, 84%): mp 83–84 °C; HPLC  $t_R$  (program 1) 29.6 min;  $R_f$  0.43 (10% CH<sub>3</sub>OH/CHCl<sub>3</sub>); UV-vis (CH<sub>3</sub>OH)  $\lambda_{max}$  360 nm; <sup>1</sup>H NMR (CDCl<sub>3</sub>)  $\delta$  1.75 [s, C(6)CH<sub>3</sub>], 2.25 [s, N(1a)CH<sub>3</sub>], 3.17 [dd,  $J$  = 1.8, 4.8 Hz, C(2)H], 3.19 [s, C(9a)OCH<sub>3</sub>], 3.46 [d,  $J$  = 4.8 Hz, C(1)H], 3.66–3.69 [m, NHCH<sub>2</sub>CO<sub>2</sub>CH<sub>3</sub>], 3.71–3.73 [m, C(3)H <sub>$\alpha$</sub> , partially overlapped], 4.35 [dd,  $J$  = 4.6, 10.7 Hz, C(9)H], 3.79 [s, CO<sub>2</sub>CH<sub>3</sub>], 4.71 [t,  $J$  = 10.7 Hz, C(10)HH'], 4.20 [d,  $J$  = 13.1 Hz, C(3)H <sub>$\beta$</sub> ], 4.96 [dd,  $J$  = 4.6, 10.7 Hz, C(10)HH'], 5.23 [br, C(7)NH<sub>2</sub>], 6.92 [br, NHCO<sub>2</sub>CH<sub>3</sub>], the assignment was further confirmed by the COSY spectrum; <sup>13</sup>C NMR (CDCl<sub>3</sub>) 7.9, 42.6, 42.7, 43.4, 46.0, 46.5, 49.7, 49.7, 52.5, 68.0, 105.0, 105.9, 109.9, 147.3, 154.7, 169.5, 175.6, 178.5, 190.3 ppm; MS (+CI),  $m/e$  (rel intensity) 306 [M<sup>+</sup> – C(S)N(H)CH<sub>2</sub>CO<sub>2</sub>CH<sub>3</sub>, 32], 288 (84), 256 (100);  $M_r$  (+CI) 437.148 80 [M + 1]<sup>+</sup> (calcd for C<sub>19</sub>H<sub>25</sub>N<sub>4</sub>O<sub>6</sub>S, 437.149 48).

**Preparation of N-[10-Decarbamoyl-10-O-thiocarbonylporfiromycin]glycine Methyl Ester (19) under Aqueous Conditions.** To a 20% methanolic/aqueous 0.05 M borax buffer solution ("pH" 8.0, 0.5 mL) containing **6** (1.6 mg, 0.004 mmol) was added **17** (2.4 mg, 0.02 mmol). The reaction was stirred at 30 °C and monitored by HPLC (program 2). After 24 h, the ratio of **19** to **6** was 18:1. Formation of **19** was confirmed by coinjection of an authentic sample of **19** with the reaction mixture in the HPLC.

**Preparation of N-[10-Decarbamoyl-10-O-carbonyl-[N(1a)-(methylthio)carbonyl]mitomycin] Methyl Ester (20).** Utilizing the procedure for **18** and using **7** (1.0 mg, 0.002 mmol), 4-(dimethylamino)pyridine (1.5 mg, 0.01 mmol), and **17** (1.4 mg, 0.01 mmol) gave **19** (0.5 mg, 48%): mp 102–103 °C (d); HPLC  $t_R$  (program 2) 27.4 min;  $R_f$  0.33 (10% CH<sub>3</sub>OH/CHCl<sub>3</sub>); UV-vis (CH<sub>3</sub>OH)  $\lambda_{max}$  360 nm; <sup>1</sup>H NMR (CDCl<sub>3</sub>)  $\delta$  1.76 [s, C(6)CH<sub>3</sub>], 2.29 [s, N(1a)C(O)SCH<sub>3</sub>], 3.18 [s, C(9a)OCH<sub>3</sub>], 3.33 [dd,  $J$  = 1.7, 4.8 Hz, C(2)H], 3.49 [d,  $J$  = 1.7 Hz, C(1)H], 3.54–3.57 [m, NHCH<sub>2</sub>CO<sub>2</sub>CH<sub>3</sub>], 3.65–3.69 [m, C(3)H <sub>$\alpha$</sub> , partially overlapped], 3.71 [dd,  $J$  = 4.6, 10.9 Hz, C(9)H], 3.76 [s, CO<sub>2</sub>CH<sub>3</sub>], 4.20 [t,  $J$  = 10.9 Hz, C(10)HH'], 4.46 [d,  $J$  = 13.5 Hz, C(3)H <sub>$\beta$</sub> ], 4.96 [dd,  $J$  = 4.6, 10.9 Hz, C(10)HH'], 5.30 [br, C(7)NH<sub>2</sub>], 5.41 [br, NHCO<sub>2</sub>CH<sub>3</sub>]; <sup>13</sup>C NMR (CDCl<sub>3</sub>) 7.9, 13.0, 41.9, 42.0, 42.7, 44.2, 48.7, 49.7, 52.3, 62.2, 105.1, 105.5, 110.4, 147.1, 154.0, 170.5, 175.8, 178.4, 179.9, 181.6 ppm; MS (+CI),  $m/e$  (rel intensity) 481 [M + 1, 6]<sup>+</sup>, 366 (10), 348 (35), 318 (27), 316 (100), 270 (22), 116 (11);  $M_r$  (+CI) 481.138 65 [M + 1]<sup>+</sup> (calcd for C<sub>20</sub>H<sub>25</sub>N<sub>4</sub>O<sub>8</sub>S, 481.139 31).

**Preparation of N-[10-Decarbamoyl-10-O-carbonyl-[N(1a)-(methylthio)carbonyl]mitomycin]glycine Methyl Ester (20) under Aqueous Conditions.** Mitomycin **7** (2.0 mg, 0.004 mmol) was added to a 20% methanolic/aqueous 0.05 M borax buffer solution ("pH"



8.0, 0.5 mL) containing **17** (2.7 mg, 0.02 mmol), and the resulting solution was stirred at room temperature (5 days). The progress of the reaction was monitored by HPLC (program 2). The HPLC analysis of the reaction mixture indicated the presence of five major peaks ( $t_R$  18.2, 25.1, 27.4, 29.6, 30.1 min). Two of these corresponded to **2** (18.2 min) and **12** (25.1 min) and were identified by coinjection of authentic samples with the reaction mixture in the HPLC. The peaks at 27.4 and 30.1 min have been tentatively assigned as **20** and **7**, respectively. The solvent was removed under reduced pressure to give a crude purple solid. Purification by preparative TLC (10% methanol/chloroform) afforded **20** (0.7 mg, 36%) as a purple solid: mp 102–103 °C (dec); HPLC  $t_R$  (program 2) 27.4 min;  $R_f$  0.33 (10% CH<sub>3</sub>OH/CHCl<sub>3</sub>); <sup>1</sup>H NMR (CDCl<sub>3</sub>)  $\delta$  1.76 [s, C(6)CH<sub>3</sub>], 2.30 [s, N(1a)C(O)SCH<sub>3</sub>], 3.19 [s, C(9a)OCH<sub>3</sub>], 3.33–3.40 [m, C(2)H], 3.51–3.52 [m, C(1)H], 3.55–3.56 [m, NHCH<sub>2</sub>CO<sub>2</sub>CH<sub>3</sub>], 3.61–3.68 [m, C(3)H <sub>$\beta$</sub> , partially overlapped], 3.70–3.75 [m, C(9)H], 3.77 [s, CO<sub>2</sub>CH<sub>3</sub>], 4.02–4.06 [m, C(10)-HH'], 4.47 [d,  $J$  = 13.5 Hz, C(3)H <sub>$\alpha$</sub> ], 4.96–5.01 [m, C(10)HH'], 5.18–5.30 [br, C(7)NH<sub>2</sub>], 5.32–5.39 [br, NHCO<sub>2</sub>CH<sub>3</sub>].

**Preparation of N-[10-Decarbamoyl-10-O-thiocarbonyl-N(1a)-(methylthio)carbonyl]mitomycin]-glycine Methyl Ester (21).** Utilizing the procedure for **18** and using **8** (14.0 mg, 0.03 mmol), 4-(dimethylamino)pyridine (21.6 mg, 0.18 mmol), and **17** (18.5 mg, 0.15 mmol) gave **21** (12.0 mg, 83%) as a purple solid: mp 95–96 °C; HPLC  $t_R$  (program 1) 35.2 min;  $R_f$  0.51 (10% CH<sub>3</sub>OH/CHCl<sub>3</sub>); UV-vis (MeOH)  $\lambda_{max}$  360 nm; <sup>1</sup>H NMR (CDCl<sub>3</sub>)  $\delta$  1.77 [s, C(6)CH<sub>3</sub>], 2.30 [s, N(1a)C(O)SCH<sub>3</sub>], 3.19 [s, C(9a)OCH<sub>3</sub>], 3.33–3.35 [m, C(2)H], 3.48–3.49 [m, C(1)H], 3.51–3.57 [m, NHCH<sub>2</sub>CO<sub>2</sub>CH<sub>3</sub>], 3.71–3.76 [m, C(3)H <sub>$\beta$</sub> , partially overlapped], 3.83–3.85 [m, C(9)H], 3.80 [s, CO<sub>2</sub>CH<sub>3</sub>], 4.35–4.40 [m, C(10)HH'], 4.44–4.51 [m, C(3)H <sub>$\alpha$</sub> ], 5.44 [dd,  $J$  = 4.9, 11.0 Hz, C(10)HH'], 5.28–5.29 [br, C(7)NH<sub>2</sub>], 6.98–7.01 [br, NHCO<sub>2</sub>CH<sub>3</sub>], the assignment was further confirmed by the COSY spectrum; <sup>13</sup>C NMR (CDCl<sub>3</sub>) 7.9, 13.0, 41.9, 42.2, 44.2, 46.6, 48.8, 49.8, 52.5, 67.0, 105.1, 105.5, 110.4, 147.1, 154.0, 169.5, 175.8, 178.4, 180.0, 190.1 ppm; MS (+CI),  $m/e$  (rel intensity) 497 [M + 1, 100]<sup>+</sup>, 348 (69), 316 (61), 182 (19);  $M_r$  (+CI) 497.116 38 [M + 1]<sup>+</sup> (calcd for C<sub>20</sub>H<sub>25</sub>N<sub>4</sub>O<sub>7</sub>S<sub>2</sub>, 497.116 46).

**Preparation of N-[10-Decarbamoyl-10-O-thiocarbonyl-N(1a)-(methylthio)carbonyl]mitomycin]-glycine Methyl Ester (21) under Aqueous Conditions.** To a 20% methanolic/aqueous 0.05 M borax buffer solution ("pH" 8.0, 0.5 mL) containing **8** (2.4 mg, 0.005 mmol) was added **17** (3.2 mg, 0.03 mmol). The reaction was stirred at 30 °C and monitored by HPLC (program 2). After 48 h, the complete consumption of **8** was observed with only the production of **21**. Formation of **21** was confirmed by coinjection of an authentic sample of **21** with the reaction mixture in the HPLC.

**Conjugation of the N-[10-Decarbamoyl-10-O-thiocarbonyl-N(1a)-(methylthio)carbonyl]mitomycin]-imidazole (8) to 5'-H<sub>2</sub>N(CH<sub>2</sub>)<sub>6</sub>-P(S)(OH)-GGC-CCCGTGGTGGCTCCAT (22) (Analytical Scale).** A stock solution (45 mM) of **8** (2.6 mg, 5.4  $\mu$ mol) in acetonitrile (120  $\mu$ L) was prepared, and then 10  $\mu$ L (646 nmol) of this solution was added to an aqueous solution (50  $\mu$ L) of **22** (22.7 nmol). The final volume of the reaction was adjusted to 100  $\mu$ L by the addition of an aqueous 0.2 M Tris·HCl solution (pH 8.3). The reaction mixture was vortexed and stored at 5–10 °C. After 24 h, the ratio of **23** to **22** was 0.6:1 (260 nm), and the ratio of **8**, **12**, and **10** was 11:27:1 (365 nm) (HPLC analysis). The identities of **8**, **12**, and **10** were verified by coinjection of authentic samples with the reaction mixture in the HPLC.

**Conjugation of 10-Des(carbamoyloxy)-10-isothiocyanatoporfirromycin (9) to Oligodeoxynucleotides 32–35.** HPLC-purified phosphorothioate oligodeoxynucleotides **32–35** (>90% purity) supplied as the ammonium salts were converted to the corresponding sodium salts by dissolution in aqueous 0.1 M NaOH, and then the solution was lyophilized. The residue was redissolved in water and then neutralized with an aqueous 0.1 M HCl solution and the final volume adjusted to 100  $\mu$ L with water. HPLC analyses of each phosphorothioate oligodeoxynucleotide prior to reaction showed only a single peak, and each was judged to be >90% pure. To an aqueous 0.2 M NaHCO<sub>3</sub> buffer solution (10  $\mu$ L, pH 9.0) was added an equal volume of the phosphorothioate oligodeoxynucleotide stock solution (**32–35**), and then a dimethylformamide solution (20  $\mu$ L) of **9** (860 nmol) was added. The solution was vortexed, centrifuged (4000 rpm, 1 min), and then allowed to stand at room temperature (24–30 h). HPLC analyses showed that the reaction gave the conjugated adduct, a small amount of the starting phosphorothioate oligodeoxynucleotide, and byproducts derived from **9**. The HPLC retention times of conjugated phosphorothioate oligodeoxynucleotides (**28–31**) were within 0.8 min of that of the starting phosphorothioate oligodeoxynucleotides (**32–35**). The conjugated product was isolated using semi-preparative HPLC (Rainin, flow rate 2.0 mL/min). The fractions containing the conjugated product were collected and combined, and then the volatile solvents were removed with a stream of N<sub>2</sub>. The remaining solution was lyophilized, and the light purple residue was redissolved in water (200  $\mu$ L) and lyophilized to remove the remaining triethylammonium acetate. The products were analyzed by HPLC, UV-vis spectroscopy, and electrospray mass spectrometry. The DNA/porfirromycin ratio was determined by UV-vis spectroscopy by measuring the relative absorbances at 260 and 360 nm, respectively. The  $\epsilon$  values for purines, pyrimidines, and porfirromycin at 260 nm were considered to be 14 000, 7000, and 12 000, respectively; the  $\epsilon$  values for porfirromycin and DNA at 360 nm were considered to be 23 000 and 0, respectively (41, 42).

**Preparation of 28.** Using **32** (158 nmol) gave **28** (158 nmol) in near quantitative yield: HPLC  $t_R$  (program 1) 21.6 min; UV-vis 260 nm:360 nm = 11.6:1 (calcd 10.9:1), which corresponds to 1.06 equiv of **32** to 1 equiv of **9**; MS (–ESI) 8511.1 [M + 1]<sup>+</sup> (calcd for **28**, 8510.6) [MS (–ESI) of **32**, 8163.3 [M]<sup>+</sup> (calcd for **32**, 8163.2)].

**Preparation of 29.** Using **33** (158 nmol) gave **29** (92 nmol, 58%): HPLC  $t_R$  (program 1) 21.9 min; UV-vis 260 nm:360 nm = 10.3:1 (calcd 10.5:1), which corresponds to 0.98 equiv of **33** to 1 equiv of **9**; MS (–ESI) 8486.5 [M + 1]<sup>+</sup> (calcd for **29**, 8486.6) [MS (–ESI) of **33**, 8139.4 [M + 1]<sup>+</sup> (calcd for **33**, 8139.2)].

**Preparation of 30.** Using **34** (149 nmol) gave **30** (111 nmol, 75%): HPLC  $t_R$  (program 1) 22.5 min; UV-vis 260 nm:360 nm = 11.0:1 (calcd 10.3:1), which corresponds to 1.07 equiv of **34** to 1 equiv of **9**; MS (–ESI) 8446.2 [M + 1]<sup>+</sup> (calcd for **30**, 8446.8) [MS (–ESI) of **34**, 8099.2 [M + 1]<sup>+</sup> (calcd for **34**, 8099.2)].

**Preparation of 31.** Using **35** (170 nmol) gave **31** (21 nmol, 12%): HPLC  $t_R$  (program 1) 23.8 min; UV-vis 260 nm:360 nm = 13.8:1 (calcd 10.3:1), which corresponds to 1.33 equiv of **35** to 1 equiv of **9**; MS (–ESI) 8462.2 [M + 2]<sup>+</sup> (calcd for **31**, 8461.5) [MS (–ESI) of **35**, 8113.9 [M]<sup>+</sup> (calcd for **35**, 8113.1)].

**Cell Binding Assay for bFGF (43).** [<sup>125</sup>I]bFGF was purchased from Biomedical Technologies Inc., Stoughton, MA. Human aortic smooth muscle cells (P3–P9; 9000 cells/well) were grown to 80–90% confluency in a 48-well

culture dish. The growth medium was removed, and cells were incubated at 37 °C for 24 h with 1  $\mu$ M inhibitor in binding buffer (25 mM Hepes, pH 7.4, 1 mg/mL BSA; 200  $\mu$ L/well). Cells were washed with 300  $\mu$ L of PBS and then incubated for 90 min at room temperature with [ $^{125}$ I]-bFGF in binding buffer. Nonspecific binding was determined by adding to a few wells 1000-fold excess of unlabeled human recombinant bFGF. Wells were then washed sequentially with 300  $\mu$ L of PBS, 300  $\mu$ L of heparin (250  $\mu$ g/mL in PBS), and again 300  $\mu$ L of PBS. The cells were lysed by incubation for 15 min at room temperature with 300  $\mu$ L of 1% Triton X-100 in water. The lysates were collected, and high-affinity binding was determined by counting the radioactivity in a Beckman LS 6000TA scintillation counter.

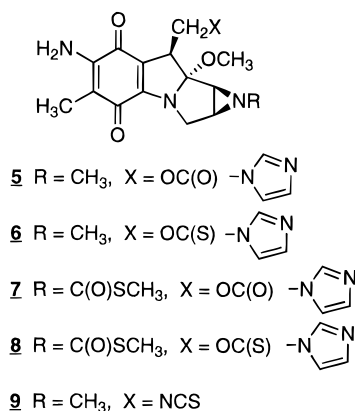
**Cell Proliferation Assay.** Human aortic smooth muscle cells were plated in 96-well plates (3000 cells/well) and grown for 24 h in Dulbecco's Modified Eagle's Medium containing 10% fetal bovine serum. The following day, wells were treated with inhibitors. Cell proliferation was quantitated 4 days later by counting cells in a Coulter counter.

## RESULTS AND DISCUSSION

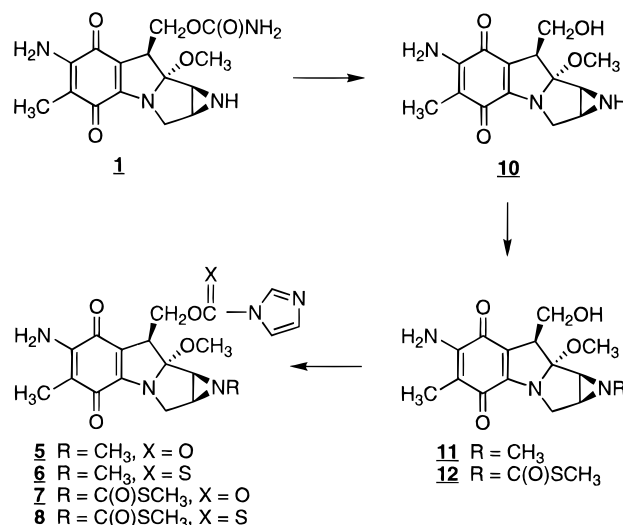
**A. Choice of Compounds and Target.** Mitomycins are multifunctional molecules (40). Although strategies to modify the C(7) (44–47) and N(1a) (48–50) loci exist, we chose to attach the antisense oligodeoxynucleotide to the C(10) site in the mitomycin (51). Preliminary molecular modeling experiments indicated that mitomycins tethered at this site would permit subsequent DNA bonding at C(1) with minimal distortion of the DNA duplex.<sup>2</sup> Unfortunately, few methods exist for the modification of the C(10) site (35, 40).

We established several criteria for synthesis of C(10) mitomycin–oligodeoxynucleotide conjugates. First, the coupling of the mitomycin with the oligodeoxynucleotide had to proceed efficiently in aqueous solutions. This led us to use a hexylamino spacer at the 5' terminus of the antisense DNA. Second, the mitomycin–oligodeoxynucleotide conjugates had to be sufficiently stable *in vitro* and *in vivo* so as to permit assessment as antisense reagents. Accordingly, we employed oligodeoxynucleotide units containing phosphorothioate units in place of the phosphodiester backbone to minimize exo- and endonuclease cleavage (52, 53). Third, the mitomycin–oligodeoxynucleotide conjugates had to be separable from the starting oligodeoxynucleotides to permit their purification and characterization.

These criteria led us, in part, to select 5–9 as substrates for DNA attachment. The porfiromycin substrates 5, 6, and 9 were designed to target mRNA



## Scheme 2. Synthesis of Mitomycins 5–8

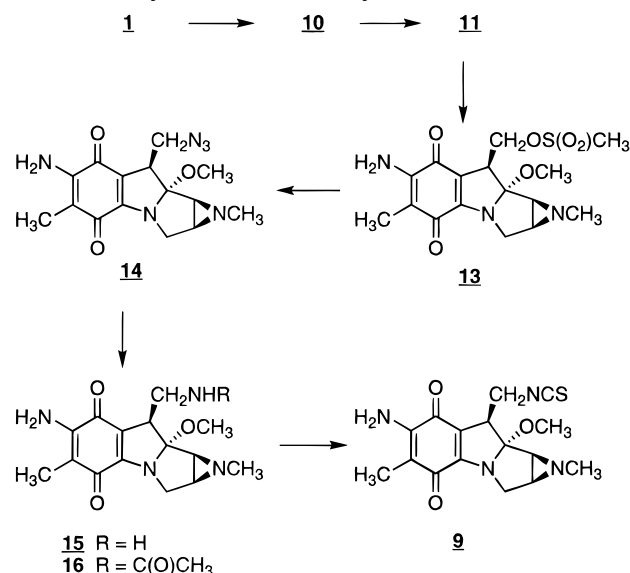


(21–23), whereas 7 and 8 were projected to react preferentially with mRNAs having an appropriately disposed 5'AG\* or 5'TG\* site (24).

We chose phosphorothioate oligodeoxynucleotides for the final mitomycin coupling reactions that were based on the sequence of human *FGFR1* gene because of the important role this gene plays in cellular proliferation, especially during vascular remodeling. Thus, it has been reported that there is an upregulation of high-affinity receptors for bFGF in proliferating vascular smooth muscle cells (54) and that the predominant form of the receptor expressed by such cells is type I (55). Furthermore, *FGFR1* expression is upregulated *in vivo* during vascular remodeling (56, 57). Finally, inhibition of the expression of *FGFR1* has been shown to result in decreased proliferation in a variety of cell types (58–60).

**B. Synthesis.** Separate strategies were developed for the synthesis of 5–8 and 9. These compounds served as mitomycin candidates for coupling to tethered hexylamine DNAs. In the first strategy, decarbamylation of mitomycin C (1) with sodium methoxide in benzene afforded 10-decarbamoylemitomycin C (35, 36) (10) (Scheme 2). Functionalization of the *N*-aziridine group with dimethyl sulfate or methyl chlorothioformate and base yielded the *N*-substituted mitomycins 11 and 12, respectively. The *N*-aziridine substituted 10-decarbamoylemitomycins were then reacted with 1,1'-carbonyldiimidazole or 1,1'-thiocarbonyldiimidazole to yield 5–8.

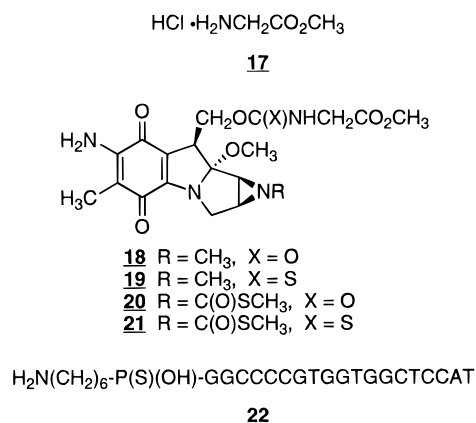
The second approach required that we replace the C(10) hydroxy group in 10-decarbamoylemitomycin (35, 36) (11) with an amino unit (Scheme 3). This interchange was accomplished in three steps in a 21% overall yield. First, the C(10) site in 11 was activated by conversion to the C(10) mesylate 13 (35, 38–40) and then treated with NaN<sub>3</sub> in dimethylformamide at 90 °C to give C(10) azide 14 in a 68% isolated yield. Reduction of the C(10) azide group in 14 to the amine 15 in the final step without concomitant reductive activation and destruction of the mitosane ring system was accomplished by catalytic reduction (PtO<sub>2</sub>, H<sub>2</sub>) in pyridine followed by air oxidation. This method took advantage of Danishefsky and co-workers' discovery that mitomycin quinone reduction in pyridine proceeds without loss of methanol at C(9) and C(9a) (61). Amine 15 was characterized as the free base and by conversion to the *N*-acetyl derivative 16. Conversion of 15 to 10-des(carbamoyloxy)-10-isothiocyanatopor-

**Scheme 3. Synthesis of Mitomycin 9**

firomycin (**9**) occurred smoothly with di-2-pyridyl thion-carbonate (DPT).

These procedures provided the C(10)-activated mitomycins **5–9**. All compounds were fully characterized, and their spectroscopic properties were consistent with those of the proposed structures. Table 1 lists the key <sup>1</sup>H and <sup>13</sup>C NMR data for **5–9** along with key synthetic precursors and derivatives. As anticipated, placement of a carbonyl or a thiocarbonylimidazole unit at C(10) led to a downfield shift in the <sup>1</sup>H NMR for the C(10) methylene protons compared with the starting alcohols **11** and **12** (62).

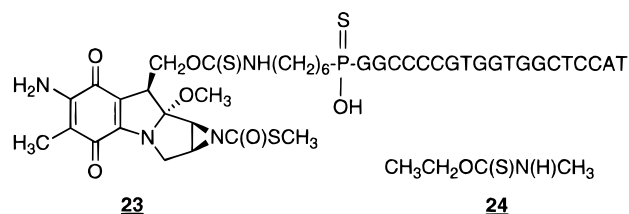
**C. Preliminary Conjugation Studies.** Prior to treatment of C(10)-activated mitomycins **5–9** with the hexyl-amino-tethered DNAs, we tested the ease and efficiency of the coupling transformation by reaction with glycine methyl ester hydrochloride (**17**) in aqueous buffered solutions. Authentic samples of the C(10)-coupled carbamates **18** and **20** and thiocarbamates **19** and **21** were prepared by the addition of **17** (3.3–5.5 equiv) to methylene chloride solutions containing **5–8** and 4-(dimethylamino)pyridine. We observed that treatment of an aqueous solution (pH 9.0) of **5** with **17** (5 equiv) gave 10-decarbamoylethylporfiromycin (**11**) as the major product rather than **18** (HPLC analysis) (data not shown). Correspond-



ingly, addition of **17** (5 equiv) to **7** in a 20% methanolic/aqueous borax solution ("pH" 8.0) furnished the desired adduct **20** in a 36% isolated yield. Improved coupling yields were observed for the two 10-decarbamoylethyl-10-O-thiocarbonylimidazoles **6** and **8**. Both **6** and **8** reacted

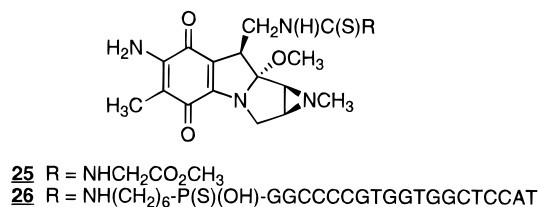
with **17** (5–6 equiv) in 20% methanolic/aqueous borax solutions ("pH" 8.0) to give **19** and **21**, respectively, in high yields (>95%, HPLC analysis).

The efficiency of **6** and **8** coupling transformations led us to react **8** with the hexylamino-tethered DNA, H<sub>2</sub>N-(CH<sub>2</sub>)<sub>6</sub>-P(S)(OH)-GGCCCCGTGGTGGCTCCAT (**22**), in our trial DNA coupling reactions. This 19-mer phosphorothioate oligodeoxynucleotide segment in **22** complements a 19-base sequence in the translation initiation region of the human *A-raf-1* gene (63). This gene product has been linked to mammalian cell proliferation. Preliminary optimization reactions indicated that use of excess amounts of **8** (28 equiv) in 10% acetonitrile/0.08 M Tris·HCl aqueous buffered solutions maintained at "pH" 8.3 gave a 36% yield of a new DNA product along with the major mitomycin byproducts **10** and **12** (HPLC analysis). The new DNA compound has been tentatively assigned as **23** on the basis of its relative absorption values at 260 and 365 nm (41). Repetition of this reaction on a semipreparative scale and separation of the product mixture by preparative HPLC satisfactorily removed the coupled mitomycin–DNA adduct from the mitomycin byproducts (i.e., **10**, **12**) but did not completely separate the product from the starting phosphorothioate oligodeoxynucleotide **22** (UV–vis analysis) (data not shown). We have attributed the observed low yield of **23** to the competitive hydrolytic cleavage of the oxy(thiocarbonyl)-imidazole moiety in **8** rather than to the instability of the product **23** under the study conditions. This assump-



tion is based, in part, upon the identification of **10** and **12** as major byproducts in the reaction and upon our observation that *N*-methyl ethylthiocarbamate (**24**) did not undergo hydrolysis in D<sub>2</sub>O solutions maintained at 22 °C for 4 days (NMR analysis, data not shown).<sup>3</sup>

The low phosphorothioate oligodeoxynucleotide coupling yields observed for *N*-[10-decarbamoylethyl-10-O-thiocarbonyl-*N*-(1a)-(methylthio)carbonyl]mitomycin]imidazole (**8**) and our inability to satisfactorily purify the DNA conjugates led us to use 10-des(carbamoyloxy)-10-isothiocyanatoporphiromycin (**9**) in place of **8**. Isothiocyanates can be coupled to amines in aqueous solutions (64). Accordingly, reaction of **9** with **17** in aqueous Tris·HCl solutions gave **25** in a 61% yield (HPLC analysis).



The DNA coupling conditions were defined by HPLC monitoring of a series of analytical reactions of **9** with **22** in which the DNA concentration, ratio of **9** to **22**, buffer composition (concentration, "pH", organic cosolvent), and reaction temperature were systematically

<sup>3</sup> Unpublished results (N.H. and H.K.).

**Table 1.** Select  $^1\text{H}$  and  $^{13}\text{C}$  NMR Data for Functionalized Mitomycins

| compd                  | $^1\text{H}$ NMR <sup>a</sup> |                            |                      | $^{13}\text{C}$ NMR <sup>b</sup> |                   |
|------------------------|-------------------------------|----------------------------|----------------------|----------------------------------|-------------------|
|                        | C(9)H                         | C(10)HH'                   | C(10)HH'             | C(9)                             | C(10)             |
| <b>5</b> <sup>c</sup>  | 3.72 (dd; 5.0, 10.4)          | 4.69 (t; 10.4)             | 5.04 (dd; 5.0, 10.4) | 43.2                             | 65.5              |
| <b>6</b> <sup>c</sup>  | 3.82 (dd; 4.9, 10.4)          | 4.91 (t; 10.4)             | 5.18 (dd; 4.9, 10.4) | 43.3                             | 70.7              |
| <b>7</b> <sup>c</sup>  | 3.82 (dd; 5.4, 10.9)          | 4.56 (t; 10.9)             | 5.15 (dd; 4.8, 10.9) | 43.8                             | 64.8              |
| <b>8</b> <sup>c</sup>  | 3.93 (dd; 4.8, 10.9)          | 4.78 (t; 10.9)             | 5.49 (dd; 4.8, 10.9) | 43.8                             | 69.4              |
| <b>9</b> <sup>d</sup>  | 3.75 (dd; 4.09, 11.1)         | 4.06 (dd; 11.1, 14.3)      | 4.43 (dd; 4.0, 14.3) | 45.9 <sup>e</sup>                | 45.6 <sup>e</sup> |
| <b>10</b> <sup>c</sup> | 3.37–3.40 (m)                 | 4.01–4.03 (br)             | 4.61–4.64 (br)       |                                  |                   |
| <b>11</b> <sup>c</sup> | 3.34 (t; 5.1)                 | 3.96–3.97 (br)             | 4.12 (dd; 8.4, 10.7) | 45.2                             | 61.4              |
| <b>12</b> <sup>c</sup> | 3.48–3.50 (m)                 | 3.93–3.96 (m)              | 4.12 (dd; 8.4, 10.7) | 45.5                             | 61.8              |
| <b>13</b> <sup>c</sup> | 3.63 (dd; 4.1, 10.9)          | 4.53 (dd; 9.7, 10.9)       | 4.82 (dd; 4.1, 9.7)  | 45.5                             | 66.4              |
| <b>14</b> <sup>c</sup> | 3.40–3.54 (m)                 | 3.40–3.54 (m)              | 4.16 (d; 11.5)       | 46.0                             | 48.9              |
| <b>15</b> <sup>d</sup> | 3.37 (t; 5.8)                 | 3.47–3.60 (m)              | 3.71 (dd; 5.8, 12.3) | 46.0                             | 48.9              |
| <b>16</b> <sup>d</sup> | 3.69 (t; 7.5)                 | 3.93 (ddd; 2.1, 7.5, 13.1) | 4.42–4.54 (m)        | 44.0 <sup>e</sup>                | 45.7 <sup>e</sup> |
| <b>18</b> <sup>c</sup> | 3.59 (dd; 4.3, 10.8)          | 4.41 (t; 10.8)             | 4.71 (dd; 4.3, 10.8) | 43.2                             | 62.7              |
| <b>19</b> <sup>c</sup> | 3.66–3.77 (m)                 | 4.71 (t; 10.7)             | 4.96 (dd; 4.6, 10.7) | 43.4                             | 68.0              |
| <b>20</b> <sup>c</sup> | 3.71 (dd; 4.6, 10.9)          | 4.20 (t; 10.9)             | 4.96 (dd; 4.6, 10.9) | 44.2                             | 62.2              |
| <b>21</b> <sup>c</sup> | 3.83–3.85 (m)                 | 4.35–4.40 (m)              | 5.44 (dd; 4.9, 11.0) | 44.2                             | 67.0              |

<sup>a</sup> The number in each entry is the chemical shift value ( $\delta$ ) observed in ppm relative to  $\text{Me}_4\text{Si}$ , followed by the multiplicity of the signal and the coupling constant(s) in Hz. All spectra were recorded at 300.1 MHz. <sup>b</sup> The number in the entry is the chemical shift value ( $\delta$ ) observed in ppm relative to the solvent peak. All spectra were recorded at 75.5 MHz. <sup>c</sup> The solvent used was  $\text{CDCl}_3$ . <sup>d</sup> The solvent was pyridine- $d_5$ . <sup>e</sup> These assignments may be interchanged.

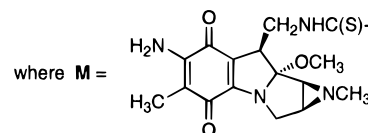
varied (data not shown).<sup>3</sup> These studies led us to use relatively high concentrations of DNA with 5–6 equiv of **9** and to choose 50% dimethylformamide/aqueous 0.1 M  $\text{NaHCO}_3$  ("pH" 9.0) as the buffer system. The reaction proceeded rapidly and gave **26** in high yield with few mitomycin byproducts.

**D. Generation of the Porfiromycin–DNA Conjugates.** The successful coupling of **9** with **22** led us to prepare a series of porfiromycin phosphorothioate oligodeoxynucleotide conjugates on a semipreparative scale. We chose to target a 30-base-long region (**27**) from the coding region of the human *FGFR1* gene (*n*566 to *n*595; Genbank sequence accession number X52833) (65). This base sequence has low C content and has a CG\*G sequence at the 3' end. We anticipated that the **9**–DNA conjugate would modify this sequence since porfiromycin (**2**) has been shown to preferentially alkylate this trinucleotide sequence upon *in vitro* reductive activation (24). Furthermore, we expected that the antisense strand would not readily undergo complementary self-base pairing due to the low G content. Since we could not confidently predict the geometric requirements needed by the hexylamino spacer unit in the antisense phosphorothioate oligodeoxynucleotides for mRNA alkylation (66, 67), we prepared a series of 25-mer DNA conjugates (**28**–**31**) in which the antisense phosphorothioate oligodeoxynucleotide was systematically displaced from the targeted CG\*G site on the sense mRNA strand. In **28** the end of the antisense sequence is one residue from the projected guanine (G\*) bonding site, whereas in **31** it is four residues away.

The phosphorothioate oligodeoxynucleotide stock solutions prepared for the porfiromycin **9** coupling reaction ranged from 52.3  $\mu\text{M}$  for **35** to 395.4  $\mu\text{M}$  for **32**. Phosphorothioate oligodeoxynucleotides **32**–**35** were treated with 5–6 equiv of **9** for 23–30 h at room temperature. HPLC analysis prior to workup indicated near complete utilization of the starting phosphorothioate oligodeoxynucleotide with the highest percentage of phosphorothioate oligodeoxynucleotide consumption observed for the most concentrated phosphorothioate oligodeoxynucleotide solutions. Parts A and B of Figure 1 show the HPLC traces at 261 and 365 nm, respectively, for reaction of **9** with **35** prior to purification. The intense signal at 23 min in Figure 1 corresponds to the product **31**, while the starting phosphorothioate oligodeoxynucleotide **35** ap-

peared at 22.7 min in Figure 1A. Semipreparative HPLC purification of the reaction mixtures provided the porfiromycin phosphorothioate oligodeoxynucleotide conjugates **28**–**31** in a 12% to near quantitative isolated yield. Figure 1C gives the HPLC trace for porfiromycin phosphorothioate oligodeoxynucleotide conjugate **31** after purification. Three experimental criteria established the identities of **28**–**31**. First, we observed a new peak in

|                                                                           |           |
|---------------------------------------------------------------------------|-----------|
| <u>Sense Strand</u>                                                       |           |
| 5'-AGATGGAAAA GAAATTGCATG CAGTGCCG*G-3'                                   | <b>27</b> |
| <u>Antisense Strands</u>                                                  |           |
| ACCTTTT CTTTAACGTAC GTCACGG-P(S)(OH)-(CH <sub>2</sub> ) <sub>6</sub> NH-M | <b>28</b> |
| TACCTTTT CTTTAACGTAC GTCACG-P(S)(OH)-(CH <sub>2</sub> ) <sub>6</sub> NH-M | <b>29</b> |
| CTACCTTTT CTTTAACGTAC GTCAC-P(S)(OH)-(CH <sub>2</sub> ) <sub>6</sub> NH-M | <b>30</b> |
| TCTACCTTTT CTTTAACGTAC GTCA-P(S)(OH)-(CH <sub>2</sub> ) <sub>6</sub> NH-M | <b>31</b> |



|                                                                                      |           |
|--------------------------------------------------------------------------------------|-----------|
| ACCTTTT CTTTAACGTAC GTCACGG-P(S)(OH)-(CH <sub>2</sub> ) <sub>6</sub> NH <sub>2</sub> | <b>32</b> |
| TACCTTTT CTTTAACGTAC GTCACG-P(S)(OH)-(CH <sub>2</sub> ) <sub>6</sub> NH <sub>2</sub> | <b>33</b> |
| CTACCTTTT CTTTAACGTAC GTCAC-P(S)(OH)-(CH <sub>2</sub> ) <sub>6</sub> NH <sub>2</sub> | <b>34</b> |
| TCTACCTTTT CTTTAACGTAC GTCA-P(S)(OH)-(CH <sub>2</sub> ) <sub>6</sub> NH <sub>2</sub> | <b>35</b> |

the HPLC profile near the starting phosphorothioate oligodeoxynucleotide (**32**–**35**) that absorbed at 365 nm in the UV–vis spectrum (42). This absorption value is characteristic for porfiromycin. Second, we determined the relative UV–vis absorption values at 360 vs 260 nm for the purified adducts. The ratio of these absorption values was in excellent agreement with the predicted values (41). Third, conjugates **28**–**31** all exhibited a parent ion in the electrospray mass spectrum consistent with their proposed structures. Moreover, each conjugate (**28**–**31**) displayed an ion  $346 \pm 2$  mass units higher than the starting unmodified oligodeoxynucleotide (**32**–**35**). This differential mass corresponded to the molecular weight of **9**.

**E. Biological Activity of the Porfiromycin–DNA Conjugates.** We chose two assays in this preliminary study to demonstrate that the porfiromycin phospho-

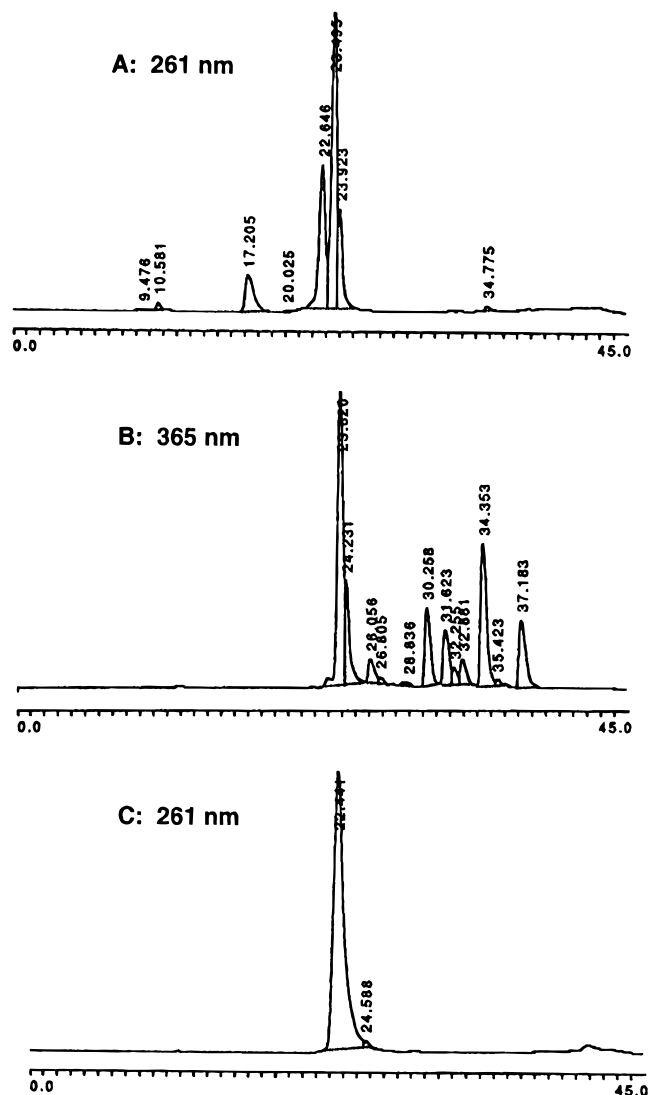


**Figure 1.** HPLC traces for reaction of **9** with **35**. A Rainin HPLC system (flow rate 2.0 mL/min) connected to a  $C_8$  reversed-phase semipreparative column (5  $\mu$ M, 25 cm, 10 mm i.d.) was used employing 90% A (0.025 M triethylammonium acetate, pH 7.0) and 10% B (acetonitrile) isocratic for 5 min and then from 90% A and 10% B to 45% A and 55% B in 40 min: (A) product mixture prior to HPLC purification (261 nm); (B) product mixture prior to HPLC purification (365 nm); (C) product **31** after HPLC purification (261 nm).

rothioate oligodeoxynucleotide conjugates were active in a biological system: (1) inhibition of HASMC proliferation and (2) inhibition of high-affinity binding of bFGF to human aortic smooth muscle cells (HASMC).

The HASMC proliferation assay (Table 2) showed that unconjugated porfiromycin (**2**) significantly inhibited cell growth at 1  $\mu$ M and was toxic at higher concentrations, as evidenced by >100% inhibition. Both the unconjugated phosphorothioate oligodeoxynucleotides and the porfiromycin phosphorothioate oligodeoxynucleotide conjugates caused significant inhibition of HASMC growth

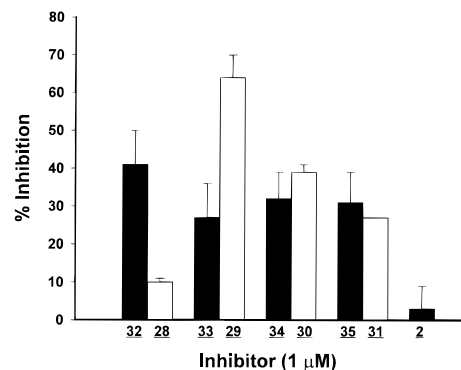


**Figure 2.** Inhibition of high-affinity bFGF binding by phosphorothioate oligodeoxynucleotide–porfiromycin conjugates. Compounds were tested at 1  $\mu$ M for their ability to inhibit high-affinity binding of bFGF to cultured human aortic smooth muscle cells. Results are expressed as percent inhibition when compared to binding in untreated control cells. Unconjugated compounds are shown as solid bars, and the adducts are shown as open bars.

but were less potent than porfiromycin alone. Porfiromycin phosphorothioate oligodeoxynucleotide conjugates **28** and **29** were more potent than the unconjugated phosphorothioate oligodeoxynucleotides **32** and **33**, respectively (Table 2). Finally, in contrast to porfiromycin, the porfiromycin phosphorothioate oligodeoxynucleotide conjugates showed no cellular toxicity even at the highest concentration used.

Figure 2 shows that at 1  $\mu$ M, unconjugated porfiromycin (**2**) did not reduce the number of high-affinity (receptor mediated) bindings of bFGF to smooth muscle cells. All of the unconjugated phosphorothioate oligodeoxynucleotides as well as the porfiromycin phosphorothioate oligonucleotide conjugates caused partial reduction of bFGF binding. However, since only one derivatized oligonucleotide inhibited bFGF binding more than the underivatized oligonucleotide (**29** vs **33**), effects on bFGF binding are not likely in general to be responsible for effects seen on proliferation.

Taken together, the results of these assays demonstrate that our newly synthesized porfiromycin phosphorothioate oligodeoxynucleotide conjugates retain activity in a biological system. As a group, the conjugates are more potent than the underivatized oligonucleotides at inhibiting proliferation. However, prevention of bFGF binding to its high-affinity receptor does not appear to account for inhibition of cell proliferation. These preliminary assays do not shed any light on the mechanism(s) involved. Thus, the inhibition seen may involve antisense as well as nonantisense modes of action, as has been demonstrated for phosphorothioate oligodeoxynucleotides in numerous instances (68–70). Future studies, which are beyond the scope of the current investigation, will have to include (1) sense and missense control phosphorothioate oligodeoxynucleotides, (2) direct measurement of the target mRNA and protein, and (3) demonstration of intracellular uptake of the compounds (71).

**Table 2. Inhibition of Proliferation of Cultured Human Aortic Smooth Muscle Cells by Porfiromycin Phosphorothioate Oligodeoxynucleotide Conjugates<sup>a</sup>**

| concn<br>( $\mu$ M) | 32          | 28         | 33          | 29          | 34         | 30         | 35         | 31          | 2           |
|---------------------|-------------|------------|-------------|-------------|------------|------------|------------|-------------|-------------|
| 1                   | 27 $\pm$ 4  | 43 $\pm$ 7 | 43 $\pm$ 11 | 45 $\pm$ 3  | 40 $\pm$ 4 | 45 $\pm$ 3 | 47 $\pm$ 4 | 37 $\pm$ 4  | 89 $\pm$ 4  |
| 3                   | 30 $\pm$ 10 | 61 $\pm$ 1 | 46 $\pm$ 2  | 72 $\pm$ 2  | 60 $\pm$ 5 | 58 $\pm$ 6 | 60 $\pm$ 2 | 40 $\pm$ 12 | 104 $\pm$ 1 |
| 10                  | 64 $\pm$ 9  | 86 $\pm$ 1 | 60 $\pm$ 6  | 100 $\pm$ 2 | 62 $\pm$ 8 | 83 $\pm$ 2 | 73 $\pm$ 1 | 78 $\pm$ 1  | 126 $\pm$ 2 |

<sup>a</sup> Values shown are percent inhibition with the standard error of the mean when compared to untreated control.

## ACKNOWLEDGMENT

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## LITERATURE CITED

- Milligan, J. F., Matteucci, M. D., and Martin, J. C. (1993) Current concepts in antisense drug design. *J. Med. Chem.* 36, 1923–1937.
- Kiely, J. S. (1994) Recent advances in antisense technology. *Annu. Rep. Med. Chem.* 29, 297–306.
- Lown, J. W. (1992) Lexitropsins: rational design of DNA sequence reading agents as novel anti-cancer agents and potential cellular probes. *Anticancer Drug. Des.* 3, 25–40.
- Nielsen, P. E. (1991) Sequence-selective DNA recognition by synthetic ligands. *Bioconjugate Chem.* 2, 1–12.
- Bailly, C., Colson, P., Houssier, C., Houssin, R., Mrani, D., Gosselin, G., Imbach, J.-L., Waring, M. J., Lown, J. W., and Hénichart, J.-P. (1992) Binding properties and DNA sequence-specific recognition of two bithiazole-linked netropsin hybrid molecules. *Biochemistry* 31, 8349–8362.
- McConnaughie, A. W., and Jenkins, T. C. (1995) Novel acridine-triazenes as prototype combilexins: synthesis, DNA binding, and biological activity. *J. Med. Chem.* 38, 3488–3501.
- Wyatt, M. D., Lee, M., Garbiras, B. J., Souhami, R. L., and Hartley, J. A. (1995) Sequence specificity of alkylation for a series of nitrogen mustard-containing analogues of distamycin of increasing binding site size: evidence for increased cytotoxicity with enhanced sequence specificity. *Biochemistry* 34, 13034–13041.
- Dervan, P. B. (1986) Design of sequence-specific DNA-binding molecules. *Science* 232, 464–471 and references cited therein.
- Sluka, J. P., Bruist, M., Horvath, S. J., Simon, M. I., and Dervan, P. B. (1987) Synthesis of a sequence-specific DNA-cleaving peptide. *Science* 238, 1129–1132.
- Steitz, T. A. (1990) Structural studies of protein-nucleic acid interaction: the sources of sequence-specific binding. *Q. Rev. Biophys.* 23, 205–280.
- Harrison, S. C., and Aggarwal, A. K. (1990) DNA recognition by proteins with the helix-turn-helix motif. *Annu. Rev. Biochem.* 59, 933–969.
- Praseuth, D., Perrouault, L., Le Doan, T., Chassignol, M., Thuong, N. T., Lhomme, J., and Helene, C. (1988) Sequence-specific binding and photocrosslinking of alpha and beta oligodeoxynucleotides to the major groove of DNA via triple-helix formation. *Proc. Natl. Acad. Sci. U.S.A.* 85, 1349–1353.
- Cooney, M., Czernuszewicz, G., Postel, E. H., Flint, S. J., and Hogan, M. E. (1988) Site-specific oligonucleotide binding represses transcription of human c-myc gene *in vitro*. *Science* 241, 456–459.
- Bergstrom, D. E., and Gerry, N. P. (1994) Precision sequence-specific cleavage of a nucleic acid by a minor-groove-directed metal-binding ligand linked through N-2 of deoxyguanosine. *J. Am. Chem. Soc.* 116, 12067–12068.
- Povsic, T. J., Strobel, S. A., and Dervan, P. B. (1992) Sequence-specific double-strand alkylation and cleavage of DNA mediated by triple-helix formation. *J. Am. Chem. Soc.* 114, 5934–5941.
- Carter, S. K., and Crooke, S. T. (1979) *Mitomycin C. Current Status and New Developments*, Academic Press, New York.
- Iyer, V. N., and Szybalski, W. (1964) Mitomycins and porfiromycin: chemical mechanism of activation and cross-linking of DNA. *Science* 145, 55–58.
- Moore, H. W., and Czerniak, R. (1981) Naturally occurring quinones as potential bioreductive alkylating agents. *Med. Res. Rev.* 1, 249–280.
- Tomasz, M. (1994) The mitomycins: Natural cross-linkers of DNA. *Molecular Aspects of Anticancer Drug-DNA Interactions* (S. Neidle and M. Waring, Eds.) Vol. 2, pp 312–348, Macmillan, Boca Raton, FL.
- Tomasz, M., Lipman, R., Chowdary, D., Pawlak, J., Verdine, G. L., and Nakanishi, K. (1987) Isolation and structure of a covalent cross-link adduct between mitomycin C and DNA. *Science* 235, 1204–1208.
- Li, V.-S., and Kohn, H. (1991) Studies on the bonding specificity for mitomycin-DNA monoalkylation processes. *J. Am. Chem. Soc.* 113, 275–283.
- Kumar, S., Lipman, R., and Tomasz, M. (1992) Recognition of specific DNA sequences by mitomycin C for alkylation. *Biochemistry* 31, 1399–1407.
- Kohn, H., Li, V.-S., and Tang, M.-s. (1992) Recognition of mitomycin C-DNA monoadducts by UVRABC nuclease. *J. Am. Chem. Soc.* 114, 5501–5509.
- Kohn, H., Li, V.-S., Schiltz, P., and Tang, M.-s. (1992) On the origins of the DNA sequence selectivity of mitomycin monoalkylation transformations. *J. Am. Chem. Soc.* 114, 9218–9220.
- De Mesmaeker, A., Häner, R., Martin, P., and Moser, H. E. (1995) Antisense oligonucleotides. *Acc. Chem. Res.* 28, 366–374.
- Agrawal, S. (1992) Antisense oligonucleotides as antiviral agents. *Trends Biotechnol.* 10, 152–158.
- Mercola, D., and Cohen, J. S. (1995) Antisense approaches to cancer gene therapy. *Cancer Gene Ther.* 2, 47–59.
- Simons, M., Edelman, E. R., DeKeyser, J.-L., Langer, R., and Rosenberg, R. D. (1992) Antisense c-myc oligonucleotides inhibit initial arterial smooth muscle cell accumulation *in vivo*. *Nature* 359, 67–70.
- Pickering, G., Weir, L., Jekanowski, J., and Isner, J. M. (1992) Inhibition of proliferation of human vascular smooth muscle cells using antisense oligonucleotides to PCNA. *J. Am. Coll. Cardiol.* 19, 165A.
- Spier, E., and Epstein, S. E. (1992) Inhibition of smooth muscle cell proliferation by an antisense oligonucleotide targeting the messenger RNA encoding proliferating cell nuclear antigen. *Circulation* 86, 538–547.
- Simons, M., and Rosenberg, R. D. (1992) Antisense non-muscle myosin heavy chain and c-myc oligonucleotides suppress smooth muscle cell proliferation *in vitro*. *Circ. Res.* 70, 835–843.
- Biro, S., Fu, Y. M., Yu, Z. X., and Epstein, S. E. (1993) Inhibitory effects of antisense oligodeoxynucleotides targeting c-myc mRNA on smooth muscle cell proliferation and migration. *Proc. Natl. Acad. Sci. U.S.A.* 90, 654–658.
- Shi, Y., Hutchinson, H. G., Hall, D. J., and Zalewski, A. (1993) Downregulation of c-myc expression by antisense oligonucleotides inhibits proliferation of human smooth muscle cells. *Circulation* 88, 1190–1195.
- Limbach, P. A., Crain, P. F., and McCloskey, J. A. (1995) Molecular mass measurement of intact ribonucleic acids via electrospray ionization quadrupole mass spectrometry. *J. Am. Soc. Mass Spectrom.* 6, 27–39.
- Choi, D., Yoo, B., Colson, K. L., Martin, G. E., and Kohn, H. (1995) C(10) Halogen 10-des(carbamoyloxy)porfiromycins: synthesis, chemistry, and biological activity. *J. Org. Chem.* 60, 3391–3396.
- Kinoshita, S., Uzu, K., Nakano, K., and Takahashi, T. (1971) Mitomycin derivatives. 2. Derivatives of decarbamoylmitosane and decarbamoylmitosene. *J. Med. Chem.* 14, 109–112.
- Han, I., Russell, D. J., and Kohn, H. (1992) Studies on the mechanism of mitomycin C(1) electrophilic transformations: structure-reactivity relationships. *J. Org. Chem.* 57, 1799–1807.
- Urakawa, C., Tsuchiya, H., Nakano, K.-I., and Nakamura, N. (1981) Preparation and biological activities of 10-decarbamoyloxy-9-dehydromitomycin B and its analogs. *J. Antibiot.* 34, 1152–1156.
- Kono, M., Kasai, M., and Shirahata, K. (1990) The derivation of 1a-demethylmitomycin G from mitomycin C. *J. Antibiot.* 43, 383–390.
- Kasai, M., and Kono, M. (1992) Studies on the chemistry of mitomycins. *Syn. Lett.* 778–790 and references therein.

- (41) The anticipated  $\epsilon$ -values for **23** at 260 nm and 360 (365) nm in the UV-visible spectrum can be predicted, see: Borowy-Borowski, H., Lipman, R., Chowdary, D., and Tomasz, M. (1990) Duplex oligodeoxyribonucleotides cross-linked by mitomycin C at a single site: Synthesis, properties, and cross-link reversibility. *Biochemistry* 29, 2992–2999.
- (42) Webb, J. S., Cosulich, D. B., Mowat, J. H., Patrick, J. B., Broschard, R. W., Meyer, W. E., Williams, R. P., Wolf, C. F., Fulmor, W., Pidacks, C., and Lancaster, J. E. (1962) The structures of mitomycins A, B and C and porfiromycin—Part I. *J. Am. Chem. Soc.* 84, 3185–3187.
- (43) Kan, M., Shi, E.-G., and McKeehan, W. (1991) Identification and assay of fibroblast growth factor receptors. *Methods Enzymol.* 198, 158–171.
- (44) Iyengar, B. S., Sami, S. M., Remers, W. A., Bradner, W. T., and Schurig, J. E. (1983) Mitomycin C and porfiromycin analogues with substituted ethylamines at position 7. *J. Med. Chem.* 26, 16–20.
- (45) Iyengar, B. S., Sami, S. M., Tarnow, S. E., Remers, W. A., Bradner, W. T., and Schurig, J. E. (1983) Mitomycin C analogues with secondary amines at position 7. *J. Med. Chem.* 26, 1453–1457.
- (46) Kono, M., Saitoh, Y., Kasai, M., Shirahata, K., Morimoto, M., and Ashizawa, T. (1993) Synthesis and structure-activity relationships of new dimeric mitomycin derivatives; 7-N,7'-N'-bis(omega-thioalkyl)dimitomycins. *J. Antibiot.* 46, 1428–1438.
- (47) Sawhney, K., and Kohn, H. (1989) Mitomycin C analogues with a substituted hydrazine at position 7. Synthesis, spectral properties, and biological activity. *J. Med. Chem.* 32, 248–252.
- (48) Fishbein, P. L., and Kohn, H. (1987) Synthesis and antineoplastic activity of (N)-1a-formyl and (N)-1a-thionoformyl derivatives of mitomycin C and (N)-2-methylaziridine. *J. Med. Chem.* 30, 1767–1773.
- (49) De Marre, A., Soye, H., Schacht, E., Shoaibi, M. A., Seymour, L. W., and Rihova, B. (1995) Synthesis and evaluation of macromolecular prodrugs of mitomycin C. *J. Controlled Release* 36, 87–97.
- (50) De Marre, A., Soye, H., and Schacht, E. (1994) Synthesis of macromolecular mitomycin C derivatives. *J. Controlled Release* 32, 129–137.
- (51) For oligodeoxynucleotide attachment at the C(7) position in mitomycin C, see: Maruenda, H., and Tomasz, M. (1995) Mitomycin C-conjugated oligonucleotides: interaction with DNA and RNA. *FASEB J.* 9, A966.
- (52) Milligan, J. F., Matteucci, M. D., and Martin, J. C. (1993) Current concepts in antisense drug design. *J. Med. Chem.* 36, 1923–1937.
- (53) Varma, R. S. (1993) Synthesis of oligonucleotide analogues with modified backbones. *Syn. Lett.* 621–637.
- (54) Saltis, J., Thomas, A. C., Agrotis, A., Campbell, J. H., Campbell, G. R., and Bobik, A. (1995) Expression of growth factor receptors in arterial smooth muscle cells. Dependency on cell phenotype and serum factors. *Atherosclerosis* 118, 77–87.
- (55) Xin, X., Johnson, A. D., Scott-Burden, T., Engler, D., and Cascells, W. (1994) The predominant form of fibroblast growth factor receptor expressed by proliferation human arterial smooth muscle cells in culture is type I. *Biochem. Biophys. Res. Commun.* 204, 557–564.
- (56) Brothers, T. E., Robison, G., Elliot, B. M., Boggs, J. M., Frankel, A. E., and Willingham, M. C. (1995) Upregulation of bFGF receptor expression after carotid bypass. *J. Surg. Res.* 58, 28–32.
- (57) Lindner, V., and Reidy, M. A. (1993) Expression of bFGF and its receptor by SMC and endothelium in injured rat arteries: An en face study. *Circ. Res.* 73, 589–595.
- (58) Becker, D., Lee, P. L., Rodeck, U., and Herlyn, M. (1992) Inhibition of the *FGFR1* gene in human melanocytes and malignant melanomas leads to inhibition of proliferation and signs indicative of differentiation. *Oncogene* 7, 2303–2313.
- (59) Kato, J., Wanebo, H., Calabresi, P., and Clark, J. W. (1992) bFGF production and growth factor receptors as potential targets for melanoma therapy. *Melanoma Res.* 2, 13–23.
- (60) Mima, T., Ueno, H., Fischman, D. A., Williams, L. T., and Mikawa, T. (1995) FGFR1 is required for *in vivo* cardiac myocyte proliferation at early embryonic stages of heart development. *Proc. Natl. Acad. Sci. U.S.A.* 92, 467–477.
- (61) Danishefsky, S., and Ciufolini, M. (1984) Leucomitomycins. *J. Am. Chem. Soc.* 106, 6424–6425.
- (62) Kohn, H., Zein, N., Lin, X. Q., Ding, J.-Q., and Kadish, K. M. (1987) Mechanistic studies on the mode of reaction of mitomycin C under catalytic and electrochemical reductive conditions. *J. Am. Chem. Soc.* 109, 1833–1840.
- (63) Beck, T. W., Huleihel, M., Gunnell, M., Bonner, T. I., and Rapp, U. R. (1987) The complete coding sequence of the human *A-raf-1* oncogene and transforming activity of a human A-raf carrying retrovirus. *Nucleic Acids Res.* 15, 595–609.
- (64) Avalos, M., Babiano, R., Cintas, P., Jimenez, J. L., Palacios, J. C., and Valencia, C. (1994) Condensation of 2-amino-2-deoxysugars with isothiocyanates. Synthesis of cis-1,2-fused glycopyrano heterocycles. *Tetrahedron* 50, 3273–3296.
- (65) Dionne, C. A., Crumley, G. R., Bellot, F., Kaplow, J. M., Searfoss, G., Ruta, M., Burgess, W. H., Jaye, M., and Schlessinger, J. (1990) Cloning and expression of two distinct high-affinity receptors cross-reacting with acidic and basic fibroblast growth factors. *EMBO J.* 9, 2685–2692.
- (66) Chatterjee, M., and Rokita, S. E. (1994) The role of a quinone methide in the sequence specific alkylation of DNA. *J. Am. Chem. Soc.* 116, 1690–1697.
- (67) Li, T., Zeng, Q., and Rokita, S. E. (1994) Target-promoted alkylation of DNA. *Bioconjugate Chem.* 5, 497–500.
- (68) Burgess, T. L., Fisher, E. F., Ross, S. L., Bready, J. V., Qian, Y.-X., Bayewitch, L. A., Cohen, A. M., Herrera, C. J., Hu, S. S.-F., Kramer, T. B., Lott, F. D., Martin, F. H., Pierce, G. F., Simonet, L., and Farrell, C. L. (1995) The antiproliferative activity of c-myc and c-myc antisense oligonucleotides in smooth muscle cells is caused by a nonantisense mechanism. *Proc. Natl. Acad. Sci. U.S.A.* 92, 4051–4055.
- (69) Guvakova, M. A., Yakubov, L. A., Vlodavsky, I., Tonkinson, J. L., and Stein, C. A. (1995) Phosphorothioate oligodeoxynucleotides bind to basic fibroblast growth factor inhibit its binding to cell surface receptors, and remove it from low affinity binding sites on extracellular matrix. *J. Biol. Chem.* 270, 2620–2627.
- (70) Bennett, C. F., Chiang, M. Y., Wilson-Lingardo, L., and Wyatt, J. R. (1994) Sequence specific inhibition of human type II phospholipase A2 enzyme activity by phosphorothioate oligonucleotides. *Nucleic Acids Res.* 22, 3202–3209.
- (71) Stein, C. A., and Krieg, A. M. (1994) Editorial: Problems in interpretation of data derived from *in vitro* and *in vivo* use of antisense oligodeoxynucleotides. *Antisense Res. Dev.* 4, 67–69.

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# Solution Structure Studies of the Cobalt Complex of a Bleomycin Functional Model Bound to d(CGCAATTGCG)<sub>2</sub> by Two-Dimensional Nuclear Magnetic Resonance Methods and Restrained Molecular Dynamics Simulation

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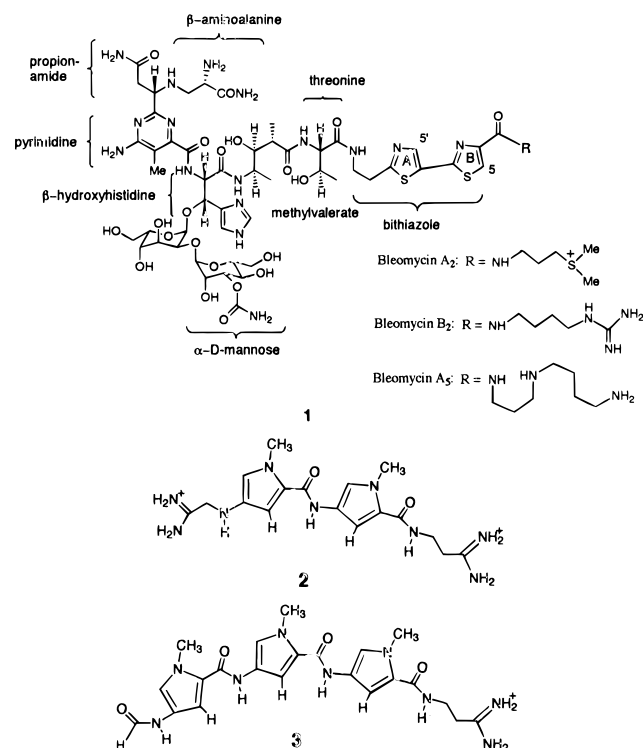
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The interaction between the cobalt(III) complex of a bleomycin functional model (AMPHIS-NET) and the oligonucleotide d(CGCAATTGCG)<sub>2</sub> and the structural features of the 1:1 ligand–DNA complex have been determined by high-resolution two-dimensional nuclear magnetic resonance methods and restrained molecular dynamics calculations. The intermolecular nuclear Overhauser effect (NOE) cross-peaks between ligand protons and the DNA minor groove protons suggest that the cobalt(III) complex of AMPHIS-NET binds in the minor groove of DNA at the central AATT site. The NOE connectivities also clearly indicate that the H<sub>8</sub> pyridine proton and the H<sub>2</sub> imidazole proton in the metal-binding domain interact with the H<sub>4'</sub> sugar proton of C<sub>19</sub> and the H<sub>4'</sub> sugar proton of A<sub>5</sub>, respectively, which defines a structure where the metal binding moiety of Co(III)·AMPHIS-NET participates in binding to the DNA and extends into the region two base pairs beyond the central AATT site in the minor groove. This binding model is in accord with the consistently observed nondiffusion DNA cleavage in locations two to three residues beyond the end of AT-rich binding sites induced by the corresponding iron(II) complexes of AMPHIS-NET and other AMPHIS–lexitropsin hybrids of the bleomycin functional model compounds.

## INTRODUCTION

The bleomycins (BLMs) are a family of glycopeptide antitumor antibiotics clinically used both alone and in combination with radiation or other chemotherapeutic agents against several types of cancer (1, 2). The structure of the bleomycins is shown in Chart 1 (1). They have been used in the treatment of squamous cell carcinoma, various lymphomas, and testicular tumors. The therapeutic effect of BLM is believed to arise from its ability to bind to and degrade DNA (3, 4) and RNA (5) selectively in the presence of certain metal ions and molecular oxygen. Recently, it has been reported that Co(III)–BLM complexes can cause photocleavage of DNA under irradiation with UV or visible light (6–8). Therefore, the investigation of the structure of cobalt–BLM complexes (9, 10), binding of metal–BLM complexes to DNA (11–16), and photocleavage (7, 17–20) of DNA by the cobalt complex of bleomycin or its synthetic analogues have received increasing attention. Wu et al. (12–14) reported the interaction of Co·bleomycin complex (green) with d(CCAGGCCTGG)<sub>2</sub> by two-dimensional nuclear magnetic resonance (2D NMR) methods, and the intermolecular nuclear Overhauser effect (NOEs) between bleomycin and the DNA suggested that the bithiazole side chain of the cobalt complex is intercalated between base pairs C<sub>6</sub>·G<sub>15</sub> and C<sub>7</sub>·G<sub>14</sub>. However, the Hecht group obtained evidence from intermolecular NOE interactions between the ligand and the DNA that support either the

**Chart 1. Structure of Bleomycins (1), Netropsin (2), and Distamycin (3)**



intercalation binding model or the minor groove binding model for the bithiazole moiety, while studying the solution structures of the Zn(II)·bleomycin A<sub>5</sub>–d(CGCTAGCG)<sub>2</sub> complex and the Zn(II)·bleomycin A<sub>2</sub>–d(CGCTAGCG)<sub>2</sub> complex by 2D NMR and molecular modeling (15, 16).

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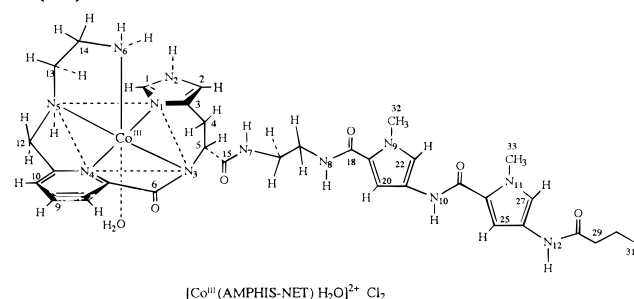
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**Chart 2. Structure and Numbering Scheme for Co(III)·AMPHIS-NET**

Netropsin (**2**) and distamycin (**3**) are two members of the naturally occurring oligomeric *N*-methylpyrrolecarboxamide peptide antibiotics with antitumor, antiviral, and antibacterial activity (22–25). Structural studies by high-resolution NMR and X-ray crystallographic methods indicate that the netropsin binds noncovalently in the minor groove at a site of four or five successive A·T base pairs in double-stranded B-DNA (26–29) in 1:1 drug–DNA complex, while distamycin containing three *N*-methylpyrrole ring fits along the minor groove of AT-rich B-DNA, not only in a 1:1 drug–DNA model (30–32) but also in an antiparallel side-by-side 2:1 drug–DNA model (33–35).

In order to gain insight into the interaction of bleomycin with DNA, we have designed and synthesized a series of functional model compounds (36–39) of bleomycin through conjugation of the simplified metal-chelating subunit AMPHIS (40, 41) [methyl[[[2-(2-aminoethyl)-amino]methyl]pyridinyl]-6-carboxylhistidinate] with oligo(*N*-methylpyrrole) lexitropsin (26, 42–45) residues which are recognized as sequence-selective DNA minor groove binding moieties (46–48). It has been assumed, but hitherto has not been proven, that metal ions are coordinated at the AMPHIS site and that the oligo(*N*-methylpyrrole) moiety of the AMPHIS–lexitropsin hybrid would provide DNA affinity and sequence selectivity, directing the metal binding moiety to sites differing from those of bleomycin. Recently, we first prepared the cobalt complexes of such hybrids which can specifically bind in the minor groove of AT-rich DNA and have elucidated the solution structure of them by 2D NMR and restrained molecular modeling calculation (49). The structure and numbering scheme of one of the cobalt complexes, Co(III)·AMPHIS-NET (netropsin), based on the hybrid of AMPHIS and the naturally occurring oligopeptide antibiotic netropsin (**2**) are shown in Chart 2. The pyridine nitrogen, imidazole nitrogen, secondary amine group, and deprotonated pyridinecarboxamide comprise the basal plane of coordination, while the primary amine group and solvent molecule occupy the axial positions. This paper reports our results on the interaction between this cobalt complex of a bleomycin functional model and the decameric oligonucleotide d(CGCAATTGCG)<sub>2</sub> and provides detailed structural features of the ligand–DNA complex essential for further drug design.

## MATERIALS AND METHODS

**Sample Preparation.** The cobalt complex of AMPHIS-NET was synthesized by reaction of AMPHIS-NET with Na<sub>3</sub>[Co(CO<sub>3</sub>)<sub>3</sub>]·3H<sub>2</sub>O which was prepared by following the published procedure (50) and then purified by strong acidic cation exchanger SP-C50-120 purchased from Sigma Chemical Co. The oligonucleotide was prepared as described previously (51, 52).

The DNA sample for the NMR titration was prepared by dissolving the decameric oligonucleotide in 0.5 mL of

D<sub>2</sub>O buffer containing 10 mM sodium phosphate (pD 6.8, uncorrected) and lyophilizing to dryness. After being lyophilized twice from 99.9% D<sub>2</sub>O, the sample was finally redissolved in 0.5 mL of 99.96% D<sub>2</sub>O. The extinction coefficient for d(CGCAATTGCG) was calculated (53) to be  $9.07 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ . The concentration of the double-stranded DNA sample was determined to be 1.62 mM by a UV spectrum at 260 nm at 80 °C. A stock solution of Co(III)·AMPHIS-NET was prepared by dissolving 1.5 mg of the ligand in 0.5 mL of 99.9% D<sub>2</sub>O and lyophilizing twice from 99.9% D<sub>2</sub>O, and then it was redissolved in 200  $\mu\text{L}$  of 99.96% D<sub>2</sub>O. The concentration of the stock solution was determined to be 10.5 mM by UV absorbance at 300 nm ( $\epsilon = 1.13 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ ).

**1D NMR Titration.** All of the 1D and 2D <sup>1</sup>H NMR experiments were performed at 30 °C on a Varian UNITY 500 MHz spectrometer equipped with a Sun workstation, unless otherwise specified. The solvent signals were used as an internal reference ( $\delta = 4.75 \text{ ppm}$  at 30 °C) in the proton NMR spectra in D<sub>2</sub>O and in the H<sub>2</sub>O/D<sub>2</sub>O (9:1) solution. NMR titration was carried out by stepwise addition of Co·AMPHIS-NET into the NMR sample containing the DNA oligomer in 0.2 molar equiv per addition. 1D NMR spectra were acquired using 16K data points, 128 scans, and a spectral width of 4450 Hz. The presaturation method was applied to suppress the residual HOD resonance during the 2.0 s recycle delay.

**2D NMR Spectra.** The NOESY experiments with mixing times of 80, 150, 250, and 350 ms for 1:1 ligand–DNA complex were carried out on nonspinning samples, and the phase-sensitive detection was accomplished by using the hypercomplex data collection method (54, States–Haberkon method). The NOESY spectra were obtained from a 2048  $\times$  512 data matrix, with 80 scans and 4 dummy scans per *t*<sub>1</sub> value for a spectral width of 4450 Hz. The relaxation delay between scans was 1.0 s, and a presaturation pulse was applied during the recycle delay to suppress the residual HDO resonance. All the final matrices were zero-filled to 2048  $\times$  2048. The Gaussian apodization function was applied in both directions for Fourier transformation. The NOESY spectrum for 1:1 ligand–DNA complex with 250 ms mixing time in 9:1 H<sub>2</sub>O/D<sub>2</sub>O solution using either presaturation and the Sklenar–Bax method to suppress the solvent resonance was acquired to study the exchangeable protons. The spectral widths and data points were similar to those acquired in D<sub>2</sub>O solution. FIDs in *t*<sub>1</sub> with 128 transients and 4 dummy scans were acquired.

The TOCSY spectra using an MLEV-17 sequence to generate the spin lock field were acquired with spin-locking periods of 35 and 75 ms. The spectra were collected with 2048 complex data points in *t*<sub>2</sub> using a spectral width of 4450 Hz. Typically, 512 *t*<sub>1</sub> experiments with 16 scans and 4 dummy scans were recorded and zero-filled to 2048. The recycle delay was 1.0 s, and the sine-bell window function was used in both directions for Fourier transformation.

**Distance Restraints and Structure Refinement.** The interproton distance restraints were estimated from the volume integrals of each cross-peak in the NOESY spectra acquired at mixing times of 80 and 250 ms, using the isolated spin pair approximation method. The cytosine H<sub>5</sub>–H<sub>6</sub> interproton distance (2.45 Å) was used as calibration (55) to calculate the interproton distance restraints. The intermolecular NOE restraints were classified semiquantitatively into three categories: strong (less than 3.0 Å), medium (3.0–4.0 Å), or weak (4.0–5.0 Å).

The starting structure of the Co(III)·AMPHIS-NET complex was constructed using the builder module of

**Insight II.** This structure was then minimized using the esff force field provided in the Discover 3 program of Biosym Technologies (San Diego, CA) restrained by NOE data obtained from the 2D ROESY spectrum of Co(III)-AMPHIS-NET. The structure of the DNA duplex was constructed using the biopolymer module of Insight II. The Co(III)-AMPHIS-NET molecule was docked onto the minor groove of the DNA decameric duplex to obtain the structure of the ligand-DNA complex until the vdW energy falls to a small negative value. This was followed by minimization through the steepest descent and conjugate gradient methods for 10 000 and 5000 steps using the esff force field provided in the Discover 3 program, respectively. A total of 16 intermolecular ligand-DNA NOEs, 20 intraligand NOEs, and 286 intra-DNA NOEs were used as distance restraint to simulate the structure of ligand-DNA complex. The minimized structure of Co(III)-AMPHIS-NET-DNA complex was then subjected to molecular dynamics computation at 298 K for 5 ps with a step size of 1 fs. In addition to the distance restraints derived from NOE data, hydrogen bonds in the outermost two base pairs of the oligonucleotide were also constrained to account for the terminal fraying in the process of minimization and molecular dynamics simulation. Counterions were set 4.5 Å from the two adjacent phosphorus atoms. The dielectric constant of 4 or distance dependent dielectric constant was utilized in the computation. Six to twelve Lenard-Jones and 10–12 potentials were used for vdW and hydrogen bond energies, respectively.

## RESULTS AND DISCUSSION

**Titration of d(5'-CGCAATTGCG-3')<sub>2</sub> with Co(III)-AMPHIS-NET.** The titration of the decadeoxyribonucleotide d(CGCAATTGCG)<sub>2</sub> with the cobalt complex of the AMPHIS-NET hybrid was carried out at 30 °C with 0, 0.2, 0.4, 0.6, 0.8, and 1.0 molar equiv of ligand addition. The titration results are shown in Figure 1. Upon addition of the ligand, a set of new signals appeared corresponding to the NMR resonances of the bound ligand molecule whose intensities increased with addition of ligand. At a ligand:DNA ratio of 1:1, there is only one set of DNA resonances and one set of bound ligand resonances. Subsequent additions of Co-AMPHIS-NET caused resonances from free Co(III)-AMPHIS-NET H<sub>20</sub> and H<sub>25</sub> pyrrole protons to appear, which implies that the drug binding sites have become saturated. The broadening and shift of the DNA resonances, especially for the adenine H<sub>2</sub> proton and H<sub>1'</sub> proton position in the minor groove of DNA, suggest minor groove binding of the Co-AMPHIS-NET in the DNA duplex. These features are consistent with similar binding in the minor groove of DNA of distamycin, netropsin, and their analogues. Comparison of the 1D proton spectra of Co(III)-AMPHIS-NET and both free and bound decamer d(CGCAATTGCG)<sub>2</sub> indicated that some protons in the metal-binding domain of Co(III)-AMPHIS-NET have experienced broadening and downfield shift upon the addition of Co-AMPHIS-NET into DNA. Broadening and shifting of these resonances lying in the metal-binding domain lends support to the idea that the metal binding moiety of Co(III)-AMPHIS-NET takes part in DNA binding. In addition, the intermolecular NOE contacts between the protons in the metal-binding domain of Co(III)-AMPHIS-NET and DNA minor groove protons, which are described below, support this inference, and it is consistent with the conclusion obtained by the Stubbe group (12–14) and the Hecht group (15, 16) on the metal-binding domain of Zn(II)-BLM A<sub>5</sub>, Zn(II)-BLM A<sub>2</sub>, and Co(III)-BLM A<sub>2</sub> participating in DNA binding. A

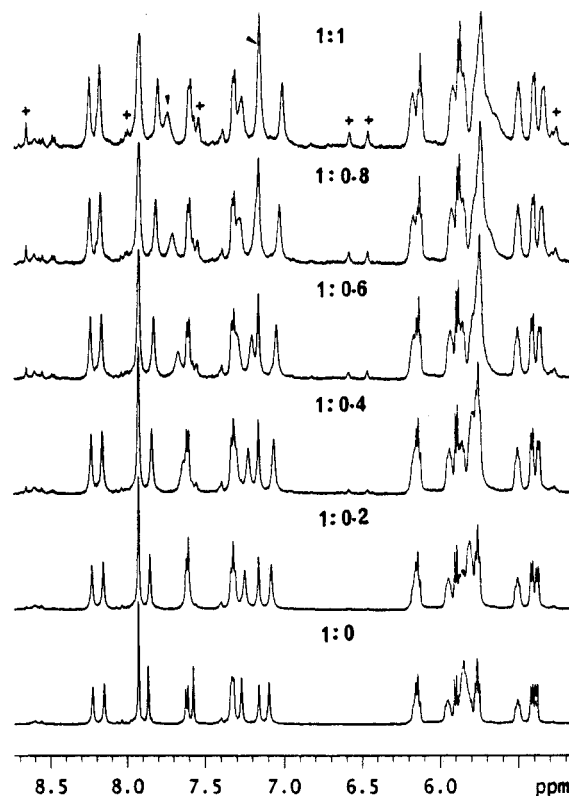


**Figure 1.** Titration of d(5'-CGCAATTGCG-3')<sub>2</sub> with the cobalt complex of AMPHIS-NET at 30 °C with 0, 0.2, 0.4, 0.6, 0.8, and 1.0 equiv of cobalt complex of AMPHIS-NET added, respectively. Aromatic and sugar H<sub>1'</sub> proton regions of <sup>1</sup>H NMR spectra. The plus signs denote the selected protons of ligand, and the chemical shifts of the two adenine H<sub>2</sub> resonances of ligand-binding DNA are indicated by the arrows.

general increase in the line widths of the resonances over much of the spectra, including the resonances of the Co(III)-AMPHIS-NET and DNA protons, implies that the ligand is in fast exchange on the NMR time scale. In addition, only one set of DNA resonances and one set of bound ligand resonances were observed throughout the titration, implying that the C<sub>2</sub> symmetry of the self-complementary duplex sequence appears to be retained in the Co(III)-AMPHIS-NET-DNA complex.

**Assignments of the Protons of Ligand Free and Ligand-Bound DNA.** The <sup>1</sup>H NMR assignment for the ligand free decadeoxyribonucleotide d(CGCAATTGCG)<sub>2</sub> has been previously reported (51, 56–57), and for comparison with that of its ligand-bound DNA counterpart, the <sup>1</sup>H NMR signals of the ligand free DNA were reassigned under similar experimental conditions as those of the ligand-DNA complex reported in this study. The proton reassignments of the ligand free DNA were established by means of standard two-dimensional experiments (58). The nonexchangeable proton resonances were analyzed by a combined use of through-bond connectivities (COSY), through-multiple-bond connectivities (TOCSY), and through-space connectivities (NOESY). The assignments of base protons and sugar H<sub>1'</sub>, H<sub>2'</sub>, and H<sub>2''</sub> protons were made by the observation of sequential connectivities from the base protons to its own sugar protons (intraresidue NOEs) and to the 5'-flanking sugar protons (interresidue NOEs) in the NOESY spectrum, confirming the overall right-handed B-type conformation of the decadeoxyribonucleotide d(CGCAATTGCG)<sub>2</sub> by comparison with previously reported sequential NOE connectivities and chemical shift data of ligand free DNA.

The assignments of the proton signals for the ligand-bound DNA were accomplished using methods similar

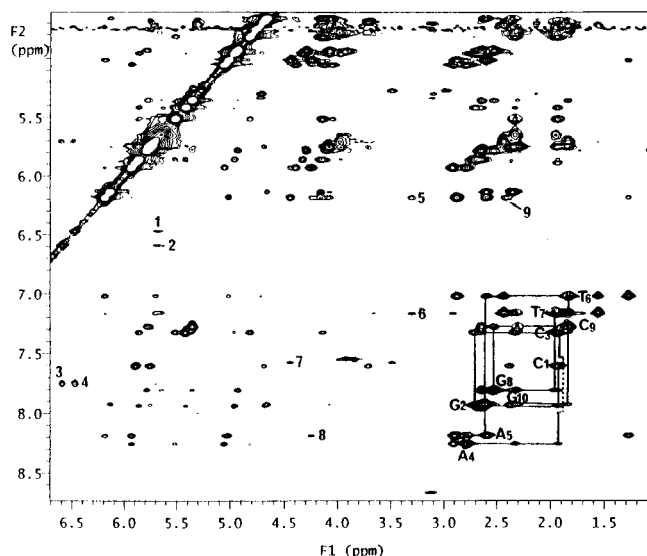


**Figure 2.** Expanded NOESY spectrum with 250 ms mixing time at 30 °C in D<sub>2</sub>O buffer for 1:1 Co·AMPHIS-NET-DNA complex. The solid line denotes the sequential connectivities in the NOESY spectrum between the base protons (H<sub>8,6</sub>) and its own and 5'-flanking sugar H<sub>2'</sub> protons. The intermolecular NOE cross-peaks between the cobalt complex and DNA were assigned to the following: (1) H<sub>20</sub>-T<sub>7</sub>-H<sub>1'</sub>, (2) H<sub>25</sub>-T<sub>17</sub>-H<sub>1'</sub>, (3) H<sub>25</sub>-A<sub>5</sub>-H<sub>2</sub>, (4) H<sub>20</sub>-A<sub>15</sub>-H<sub>2</sub>, (5) H<sub>16</sub>-A<sub>5</sub>-H<sub>1'</sub>, (6) H<sub>16</sub>-A<sub>14</sub>-H<sub>2</sub>, (7) H<sub>2</sub>-A<sub>15</sub>-H<sub>4</sub>, (8) H<sub>8</sub>-G<sub>9</sub>-H<sub>4</sub>, and (9) H<sub>29</sub>-A<sub>5</sub>-H<sub>1'</sub>.

to those used for the ligand free DNA. The NOESY spectrum (Figure 2) with 250 ms of mixing time in 10 mM phosphate buffer in D<sub>2</sub>O solution (pD 6.8, uncorrected) was primarily used to assign the base and the sugar protons for the ligand-bound DNA and to identify the intermolecular ligand-DNA contacts between non-labile protons. The aromatic base protons (H<sub>8</sub>, purine; H<sub>6</sub>, pyrimidine) and sugar H<sub>1'</sub>, H<sub>2'</sub>, and H<sub>2''</sub> protons were assigned through two independent magnetization transfer pathways involving the NOE interactions of purine/pyrimidine base protons to their own and 5'-flanking sugar H<sub>1'</sub> protons and to their own and 5'-flanking sugar H<sub>2'</sub> and H<sub>2''</sub> protons. The solid line denotes the sequential connectivity pathway from base protons to their own and 5'-flanking sugar H<sub>2'</sub> protons, and partial intermolecular NOE cross-peaks between the ligand and the DNA were labeled using numbers.

The further correct assignments of the adenine base H<sub>2</sub> resonances would be of use to establish the orientation of the ligand in the DNA complex later. These assignments were obtained from the interstrand and intras-trand cross-peaks between adenine H<sub>2</sub> protons and sugar H<sub>1'</sub> protons in the NOESY spectra in D<sub>2</sub>O solution. In general, for an AT-rich B-DNA sequence, the NOE interactions could be observed from the H<sub>2</sub> of an adenine residue to the H<sub>1'</sub> proton of its 3' neighbor and to the 3' neighbor of its complementary base due to highly propeller-twisted AT base pairs and the narrow minor groove width of the AT tract. The expansion of the aromatic base protons to H<sub>1'</sub> proton regions of NOESY spectrum of the AATT duplex taken with 1 equiv of added ligand is shown in Figure 3. The resonance at 7.16 ppm with NOESY cross-peaks to H<sub>1'</sub> protons of A<sub>5</sub> (the 3' neighbor of A<sub>4</sub>) and to G<sub>18</sub> residues (the 3' neighbor of the complementary base of A<sub>4</sub>) may be attributed to A<sub>4</sub> H<sub>2</sub>. Similarly, the resonance at 7.75 ppm generated NOESY cross-peaks to H<sub>1'</sub> protons of T<sub>6</sub> and T<sub>17</sub> residues and therefore must belong to A<sub>5</sub> H<sub>2</sub>. The assignments of the adenine base H<sub>2</sub> resonances were further confirmed through the sequential adenine H<sub>2</sub>-adenine H<sub>2</sub> NOE connectivities, as well as their 2D NOE cross-peaks with

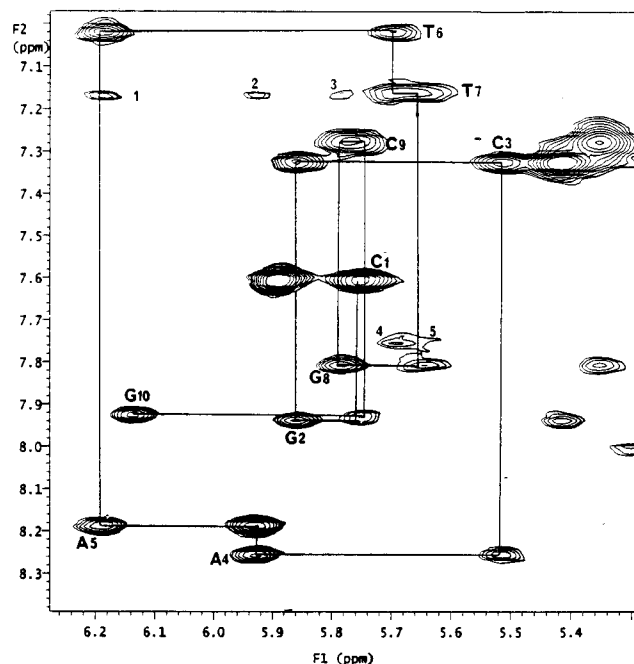


**Figure 3.** Expanded NOESY spectrum of 1:1 Co·AMPHIS-NET-DNA complex. The tracing outlines the sequential connectivities in the NOESY spectrum between the base protons (H<sub>8,6</sub>) and its own and 5'-flanking sugar H<sub>1'</sub> protons. The intra- and interstrand NOE cross-peaks were labeled as follows: (1) A<sub>4</sub>-H<sub>2</sub>-A<sub>5</sub>-H<sub>1'</sub> (or A<sub>14</sub>-H<sub>2</sub>-A<sub>15</sub>-H<sub>1'</sub>), (2) A<sub>4</sub>-H<sub>2</sub>-A<sub>4</sub>-H<sub>1'</sub> (or A<sub>14</sub>-H<sub>2</sub>-A<sub>14</sub>-H<sub>1'</sub>), (3) A<sub>4</sub>-H<sub>2</sub>-G<sub>18</sub>-H<sub>1'</sub> (or A<sub>14</sub>-H<sub>2</sub>-G<sub>8</sub>-H<sub>1'</sub>), (4) A<sub>5</sub>-H<sub>2</sub>-T<sub>6</sub>-H<sub>1'</sub> (or A<sub>15</sub>-H<sub>2</sub>-T<sub>16</sub>-H<sub>1'</sub>), and (5) A<sub>5</sub>-H<sub>2</sub>-T<sub>17</sub>-H<sub>1'</sub> (or A<sub>15</sub>-H<sub>2</sub>-T<sub>7</sub>-H<sub>1'</sub>).

**Table 1.** <sup>1</sup>H Chemical Shifts of DNA within the Co(III)·AMPHIS-NET Complex, Obtained by Phase-Sensitive NOESY with 250 ms of Mixing Time at 30 °C in D<sub>2</sub>O Solution<sup>a</sup>

|                 | H <sub>8</sub> /H <sub>6</sub> | H <sub>5</sub> /H <sub>2</sub> /Me | H <sub>1'</sub> | H <sub>2''</sub> | H <sub>2'</sub> | H <sub>3'</sub> | H <sub>4'</sub> | H <sub>5'</sub> | H <sub>5''</sub> |
|-----------------|--------------------------------|------------------------------------|-----------------|------------------|-----------------|-----------------|-----------------|-----------------|------------------|
| C <sub>1</sub>  | 7.60                           | 5.89                               | 5.75            | 2.36             | 1.93            | 4.69            | 4.05            | 3.71            |                  |
| G <sub>2</sub>  | 7.94                           |                                    | 5.86            | 2.70             | 2.65            | 4.96            | 4.33            | 4.07            | 3.96             |
| C <sub>3</sub>  | 7.32                           | 5.42                               | 5.51            | 2.33             | 1.93            | 4.82            | 4.14            | 4.04            |                  |
| A <sub>4</sub>  | 8.26                           | 7.16                               | 5.93            | 2.91             | 2.79            | 5.05            | 4.39            | 4.14            | 4.04             |
| A <sub>5</sub>  | 8.19                           | 7.75                               | 6.19            | 2.87             | 2.59            | 5.02            | 4.44            | 4.24            |                  |
| T <sub>6</sub>  | 7.02                           | 1.27                               | 5.70            | 2.43             | 1.83            | 4.72            | 4.09            |                 |                  |
| T <sub>7</sub>  | 7.16                           | 1.55                               | 5.64            | 2.35             | 1.95            | 4.79            | 4.05            |                 |                  |
| G <sub>8</sub>  | 7.81                           |                                    | 5.78            | 2.64             | 2.52            | 4.93            | 4.29            | 4.07            | 3.94             |
| C <sub>9</sub>  | 7.73                           | 5.41                               | 5.75            | 2.32             | 1.90            | 4.75            | 4.25            |                 |                  |
| G <sub>10</sub> | 7.93                           |                                    | 6.14            | 2.59             | 2.35            | 4.66            | 4.15            | 4.07            |                  |

<sup>a</sup> The solvent signal (δ = 4.75 ppm) was used as reference.

the flanking exchangeable imino protons that were observed in the NOESY spectrum acquired in H<sub>2</sub>O/D<sub>2</sub>O (90:10) solution. The chemical shifts of Co(III)·AMPHIS-NET-bound DNA are shown in Table 1.

By comparison of the intraresidue NOE cross-peak patterns and the interresidue NOE cross-peak patterns of the ligand-bound DNA with that of the ligand free DNA, there is no discernible distortion of the DNA helix as a result of ligand binding, and the DNA retained the B-form conformation in the complex. The MINSY (59) and the ROESY (60) experiments of the ligand-binding DNA (data not shown) also confirm the B-form conformation of the ligand-bound DNA (61). The binding of the Co(III)·AMPHIS-NET did not disrupt the C<sub>2</sub> symmetry of the DNA duplex, indicative of fast exchange and possibly relatively weak binding and consistent with the NMR titration results.

**Assignments of the Ligand Protons within the Ligand-DNA Complex.** The assignments of the ligand protons in the Co(III)·AMPHIS-NET-DNA complex, including the metal-binding domain and DNA-binding

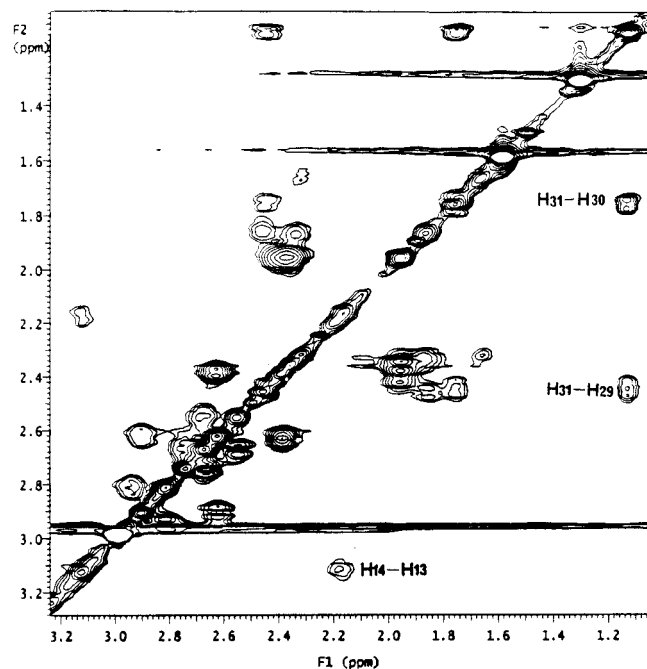


**Figure 4.** Expanded TOCSY spectrum of 1:1 Co·AMPHIS-NET-DNA complex shown with partial COSY and RELAY connectivities of ligand in DNA complex.

domain, have also been made by COSY, NOESY, and TOCSY spectra analysis. The TOCSY spectrum of the ligand-DNA complex with 75 ms of mixing time in upfield regions is shown in Figure 4. The aliphatic protons of the propyl end group in the DNA-binding domain of the ligand were identified according to the couplings and relay connectivities in the COSY and TOCSY spectra of the ligand-DNA complex. The assignments of H<sub>20</sub> and H<sub>25</sub> pyrrole protons lining the convex edge of the ligand were identified according to their intra- and intermolecular NOE cross-peaks. Figure 8 shows the NOESY spectrum of Co(III)·AMPHIS-NET-DNA complex in water solution (H<sub>2</sub>O:D<sub>2</sub>O = 9:1). For the exchangeable resonance at 9.34 ppm, a NOE cross-peak to the H<sub>29</sub> proton at the end propyl group of the ligand was observed. This exchangeable resonance was assigned to the NH-12 amide proton. This amide proton also had a NOE cross-peak to the resonance at 6.63 ppm, which was assigned to H<sub>25</sub> pyrrole proton at 6.63 ppm. Corresponding to the exchangeable resonance at 9.01 ppm, it was assigned to NH-10 amide proton from two NOE cross-peaks at 9.01/6.63 and 9.01/6.51 ppm which resulted from the cross-relaxation interaction between NH-10 amide proton and H<sub>25</sub> pyrrole proton and between NH-10 amide proton and H<sub>20</sub> pyrrole proton. Thus, the resonance at 6.51 ppm was assigned to the H<sub>20</sub> pyrrole proton. Weak cross-peaks at 6.51/7.55 and 6.63/7.55 ppm observed in the TOCSY spectrum, which resulted from the small long range coupling between two pairs of pyrrole protons, permitted the assignment of H<sub>22</sub> and H<sub>27</sub> pyrrole protons with an identical chemical shift at 7.55 ppm. Subsequently, the resonances of two *N*-methyl protons of pyrrole were identified unequivocally as H<sub>32</sub> *N*-methyl at 3.92 ppm and H<sub>33</sub> *N*-methyl at 3.90 ppm by the cross-peak with the pyrrole protons lying along the concave edge of the ligand in the NOESY spectrum.

The proton assignments of the metal-binding domain of ligand were also obtained from the NOESY and TOCSY experiments. The intraligand NOE interactions provide useful guidance for the assignments of the protons of the metal-binding domain within Co(III)·AMPHIS-NET-DNA complex. The chemical shifts of the

**Table 2.** Chemical Shifts of Ligand Protons within 1:1 Co·AMPHIS-NET-DNA Complex

| proton           | chemical shift (ppm) | proton                             | chemical shift (ppm) |
|------------------|----------------------|------------------------------------|----------------------|
| H <sub>1</sub>   | 8.66                 | H <sub>14</sub>                    | 2.18                 |
| H <sub>2</sub>   | 7.58                 | H <sub>16</sub>                    | 3.30                 |
| H <sub>4'</sub>  | 3.48                 | H <sub>20</sub>                    | 6.51                 |
| H <sub>4</sub>   | 2.98                 | H <sub>22</sub>                    | 7.55                 |
| H <sub>5</sub>   | 5.27                 | H <sub>25</sub>                    | 6.63                 |
| H <sub>8</sub>   | 8.20                 | H <sub>27</sub>                    | 7.55                 |
| H <sub>9</sub>   | 8.49                 | H <sub>29</sub>                    | 2.44                 |
| H <sub>10</sub>  | 8.01                 | H <sub>30</sub>                    | 1.74                 |
| H <sub>12</sub>  | 4.72                 | H <sub>31</sub>                    | 1.13                 |
| H <sub>12'</sub> | 5.31                 | H <sub>32</sub> (CH <sub>3</sub> ) | 3.92                 |
| H <sub>13</sub>  | 3.12                 | H <sub>33</sub> (CH <sub>3</sub> ) | 3.90                 |
| H <sub>13'</sub> | 3.15                 | NH-10                              | 9.01                 |
| NH-12            | 9.34                 |                                    |                      |

**Table 3.** Intermolecular NOE Restraints of DNA Generated from the Volume Integrals of the Cross-Peaks in the NOESY Spectrum of the DNA Complex with the Cobalt Complex of AMPHIS-NET

| DNA                             | ligand          | NOE classification |
|---------------------------------|-----------------|--------------------|
| A <sub>15</sub> H <sub>2</sub>  | H <sub>25</sub> | s                  |
| A <sub>5</sub> H <sub>2</sub>   | H <sub>20</sub> | s                  |
| A <sub>5</sub> H <sub>4'</sub>  | H <sub>2</sub>  | m                  |
| A <sub>4</sub> H <sub>2</sub>   | H <sub>20</sub> | w                  |
| A <sub>14</sub> H <sub>2</sub>  | H <sub>25</sub> | w                  |
| A <sub>4</sub> H <sub>2</sub>   | H <sub>16</sub> | m                  |
| A <sub>5</sub> H <sub>1'</sub>  | H <sub>16</sub> | m                  |
| A <sub>15</sub> H <sub>1'</sub> | H <sub>29</sub> | s                  |
| C <sub>19</sub> H <sub>4'</sub> | H <sub>8</sub>  | m                  |
| T <sub>7</sub> H <sub>1'</sub>  | H <sub>25</sub> | m                  |
| T <sub>17</sub> H <sub>1'</sub> | H <sub>20</sub> | m                  |
| A <sub>15</sub> H <sub>2</sub>  | NH-12           | s                  |
| A <sub>5</sub> H <sub>2</sub>   | NH-10           | s                  |

**Table 4.** Intramolecular NOE Restraints of Ligand Generated from the Volume Integrals of the Cross-Peaks in the NOESY Spectrum of the DNA Complex with the Cobalt Complex of AMPHIS-NET

| proton pairs                      | classification | proton pairs                      | classification |
|-----------------------------------|----------------|-----------------------------------|----------------|
| H <sub>10</sub> -H <sub>12'</sub> | s              | H <sub>1</sub> -H <sub>13</sub>   | s              |
| H <sub>22</sub> -H <sub>32</sub>  | s              | H <sub>27</sub> -H <sub>33</sub>  | s              |
| H <sub>2</sub> -H <sub>4'</sub>   | s              | H <sub>12</sub> -H <sub>12'</sub> | s              |
| H <sub>5</sub> -H <sub>4'</sub>   | s              | H <sub>5</sub> -H <sub>4</sub>    | s              |
| N <sub>12</sub> -H <sub>13'</sub> | s              | H <sub>4</sub> -H <sub>4'</sub>   | s              |
| H <sub>13'</sub> -H <sub>14</sub> | s              | H <sub>9</sub> -H <sub>8</sub>    | s              |
| H <sub>9</sub> -H <sub>10</sub>   | s              |                                   |                |

ligand protons within the Co(III)·AMPHIS-NET-DNA complex are listed in Table 2. Most of these intraligand NOE interactions were also observed in the NOESY spectrum of the unbound Co(III)·AMPHIS-NET (49) which suggests that the structure of the metal-binding domain of the Co(III)·AMPHIS-NET does not significantly change upon complexation with the DNA.

**Intermolecular Contacts.** As discussed above, despite the asymmetry of the whole complex induced by the asymmetric ligand, pseudo-C<sub>2</sub> symmetry-related protons are not sufficiently different to allow a definite assignment to individual strands. Thus, the following assignment takes into account the geometry of the overall complex. One of two equivalent complex structures is presented schematically in Figure 5.

The close contacts between the ligand protons and the DNA minor groove marker protons, for example the sugar H<sub>1'</sub> protons and the adenine H<sub>2</sub> protons on the groove floor, are generally identified by the associated intermolecular nuclear Overhauser interactions. Accordingly, the NOE cross-peaks between the H<sub>20</sub> pyrrole proton and the sugar H<sub>1'</sub> proton of T<sub>17</sub>, between H<sub>25</sub> pyrrole proton and the sugar H<sub>1'</sub> proton of T<sub>7</sub>, and between H<sub>29</sub> proton of the propyl end group and the sugar H<sub>1'</sub> proton of A<sub>15</sub> were observed. Strong NOE cross-peaks of H<sub>20</sub> and H<sub>25</sub>

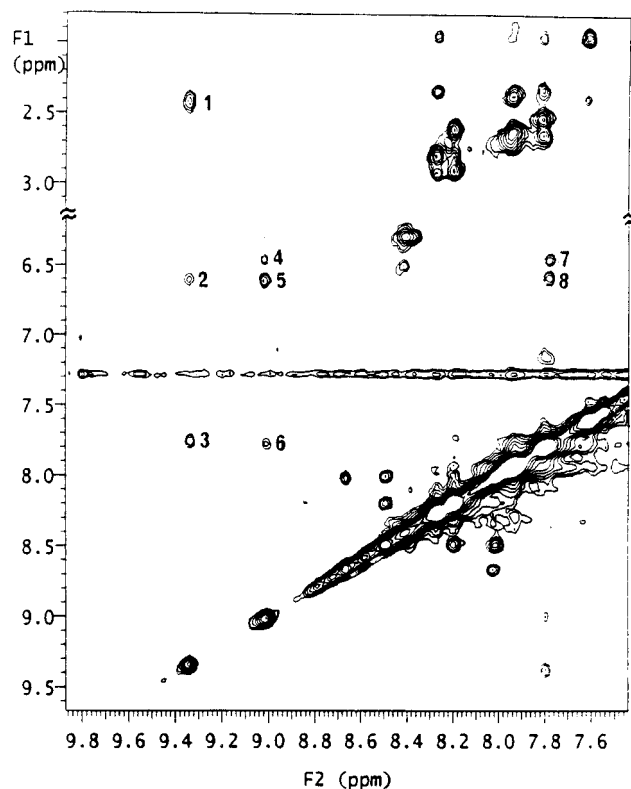


**Figure 5.** Expanded NOESY spectrum with 250 ms mixing time at 30 °C in H<sub>2</sub>O/D<sub>2</sub>O (9:1) solution for 1:1 Co-AMPHIS-NET-DNA complex. The intra- and intermolecular NOE cross-peaks were assigned to the following: (1) NH-12-H<sub>29</sub>, (2) NH-12-H<sub>25</sub>, (3) NH-12-A<sub>15</sub>-H<sub>2</sub>, (4) NH-10-H<sub>20</sub>, (5) NH-10-H<sub>25</sub>, (6) NH-10-A<sub>5</sub>-H<sub>2</sub>, (7) H<sub>20</sub>-A<sub>15</sub>-H<sub>2</sub>, and (8) H<sub>25</sub>-A<sub>5</sub>-H<sub>2</sub>.

pyrrole protons of ligand with adenine H<sub>2</sub> of DNA were also observed. The assignment of the NOE cross-peak between H<sub>20</sub> pyrrole proton of ligand and A<sub>5</sub> H<sub>2</sub> proton at the minor groove floor of DNA instead of the cross-peak between H<sub>20</sub> proton of ligand and A<sub>15</sub> H<sub>2</sub> proton is based on the consistency between the distance derived from NOE cross-peak intensity and the expected proton-proton distances. This assignment is also consistent with previous structural studies on lexitropsin-DNA interactions (51, 56, 57). The same situation applies to the assignment of the NOE interactions from H<sub>25</sub> pyrrole proton of ligand to A<sub>15</sub> H<sub>2</sub> DNA proton as well. Similarly, the intermolecular NOE contacts between NH-12 amide proton and A<sub>15</sub> H<sub>2</sub> proton and between NH-10 amide proton and A<sub>5</sub> H<sub>2</sub> proton were identified.

These intermolecular NOE interactions, in combination with the NOE interactions from the linker protons of the cobalt complex to both A<sub>4</sub> H<sub>2</sub> proton and A<sub>5</sub> H<sub>1'</sub> sugar proton provided strong support for minor groove binding of the ligand and suggest that the lexitropsin moiety of the cobalt complex is located in the minor groove at the central (AATT)<sub>2</sub> segment of DNA. The plot of the ligand-induced chemical shift changes of both the base protons and minor groove protons versus the DNA sequence, so-called "NMR footprinting", also demonstrated that the ligand binds in the minor groove at the central (AATT)<sub>2</sub> core of the DNA, because the ligand binding in the minor groove of the DNA strongly influenced the chemical shifts of nucleotides A<sub>4</sub>-T<sub>7</sub> but only weakly influenced nucleotides of G<sub>8</sub> and C<sub>3</sub>.

Significantly, NOE cross-peaks were observed between protons in the metal-binding domain of the ligand and sugar protons in the minor groove. The NOE cross-peaks between the H<sub>8</sub> pyridine proton in the metal-binding domain of the ligand with the H<sub>4'</sub> sugar proton of

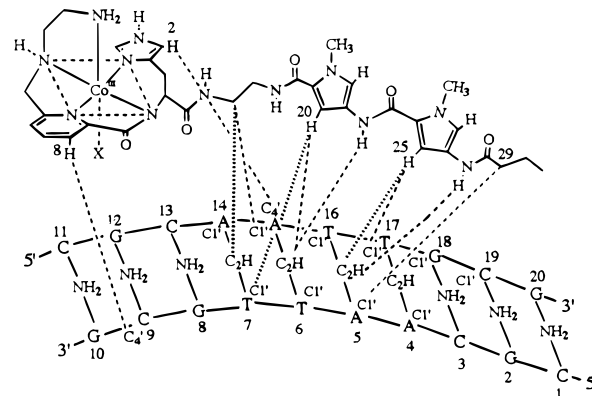


**Figure 6.** Schematic representation of selected intermolecular NOEs between the cobalt complex of AMPHIS-NET and d(5'-CGCAATTGCG-3')<sub>2</sub> in the 1:1 Co-AMPHIS-NET-DNA complex.

nucleotides C<sub>19</sub> of the DNA and between the H<sub>2</sub> imidazole proton in the metal-binding domain of the ligand and the H<sub>4'</sub> sugar proton of nucleotides A<sub>5</sub> on the other strand of the DNA were observed in the NOESY spectrum. These NOE interactions suggest that the metal-binding domain of Co(III)-AMPHIS-NET participates in DNA binding. Since the pyridine and imidazole groups are positioned rigidly on the basal plane of coordination, these NOE contacts effectively define the orientation of the metal-binding domain with respect to the minor groove, allowing a detailed structure to be built by the following molecular modeling. The intermolecular NOE contacts between ligand and DNA are summarized diagrammatically in Figure 6.

**Structure of Ligand-DNA Complex and the Further Design Direction.** The distance restraints derived from NOE experiments were utilized to obtain a refined complex structure for the Co(III)-AMPHIS-NET-DNA complex using a molecular dynamics approach. The structure of the Co(III)-AMPHIS-NET-d(CGCAATTGCG)<sub>2</sub> complex as refined by restrained molecular dynamics calculation and energy minimization is shown in Figure 6. The molecular modeling results clearly indicate that the Co(III)-AMPHIS-NET ligand fits snugly into the minor groove of the DNA and its metal binding moiety indeed participates in DNA binding in the minor groove. The orientation of the hydrophobic propyl end of the ligand molecule is somewhat free with respect to the minor groove in that limited rotation along C<sub>28</sub>-C<sub>29</sub> and along C<sub>29</sub>-C<sub>30</sub> is permitted.

Figure 7 shows a detailed picture of how the metal-binding domain resides within the minor groove of the DNA. The metal-binding moiety of Co(III)-AMPHIS-NET is located between the A<sub>4</sub>·T<sub>17</sub> base pair and the G<sub>2</sub>·C<sub>19</sub> base pair. The basal plane projects into the minor groove with the pyridinecarboxamide edge at a certain angle such that the coordinating water bound to the metal atom points into the floor of the minor groove. The central metal is roughly two residues away from the AATT site. The ligand molecule causes slight widening of the DNA minor groove in its proximity, and distortion of the C<sub>3</sub>·G<sub>18</sub> base pair probably results from the bulky and rigid moiety (metal-binding domain) of Co(III)-AMPHIS-NET. The energy-minimized structure shows hydrogen bonds between a primary amine proton in the axial position and a DNA backbone phosphate oxygen of G<sub>20</sub> (O-N distance is 2.87 Å) and between the amino proton of G<sub>18</sub> and the carbonyl oxygen atom of the carboxyl group attached to the pyridine ring (N-O distance is 2.78 Å). These hydrogen bonds are important in stabilizing the Co(III)-AMPHIS-NET-DNA complex. In addition, it is likely that the inserted portion of the

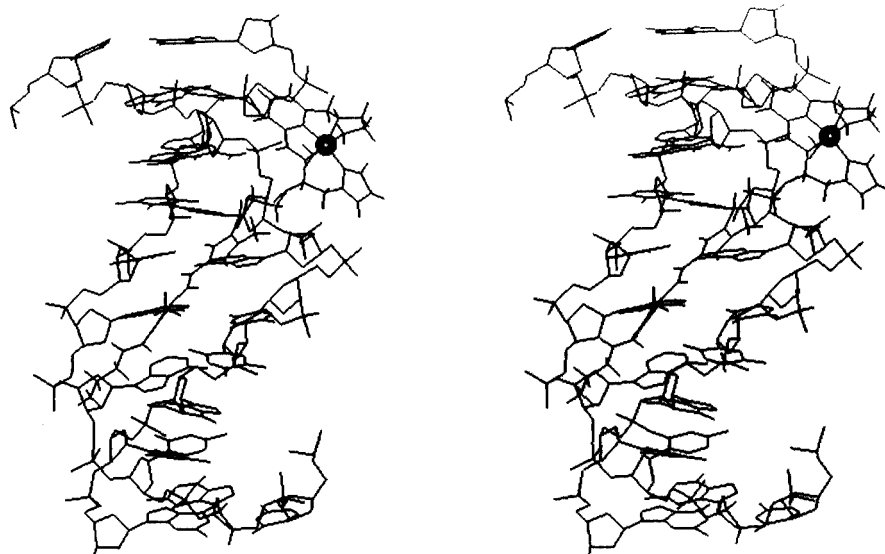


**Figure 7.** Stereodrawing of the structure of the Co(III)-AMPHIS-NET-d(CGCAATTGCG)<sub>2</sub> complex obtained by energy minimization and restrained molecular dynamics. For clarity, hydrogen atoms are omitted from the DNA structure but not from the Co(III)-AMPHIS-NET structure. The cobalt ion is depicted as a gray ball. A slight widening of the DNA minor groove is apparent.

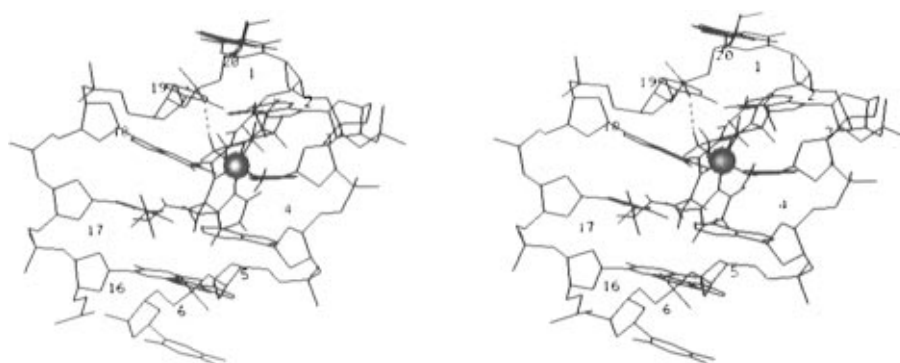


**Figure 8.** Stereodrawing of the metal-binding domain of the Co(III)-AMPHIS-NET complex in the minor groove of the DNA.

metal binding domain may help stabilize the anchoring by van der Waals interactions with the sugar moiety in the groove.

The binding model of the Co(III)-AMPHIS-NET to the DNA differs entirely from that of metal-bleomycin-DNA complex (12–16, 62). The metal-binding moiety of bleomycin within Zn(II)-bleomycin A<sub>2</sub>-DNA complex and Zn(II)-bleomycin A<sub>5</sub>-DNA complex (16) folds back to the bithiazole residue through a turn at the junction between the threonine and methyl valerate as deduced from 2D NMR and restrained molecular dynamics computation. The structure studies of the cobalt complex of bleomycin A<sub>2</sub> (green) binding to DNA also indicated that the DNA-binding moiety intercalates into the binding site, while the metal-binding moiety folds back to bind in the DNA minor groove and plays a key role in the DNA cleavage specificity. However, there is no evidence that the cobalt complex of the AMPHIS-NET hybrid forms a folded structure. The DNA binding moiety of the cobalt complex of AMPHIS-NET is a minor groove binder and tends to enter the minor groove of DNA, while the linker moiety joined to the metal-binding moiety and carrier is too short to form a folded structure like bleomycin. So it is reasonable that the metal-binding moiety with square pyramid coordinate geometry for Co(III)-AMPHIS-NET extends farther in the region two base pairs beyond the central AATT binding site into the minor groove of the DNA. This structural feature of the Co(III)-AMPHIS-NET-d(CGCAATTGCG)<sub>2</sub> complex provides a reasonable interpretation for the observed DNA cleavage induced by the corresponding iron(II) complexes of AMPHIS-NET

and other AMPHIS-lexitropsin hybrids of the bleomycin functional model compounds that occurs consistently two to three bases beyond the end of AT-rich binding sites. It also explains the size of the binding site (beyond the four-base minimum requirement (38) and why the Fe(II)-AMPHIS-lexitropsin exhibits different sequence selectivity and different cleavage products compared with the iron(II)-bleomycin complex (38, 39).

As mentioned above, Co(III)-AMPHIS-NET binds to the DNA in a 1:1 stoichiometry throughout the titration and undergoes fast exchange on the NMR time scale, while the parent compound netropsin has been shown to bind to the DNA in slow exchange. This fact suggests that the binding affinity of Co(III)-AMPHIS-NET to the DNA is relatively low in comparison with that of netropsin. The binding constants for Co(III)-AMPHIS-NET and metal free AMPHIS-NET to poly(dA)·poly(dT) DNA, measured with the ethidium displacement assay (63, 64), are  $2.26 \times 10^5$  and  $4.75 \times 10^4$  M<sup>-1</sup>, respectively.<sup>1</sup> The relatively lower DNA binding affinities of Co(III)-AMPHIS-NET result at least partially from the rigid and bulky structure of the metal-binding domain. Netropsin is a

<sup>1</sup> The binding constants were measured exploiting the ability of the ligand to compete with binding of EtBr on DNAs. The experiments were done under the condition used previously (64). The binding constants of Co(III)-AMPHIS-NET and metal free AMPHIS-NET to poly(dA)·poly(dT) were calculated using C50 (concentration of the compound required to reduce fluorescence of DNA bound EtBr to 50%) values assuming a binding site size of four base pairs and the binding constant of EtBr as  $1.0 \times 10^6$  M<sup>-1</sup> for poly(dA)·poly(dT).

dicationic minor groove binder, and its two cationic termini may assist in anchoring the ligand molecule firmly in the minor groove of DNA by electrostatic interaction. Although Co(III)-AMPHIS-NET has two net positive charges in the metal-binding domain located in the carboxyl terminus, they are very much shielded from the DNA backbone by the surrounding bulky functional group. In addition, Co(III)-AMPHIS-NET has an uncharged and hydrophobic propyl group in the amino terminus. The hydrophobic and neutral propyl group cannot contribute to the Co(III)-AMPHIS-NET binding to DNA by either electrostatic attraction or H-bond formation, although it may improve the cell-uptake properties and eventually increase the cytotoxicity (65). The shorter linker moiety joining the metal-binding domain and the DNA-binding domain also may reduce the overall DNA-binding affinity, which has been demonstrated by our studies on the linker-modified bleomycin functional model compounds (39). Therefore, the structure studies of the Co(III)-AMPHIS-NET binding to DNA not only account for the binding affinity and DNA cleavage specificity of the metal complex of AMPHIS-lexitropsin hybrid compounds but also provide new insight into the further molecular design. The Co(III)-AMPHIS-NET molecules demonstrate that the cobalt complexes of AMPHIS-lexitropsins can be used to cleave DNA under the irradiation of UV or visible light. The binding affinity and the DNA cleavage selectivity of the metal complexes of AMPHIS-lexitropsin hybrids could be optimized by increasing the number of pyrrole carboxamide as well as the length of the linker moiety. The modification of the end group of the lexitropsin and the DNA binding moiety also provide the possibility to improve the DNA binding affinity and cleavage specificity. On the basis of the structure studies of the Co(III)-AMPHIS-NET-DNA complex, a new type of AMPHIS-lexitropsin hybrid compound with a positively charged end group and its cross-linked dimer was synthesized by our group (66). The structure studies and the biochemical investigation of these new compounds are in progress in our laboratory. The initial results show that these molecules have very good DNA binding affinity and cleavage specificity.

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#### LITERATURE CITED

- (1) Blum, R. F., Carter, S. K., Agre, K. A. (1973) A clinical review of bleomycin-a new antineoplastic agent. *Cancer* 31, 903-914.
- (2) Stubbe, J., and Kozarich, J. W. (1987) Mechanism of bleomycin-induced DNA degradation. *Chem. Rev.* 87, 1107-1136.
- (3) D'Andrea, A. D., and Haseltine, W. A. (1978) Sequence specific cleavage of DNA by the antitumor antibiotics neocarzinostatin and bleomycin. *Proc. Natl. Acad. Sci. U.S.A.* 75, 3608-3612.
- (4) Sausville, E. A., Peisach, J., and Horwitz, S. B. (1978) Effects of chelating agents and metal ions on the degradation of DNA by bleomycin. *Biochemistry* 17, 2740-2746.
- (5) Hecht, S. M. (1994) RNA degradation by bleomycin, a naturally occurring bioconjugate *Bioconjugate Chem.* 5, 513-526.
- (6) Saito, I., Morii, T., Sugiyama, H., Matsuura, T., Meares, C. F., and Hecht, S. M. (1989) Photoinduced DNA strand scission by cobalt bleomycin green complex. *J. Am. Chem. Soc.* 111, 2307-2308.
- (7) Chang, C. H., and Meares, C. F. (1982) Light-induced nicking of deoxyribonucleic acid by cobalt(III) bleomycin. *Biochemistry* 21, 6332-6334.
- (8) Subramanian, R., and Meares, C. F. (1986) Photosensitization of cobalt bleomycin. *J. Am. Chem. Soc.* 108, 6427-6428.
- (9) Dabrowiak, J. C., and Tsakayama, M. (1981) Cobalt(III) complex of pseudotetrapeptide A of bleomycin. *J. Am. Chem. Soc.* 103, 7543-7550.
- (10) Xu, R. X., Nettesheim, D., Otvos, J. D., and Petering, D. H. (1994) *Biochemistry* 33, 907-916.
- (11) Tan, J. D., Farinas, E. T., Davis, S. S., and Mascharak, P. K. (1994) NMR evidence of sequence specific DNA binding by a cobalt(III)-bleomycin analogue with tethered acridine. *Inorg. Chem.* 33, 4295-4308.
- (12) Wu, W., Vanderwall, D. E., Stubbe, J., Kozarich, J. W., and Turner, C. J. (1994) Interaction of Co-bleomycin A2 (green) with d(CCAGGCCTGG)<sub>2</sub>: Evidence for intercalation using 2D NMR. *J. Am. Chem. Soc.* 116, 10843-10845.
- (13) Wu, W., Vanderwall, D. E., Lui, S. M., Tang, X.-J., Turner, C. J., Kozarich, J. W., and Stubbe, J. (1996) *J. Am. Chem. Soc.* 118, 1268-1280.
- (14) Wu, W., Vanderwall, D. E., Turner, C. J., Kozarich, J. W., and Stubbe, J. (1996) Solution structure of Co-bleomycin A2 (green) complexed with d(CCAGGCCTGG). *J. Am. Chem. Soc.* 118, 1281-1294.
- (15) Manderville, R. A., Ellena, J. F., and Hecht, S. M. (1994) Solution structure of a Zn(II)-Bleomycin A<sub>5</sub>-d(CGCTAGCG)<sub>2</sub>. *J. Am. Chem. Soc.* 116, 10851-10852.
- (16) Manderville, R. A., Ellena, J. F., and Hecht, S. M. (1995) Interaction of Zn(II)-bleomycin with d(CGCTAGCG)<sub>2</sub>. A binding model based on NMR experiments and restrained molecular dynamics calculations. *J. Am. Chem. Soc.* 117, 7891-7903.
- (17) Chang, C. H., and Meares, C. F. (1984) Cobalt-bleomycin and deoxyribonucleic acid: Sequence-dependent interactions, actions spectrum for nicking and indifference to oxygen. *Biochemistry* 23, 2268-2274.
- (18) Nightingale, K. P., and Fox, K. R. (1994) Light-activated cleavage of DNA by cobalt-bleomycin. *Eur. J. Biochem.* 220, 173-181.
- (19) Tan, J. D., Hudson, S. E., Brown, S. J., Olmstead, M. M., and Mascharak, P. K. (1992) Synthesis, structures and reactivities of synthetic analogues of the three forms of Co(III)-bleomycin: proposed mode of light-induced DNA damage by the Co(III) chelate of the drug. *J. Am. Chem. Soc.* 114, 3841-3853.
- (20) Farinas, E. T., Tan, J. D., Baidya, N., and Mascharak, P. K. (1993) A designed synthetic analogue of Co(III)-bleomycin with enhanced DNA-binding and photocleaving activity. *J. Am. Chem. Soc.* 115, 2996-2997.
- (21) Julia, M., and Préau-Joseph, N. (1967) Amidines et guanidines apparentées à la congocidine. I. Structure de la congocidine (Amidines et guanidines related to congocidine. I. Structure of congocidine). *Bull. Soc. Chim. Fr.*, 4348-4356.
- (22) Huang, L., Quada, J. C., and Lown, J. W. (1995) Functional models of the antitumor antibiotic bleomycin. *Curr. Med. Chem.* 2, 543-560.
- (23) Hahn, F. E. (1975) Distamycin A and netropsin. In *Antibiotics III. Mechanism of Action of Antimicrobial and Antitumor Agents* (J. W. Corcoran and F. E. Hahn, Eds.) p 79, Springer-Verlag, New York.
- (24) Zimmer, C., and Wahnert, U. (1986) Non-intercalating DNA-binding ligand: Specificity of the interaction and their use as tools in biophysical and biological investigations of the genetic material. *Prog. Biophys. Mol. Biol.* 47, 31-112.
- (25) Dervan, P. B. (1986) Design of sequence-specific DNA-binding molecules. *Science* 232, 464-471.
- (26) Kopka, M. L., Yoon, C., Godsell, D., Pjura, P., and Dickerson, R. E. (1985) The molecular origin of DNA-drug specificity in netropsin. *Proc. Natl. Acad. Sci. U.S.A.* 82, 1376-1380.

- (27) Patel, D. J., and Shapiro, L. (1985) Molecular recognition in noncovalent antitumor agent-DNA complexes: NMR studies of the base and sequence dependent recognition of the DNA minor groove by netropsin. *Biochimie* 67, 887–915.
- (28) Pardi, A., Morden, D. M., Patel, D. J., and Tinoco, I. (1983) Kinetics for exchange of imino protons of the d(CGCGAATTCGCG) double helix in complex with the antibiotics netropsin and/or actinomycin. *Biochemistry* 22, 1107–1113.
- (29) Coll, M., Aymami, J., van der Marel, G. A., van Boom, J. H., Rich, A., and Wang, A. H.-J. (1989) Molecular structure of the netropsin-d(CGCGATATCGCG)<sub>2</sub> complex: DNA conformation in an alternating AT segment. *Biochemistry* 28, 310–320.
- (30) Kleivit, R. E., Wemmer, D. E., and Reid, B. R. (1986) <sup>1</sup>H NMR studies on the interaction between distamycin A and a symmetrical DNA dodecamer. *Biochemistry* 25, 3296–3303.
- (31) Pelton, J. G., and Wemmer, D. E. (1988) Structural modeling of the distamycin A-d(CGCAATTGGC) complex using 2D NMR and molecular mechanics. *Biochemistry* 27, 8088–8096.
- (32) Coll, M., Frederick, C. A., Wang, A. H.-J., and Rich, A. (1987) A bifurcated hydrogen-bonded conformation in the d(AT) base pair of the DNA dodecamer d(CGCAAATTTGCG) and its complex with distamycin. *Proc. Natl. Acad. Sci. U.S.A.* 84, 8385–8389.
- (33) Pelton, J. G., and Wemmer, D. E. (1989) Structural characterization of a 2:1 distamycin A-d(CGCAAATTTGGC) complex by two dimensional NMR. *Proc. Natl. Acad. Sci. U.S.A.* 86, 5723–5727.
- (34) Pelton, J. G., and Wemmer, D. E. (1990) Binding models of distamycin A with d(CGCAAATTTGCG)<sub>2</sub> determined by two-dimensional NMR. *J. Am. Chem. Soc.* 112, 1393–1399.
- (35) Chen, X., Ramakrishnan, B., Rao, S. T., and Sundaralingam, M. (1994) Binding of two distamycin A molecules in the minor groove of an alternating B-DNA duplex. *Nat. Struct. Biol.* 1, 169–175.
- (36) Huang, L., Morgan, A. R., and Lown, J. W. (1993) Design of DNA-cleaving molecules which incorporate a simplified metal-complexing moiety of bleomycin and lexitropsin carriers. *Biomed. Chem. Lett.* 3, 1751–1756.
- (37) Huang, L., Quada, J. C., and Lown, J. W. (1994) Design and synthesis of DNA cleaving bleomycin models: 1,2-trans-Disubstituted cyclopropane units as novel linkers. *Tetrahedron Lett.* 35, 5323–5326.
- (38) Huang, L., Quada, J. C., and Lown, J. W. (1995) Design, synthesis, and sequence selective DNA cleavage of functional models of models of bleomycin. I. Hybrids incorporating a simple metal-complexing moiety of bleomycin and lexitropsin carriers. *Bioconjugate Chem.* 6, 21–33.
- (39) Huang, L., Quada, J. C., and Lown, J. W. (1995) Design, synthesis, and sequence selective DNA cleavage of functional models of models of bleomycin. II. 1,2-trans-disubstituted cyclopropane units as novel linker. *Bioorg. Med. Chem.* 3, 647–657.
- (40) Hénichart, J. P., Houssin, R., Bernier, J. L., and Catteau, J. P. (1982) Synthetic model of a bleomycin metal complex. *J. Chem. Soc., Chem. Commun.* 1295–1297.
- (41) Hénichart, J. P., Bernier, J. L., Houssin, R., Lohez, M., Kenani, A., and Catteau, J. P. (1985) Copper(II)- and iron(II)-complexes of methyl 2-(2-aminoethyl)-aminomethyl-pyridine-6-carboxyl-histidinate (AMPHIS), a peptide mimicking the metal-chelating moiety of bleomycin. An ESR investigation. *Biochem. Biophys. Res. Commun.* 126, 1036–1039.
- (42) Lown, J. W., Krowicki, K., Bhat, U. G., Skorobogaty, A., Ward, B., and Dabrowiak, J. C. (1986) Molecular recognition between oligopeptides and nucleic acids: Novel imidazole-containing oligopeptides related to netropsin that exhibit altered DNA sequence specificity. *Biochemistry* 25, 7408–7416.
- (43) Lown, J. W. (1988) Lexitropsins: Rational Design of DNA Sequence Reading Agents as Novel Anti-cancer Agents and Potential Cellular Probes. *Anti-Cancer Drug Des.* 3, 25–40.
- (44) Lown, J. W. (1994) DNA recognition by lexitropsins, minor groove binding agents. *J. Mol. Recognit.* 7, 79–88.
- (45) Lown, J. W. (1993) Targeting the DNA minor groove for control of biological function: Progress, challenges, and prospects. *Chemtracts: Org. Chem.* 6, 205–237.
- (46) Lee, M., Krowicki, K., Shea, R. G., Lown, J. W., and Pon, R. T. (1989) Molecular recognition between oligopeptides and nucleic acids. Sequence specific binding of (4S)-(+)- and (4R)-(-)-dihydrokikumycin B to DNA deduced from <sup>1</sup>H NMR, footprinting studies and thermodynamic data. *J. Mol. Recognit.* 2, 84–93.
- (47) Rao, K. E., Shea, R. G., Yadagiri, B., and Lown, J. W. (1990) Molecular recognition between oligopeptides and nucleic acids: DNA sequence specificity and binding properties of thiazole-lexitropsins incorporating the concepts of base site acceptance and avoidance. *Anti-Cancer Drug Des.* 5, 3–20.
- (48) Zimmerman, J., Rao, K. E., Joseph, T., Sapse, A. M., and Lown, J. W. (1991) Amide isosteres of lexitropsins: Synthesis, DNA binding characteristics and sequence selectivity of thioformyl-distamycin. *J. Biomol. Struct. Dyn.* 3, 599–611.
- (49) Yang, Y., Huang, L., Cheng, S.-F., Chang, D.-K., and Lown, J. W. (1996) (manuscript in preparation).
- (50) Bauer, H. F., and Drinkard, W. C. (1966) Sodium tricarboxonatecobaltate(III) 3-hydrate. *Inorg. Synth.* 8, 202–204.
- (51) Lee, M., Krowicki, K., Hartley, J. A., Pon, R. T., and Lown, J. W. (1988) Molecular recognition between oligopeptides and nucleic acids: Influence of van der Waals contacts in determining the 3'-terminus of DNA sequences read by monocationic lexitropsins. *J. Am. Chem. Soc.* 110, 3641–3649.
- (52) Singh, M. P., Kumar, S., Joseph, T., Pon, R. T., Lown, J. W. (1992) A <sup>1</sup>H NMR study of the DNA binding characteristics of thioformyl-distamycin, a amide isosteric lexitropsin. *Biochemistry* 31, 6453–6461.
- (53) Cantor, C. R., Warshaw, M. M., and Shapiro, H. (1970) Oligonucleotide interactions. III. Circular dichroism studies of the conformation of deoxyoligonucleotides. *Biopolymers* 9, 1059–1077.
- (54) States, D. J., Haberkorn, R. A., and Ruben, D. J. (1982) A two-dimensional nuclear Overhauser experiment with pure absorption phase in four quadrants. *J. Magn. Reson.* 48, 286–292.
- (55) Baleja, J. D., Moul, J., and Sykes, B. D. (1990) Distance measurement and structure refinement with NOE data. *J. Magn. Reson.* 87, 375–384.
- (56) Lee, M., Krowicki, K., Pon, R. T., and Lown, J. W. (1988) Structural and dynamic aspects of the sequence specific binding of netropsin and its bis-imidazole analogue on the decadeoxyribonucleotide d(CGCAATTGCG)<sub>2</sub>. *J. Biomol. Struct. Dyn.* 5, 939–949.
- (57) Lee, M., Chang, D. K., Hartley, J. A., Pon, R. T., Krowicki, K., and Lown, J. W. (1988c) Structural and dynamic aspects of binding of a prototype lexitropsin to the decadeoxyribonucleotide d(CGCAATTGCG)<sub>2</sub> deduced from high-resolution <sup>1</sup>H NMR studies. *Biochemistry* 27, 445–455.
- (58) Wüthrich, K. (1986) in *NMR of Proteins and Nucleic Acids*, John Wiley & Sons, Inc., New York.
- (59) Bax, A., and Davis, D. G. (1985) Practical aspect of two-dimensional transverse NOE spectroscopy. *J. Magn. Reson.* 63, 207–213.
- (60) Bothner-by, A. A., Stephens, R. L., Lee, J.-M., Warren, C. D., and Jeanloz, R. W. (1984) Structure determination of a tetrasaccharide: Transient nuclear Overhauser effects in the rotating frame. *J. Am. Chem. Soc.* 106, 811–813.
- (61) Fregeau, N. L., Wang, Y., Pon, R. T., Wylie, W. A., and Lown, J. W. (1995) Characterization of a CPI-lexitropsin conjugate: oligonucleotide covalent complex by <sup>1</sup>H NMR and restrained molecular dynamics simulation. *J. Am. Chem. Soc.* 117, 8917–8925.
- (62) Wu, W., Vanderwall, D. E., Turner, C. J., Kozarich, J. W., and Stubbe, J. (1996) Solution structure of Co-bleomycin A2 (green) complexed with d(CCAGGCCTGG)<sub>2</sub>. *J. Am. Chem. Soc.* 118, 1281–1294.



- (63) Bauley, B. C., Denny, W. A., Atwell, G. J., and Cain, B. F. (1981) Potential antitumor agents. 34. Quantitative relationships between DNA binding and molecular structure for 9-anilinoacridines substituted in the anilino ring. *J. Med. Chem.* 24, 170–179.
- (64) Chen, Y.-H., and Lown, J. W. (1994) A new DNA minor groove binding motif: Cross-linked lexitropsins. *J. Am. Chem. Soc.* 116, 6995–7005.
- (65) The binding constants of Co(III)•AMPHIS-NET and metal free AMPHIS-NET to poly(dA)•poly(dT) were calculated using C50 (concentration of the compound required to reduce fluorescence of DNA bound EtBr to 50%) values assuming a binding site size of four base pairs and the binding constant of EtBr as  $1.0 \times 10^6 \text{ M}^{-1}$  for poly(dA)•poly(dT).
- (66) Wang, Y., Gupta, R., Huang, L., Lou, W., and Lown, J. W. (1996) Design, synthesis, cytotoxic properties and preliminary DNA sequencing evaluation of CPI-N-methylpyrrole hybrids. Enhancing effect of a trans double bond linker and role of the terminal amide functionality on cytotoxic potency. *Anti-Cancer Drug Des.* (in press).
- (67) Chen, Y.-H., Huang, L., and Lown, J. W. (1996) (manuscript in preparation).

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# Structure of a DNA Duplex Containing a Single 2'-O-Methyl- $\beta$ -D-araT: Combined Use of NMR, Restrained Molecular Dynamics, and Full Relaxation Matrix Refinement

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Two-dimensional <sup>1</sup>H NMR spectroscopy was used to determine the solution structure of the double-stranded DNA oligonucleotide d(5'-CGCATATAGCC-3'): d(5'-GGCTAXATGCG-3'), where X is 1-(2-O-methyl- $\beta$ -D-arabinofuranosyl)thymine. The structure determination was based on a total relaxation matrix analysis of NOESY cross-peak intensities using the MARDIGRAS program. The improved RANDMARDI procedure was used during the calculations to include the experimental "noise" in the NOESY spectra. The NOE-derived distance restraints were applied in restrained molecular dynamics calculations. Twenty final structures each were generated for the modified DNA duplex from both A-form and B-form DNA starting structures. The root-mean-square deviation of the coordinates for the 40 structures was 0.82 Å. The duplex adopts a normal B-DNA-type helix, and the spectra as well as the structure show that the modified nucleotide X adopts a C2'-endo (*S*) sugar conformation. There are no significant changes in the helix originating from the modified nucleotide. The CH<sub>3</sub>O group on X is directed toward the major groove, and there seems to be free space for further modifications at this position.

## INTRODUCTION

A potential gene inhibitor must possess an enhanced stability toward cellular nucleases, the ability to penetrate the cell membrane, and an efficient hybridization to the target RNA/DNA<sup>1</sup> (Hélène and Toulmé, 1990; Uhlmann and Peyman, 1990). Several chemically modified DNA and RNA oligonucleotides have been synthesized, and a few of them fulfill some of these requirements (Inoue et al., 1987; Sproat et al., 1989; Egholm et al., 1993; Jørgensen et al., 1994; Lesnik et al., 1993). Among these are 2'-O-methylated ribonucleotides (2'-OCH<sub>3</sub>-RNA) (Figure 1), which are resistant toward DNA- and RNA-specific nucleases and have improved hybridization properties. However, they are degradable by a dual RNA/DNA active enzyme such as snake venom phosphodiesterase (SV PDE) (Inoue et al., 1987; Sproat et al., 1989; Lesnik et al., 1993). Structural studies on oligodeoxynucleotides containing the 2'-O-methylated ribonucleotides show that introduction of only one modified nucleotide causes a local change in the DNA conformation (Lubini et al., 1994). A CH<sub>3</sub>O group at the 2'-ribo position on a single residue or an all-modified strand causes changes from a B-DNA to an A-DNA-type conformation (Lubini et al., 1994; Blommers et al., 1994) and forces it into a C3'-endo sugar pucker conformation

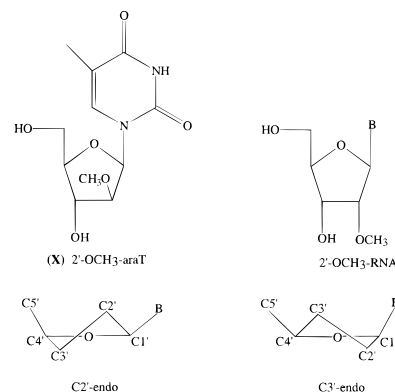


**Figure 1.** Structure of 1-(2-O-methyl- $\beta$ -D-arabinofuranosyl)thymine and 2'-OCH<sub>3</sub>-RNA together with their predominant sugar conformations. B denotes a nucleobase.

(Guschlbauer and Jankowski, 1980). The structure of an araC-modified double-stranded oligonucleotide has been reported in the literature with significant structural changes at the modification site compared to the unmodified oligonucleotide (Schweitzer et al., 1994; Gao et al., 1991).

Recently, we have described the design and synthesis of an oligonucleotide containing the thymidine analog 1-(2-O-methyl- $\beta$ -D-arabinofuranosyl)thymine (2'-OCH<sub>3</sub>-araT, X) (Figure 1) (Gotfredsen et al., 1994, 1996). The aim of this work is fourfold: (i) to minimize the structural distortions caused by the modification relative to the B-DNA structure of an unmodified duplex, (ii) to maintain normal Watson–Crick sequence selectivity of the modified oligonucleotide, (iii) to create an unhindered point of attachment for functional groups oriented into the major groove of regular B-form DNA, and (iv) to enhance the nuclease resistance of the oligonucleotide.

In this work we have used NMR spectroscopy to evaluate the success of the design of the modified oligonucleotide containing a 2'-OCH<sub>3</sub>-araT nucleotide. We have determined the solution structure of the undecanucleotide duplex d(5'-CGCATATAGCC-3'):d(5'-GGCTAX-

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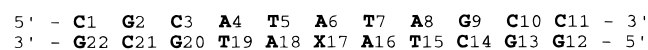
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<sup>1</sup> Abbreviations: AMBER, assisted model building with energy refinement; DNA, deoxyribonucleic acid; dsDNA, double-stranded DNA; EDTA, ethylenediaminetetraacetic acid; MARDIGRAS, matrix analysis of relaxation for discerning the geometry of an aqueous structure; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; NOESY, nuclear Overhauser effect spectroscopy; RANDMARDI, randomized MARDIGRAS; RMD, restrained molecular dynamics; SV PDE, snake venom phosphodiesterase; TAXA, d(5'-CGCATATAGCC-3'):d(5'-GGCTAXATGCG-3'); TOCSY, total correlation spectroscopy; TPPI, time proportional phase incrementation.

ATGCG-3') (TAXA) using <sup>1</sup>H NMR. Data from NOESY spectra were used to obtain interproton distance bounds using the RANDMARDI procedure (Liu et al., 1995), an improved version of the MARDIGRAS complete relaxation matrix approach (Borgias et al., 1990; Borgias and James, 1990). These distance bounds were used as restraints in molecular dynamics (RMD) calculations. Since many NOE contacts were observed between the modified nucleotide and the surrounding DNA, the resulting structures have fairly high resolution and allowed the determination of some local conformational features in the DNA.

## EXPERIMENTAL PROCEDURES

**Sample Preparation.** The synthesis of oligonucleotides containing the thymidine analog 1-(2-*O*-methyl-β-D-arabinofuranosyl)thymine (2'-OCH<sub>3</sub>-araT, **X**) has previously been described (Gotfredsen et al., 1994, 1996). A standard phosphoramidite method on a Pharmacia Gene Assembler Special DNA synthesizer was used. The crude product was purified by ethanol precipitation. The numbering scheme for the double-stranded oligonucleotide is



The duplex was formed by mixing (1:1) and annealing the complementary single-stranded oligomers at 80 °C and cooling to room temperature over a 2 h period. The samples of the modified and unmodified oligonucleotides were prepared by dissolving the lyophilized complex in 0.5 mL of 10 mM sodium phosphate buffer (pH 7.0) and 0.05 mM sodium EDTA. For experiments carried out in D<sub>2</sub>O the samples were lyophilized twice from D<sub>2</sub>O on a SpeedVac evaporator and redissolved in 99.96% D<sub>2</sub>O (Cambridge Isotope Laboratories). A 90% H<sub>2</sub>O/10% D<sub>2</sub>O mixture was used to obtain NMR spectra of exchangeable protons. The dsDNA concentrations of the modified and unmodified oligonucleotide were 1.7 and 2.0 mM, respectively.

UV and NMR studies were performed to compare the relative stabilities of the unmodified and the modified oligonucleotides as reported elsewhere (Gotfredsen et al., 1994, 1996).

**NMR Experiments.** NOESY and TOCSY NMR spectra were obtained on a Varian Unity 500 spectrometer. The NOESY buildup experiments in D<sub>2</sub>O of TAXA were acquired with mixing times of 25, 50, 100, 150, and 200 ms. The States phase cycle scheme was used to obtain 512 *t*<sub>1</sub> experiments; 64 scans were acquired for each *t*<sub>1</sub> value using 1024 complex points in *t*<sub>2</sub>, a spectral width of 5000 Hz, and a recycle delay of 2 s. These NOESY spectra were obtained repetitively without removing the sample from the magnet. The TOCSY spectra were obtained in D<sub>2</sub>O with mixing times of 30, 90, and 140 ms in the TPPI mode using 1024 complex points in *t*<sub>2</sub> and 512 *t*<sub>1</sub> experiments each acquired with 80 scans. The spectral width was 5000 Hz. NOESY spectra in H<sub>2</sub>O with mixing times 100 and 200 ms were obtained with a NOESY pulse sequence in which the last 90° pulse was replaced by a jump and return sequence (Guéron et al., 1991) to suppress the solvent signal. The spectra were acquired with a spectral width of 10 000 Hz in 2048 complex points using 512 *t*<sub>1</sub> experiments with 64 scans each. The data were processed using FELIX (version 2.1, Biosym Technologies, San Diego).

**Structure Calculation.** The upper and lower halves of the diagonal of each of the five assigned NOESY spectra were integrated separately with FELIX. The

integrated volumes of symmetry-related cross peaks above and below the diagonal were treated as independent measurements to more accurately reflect the scatter in the data. This gave a total of 10 peak intensity sets, 2 for each of the five mixing times. The spin-lattice relaxation times were measured for representative protons by the inversion recovery method. The measured intensities of the NOESY cross peaks were scaled for the effects of incomplete relaxation. The RANDMARDI procedure (Liu et al., 1995) of the complete relaxation matrix analysis method, MARDIGRAS (Borgias and James, 1990; Borgias et al., 1990; Schmitz et al., 1992; Schmitz and James, 1996), was used to calculate interproton distance RMD bounds from the resulting integrated peak intensities. The RANDMARDI procedure was used to include the effects of experimental noise in the relaxation matrix calculations. RANDMARDI adds a random number from within a specified range to each input intensity used in a MARDIGRAS calculation. The noise level used for the TAXA sequence was 0.65% of the largest integrated cross peak or ~5 times the intensity of the smallest peak. In the RANDMARDI procedure, 30 different intensity sets from each experimental data set were generated and MARDIGRAS calculations were performed on all of them. Resulting distances were then averaged together to form one bounds file for each of the 10 data sets. The bounds files from all five mixing times are then combined into a single bounds file from which the RMD restraint file is generated. Upper and lower bounds were average interproton distance ± 1 standard deviation calculated from all of the MARDIGRAS runs. The RANDMARDI procedure accurately accounts for the effects of random experimental noise and integration errors. A complete description of the RANDMARDI theory and procedure is given elsewhere (Liu et al., 1995).

The distance restraints obtained from the MARDIGRAS calculation were incorporated into a restrained molecular dynamics (RMD) procedure. The RMD and energy minimization calculations were performed using DISCOVER (version 2.95) with the AMBER force field potentials, and the models were displayed using INSIGHTII (version 2.3.0) (Biosym Technologies). A starting model of either A-form or B-form DNA was built using standard Arnott parameters (Biosym Technologies), and the H2' proton of the X17 residue was replaced with a methoxy group using the BUILDER module of INSIGHTII. The NOE-derived distance restraints were then applied, and the model was energy minimized to a maximum derivative of 0.1 Å. This was followed by 19 ps of RMD with the following temperature profile: 450 K for 4 ps cooled to 200 K in 50 K steps of 3 ps each. The final structure was then energy minimized to a maximum derivative of 0.01 Å.

The pseudo-energy term used to enforce the distance restraints was

$$E_{\text{constr}} \begin{cases} = k_1(r - r_2)^2 & \text{when } r^2 > r \\ = 0 & \text{when } r_3 \geq r \geq r_2 \\ = k_2(r - r_3)^2 & \text{when } r_3 > r \end{cases}$$

where *r*<sub>2</sub> and *r*<sub>3</sub> are the upper and lower distance bounds determined from the cross-peak volumes, and *k*<sub>1</sub> and *k*<sub>2</sub> are the force constants that can be independently adjusted for each restraint. An upper and lower bound force constant of 50 kcal/mol Å<sup>2</sup> was assigned for all NOE-derived distance restraints; a set of calculations with a

**Table 1. Chemical Shifts (ppm) for the TAXA Sequence<sup>a</sup>**

| TAXA | H(1') | H(2')             | H(2'') | H(3') | H(4')             | H(5')             | H(5'')            | H(6)/H(8) | H(5)/H(2)/CH <sub>3</sub> | H4/H6 | H4/H6 | H1/H3 |
|------|-------|-------------------|--------|-------|-------------------|-------------------|-------------------|-----------|---------------------------|-------|-------|-------|
| C1   | 5.78  | 1.95              | 2.39   | 4.69  | 4.16              | 3.71              | 3.71              | 7.63      | 5.88                      |       |       |       |
| G2   | 5.91  | 2.65              | 2.74   | 4.97  | 4.35              | 3.74 <sup>b</sup> | 3.98 <sup>b</sup> | 7.95      |                           |       |       | 13.01 |
| C3   | 5.63  | 2.06              | 2.41   | 4.85  | 4.19              |                   | 4.22              | 7.37      | 5.42                      | 8.31  | 6.43  |       |
| A4   | 6.27  | 2.70              | 2.96   | 5.01  | 4.44              | 4.12 <sup>b</sup> | 4.16 <sup>b</sup> | 8.33      | 7.62                      |       |       |       |
| T5   | 5.80  | 2.19              | 2.59   | 4.88  | 4.24              |                   | 4.29              | 7.19      | 1.52                      |       |       | 13.23 |
| A6   | 6.13  | 2.48              | 2.84   | 4.93  | 4.38              |                   |                   | 8.08      | 6.85                      |       |       |       |
| T7   | 5.64  | 1.99              | 2.40   | 4.87  | 4.16 <sup>d</sup> |                   | 4.22              | 7.14      | 1.22                      |       |       | 13.28 |
| A8   | 6.01  | 2.67              | 2.85   | 5.02  | 4.37              |                   | 4.06              | 8.12      | 7.21                      |       |       |       |
| G9   | 5.74  | 2.50              | 2.60   | 4.93  | 4.36              |                   | 4.20              | 7.63      |                           |       |       | 12.79 |
| C10  | 5.98  | 2.12              | 2.44   | 4.75  | 4.16              |                   | 4.36              | 7.40      | 5.29                      | 8.14  | 6.41  |       |
| C11  | 6.24  | 2.24              | 2.24   | 4.54  | 4.03              |                   | 4.12 <sup>c</sup> | 7.68      | 5.76                      |       |       |       |
| G12  | 5.71  | 2.52              | 2.70   | 4.83  | 4.18              | 3.67              | 3.67              | 7.85      |                           |       |       |       |
| G13  | 5.97  | 2.52              | 2.73   | 4.99  | 4.41              |                   | 4.09              | 7.85      |                           |       |       | 13.00 |
| C14  | 5.98  | 2.07              | 2.53   | 4.75  | 4.26              |                   |                   | 7.42      | 5.29                      | 8.14  | 6.50  |       |
| T15  | 5.76  | 2.19              | 2.59   | 4.91  | 4.19              |                   | 4.10              | 7.41      | 1.63                      |       |       | 13.57 |
| A16  | 6.32  | 2.65              | 3.06   | 5.05  | 4.46              | 4.14 <sup>b</sup> | 4.19 <sup>b</sup> | 8.37      | 7.22                      |       |       |       |
| X17  | 5.63  | 3.54 <sup>e</sup> | 4.15   | 4.69  |                   |                   | 4.39              | 7.14      | 1.43                      |       |       | 13.00 |
| A18  | 6.16  | 2.59              | 2.92   | 4.99  | 4.35              |                   | 4.22              | 7.93      | 7.07                      |       |       |       |
| T19  | 5.76  | 1.99              | 2.40   | 4.70  | 4.16 <sup>d</sup> |                   | 4.25              | 7.06      | 1.25                      |       |       | 13.46 |
| G20  | 5.82  | 2.58              | 2.65   | 4.96  | 4.35              |                   | 4.07              | 7.84      |                           |       |       | 12.59 |
| C21  | 5.78  | 1.90              | 2.33   | 4.81  | 4.16              |                   |                   | 7.34      | 5.39                      | 8.38  | 6.52  |       |
| G22  | 6.15  | 2.60              | 2.35   | 4.67  | 4.16              |                   |                   | 7.95      |                           |       |       |       |

<sup>a</sup> The values are given at 25 °C relative to DSS. Hydrogen-bonded amide protons are underlined. <sup>b</sup> We are unable to assign H5' and H5'' specifically; they may be interchanged. <sup>c</sup> This could be a H5' proton instead of a H5'' proton. <sup>d</sup> We are unable to decide whether it is T7H4', T19H4', or both overlapping. <sup>e</sup> This is the value of the 2'-OCH<sub>3</sub>.

force constant of 25 kcal/mol Å<sup>2</sup> was also performed but without any changes in the output structure. An additional 28 distance restraints were included to enforce Watson–Crick hydrogen bonding throughout the calculations. Three hydrogen bonds were included for each of the six G–C base pairs and two hydrogen bonds for each of the five A–T base pairs with lower and upper bounds of 1.74 and 2.10 Å, respectively. Helix parameters were calculated with the program CURVES 3.1 (Lavery and Sklenar, 1988, 1989).

## RESULTS

**Spectral Analysis.** The line widths of resonances observed in the <sup>1</sup>H NMR spectra of TAXA are similar to the ones found for the unmodified double-stranded oligonucleotide. The X17 2'-OCH<sub>3</sub> group resonates at 3.54 ppm in a region of the spectrum normally devoid of DNA signals (Figure 2).

The assignment of the nonexchangeable protons in both the unmodified undecamer, d(5'-CGCATATAGCC-3'):d(5'-GGCTATATGCG-3') and the 2'-OCH<sub>3</sub>-araT-modified TAXA undecamer was performed using standard methods (Hare et al., 1983; Wüthrich, 1986; Scheek et al., 1983). Aromatic (H6, H5, H8, and H2) and sugar protons (H1', H2', H2'', H3', most H4', and some of the H5', H5'') were assigned. The assigned resonances of the modified oligonucleotide are given in Table 1. The assignments of the exchangeable protons were obtained from the NOESY spectrum in H<sub>2</sub>O (Wüthrich, 1986; Boelens et al., 1985). The chemical shift values and the NOE connectivity pattern of the imino protons were observed to be in accordance with normal Watson–Crick base pairing. Except for the 2'-OCH<sub>3</sub>-araT nucleotide there are no significant differences in the chemical shift values between the unmodified undecamer duplex and the modified TAXA duplex.

The NOESY spectrum exhibits a few characteristic features originating from the 2'-OCH<sub>3</sub>-araT. Figure 2 shows the sequential connectivities from aromatic H6/H8 protons to both intra- and inter-residue H2'/H2''. The connectivities from the modified X17 OCH<sub>3</sub>/H2'' to A18 H8 are shifted downfield compared to the normal deoxyribose H2'/H2''. The presence of weak NOESY cross peaks from the H6/H8 base protons to H1' protons

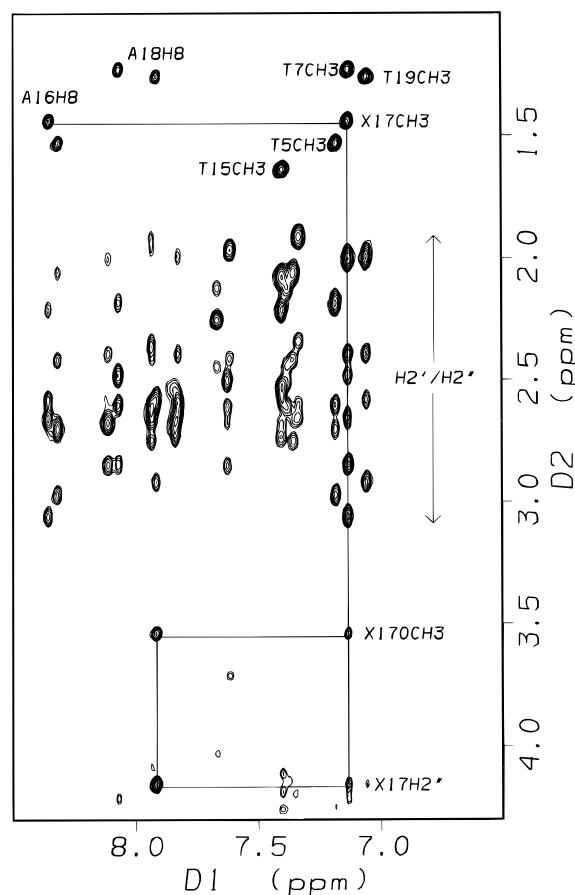


**Figure 2.** H2'/H2'' to aromatic part of the 200 ms NOESY spectrum of TAXA. A solid line indicates the sequential connectivity pathway from X17 H2'' and OCH<sub>3</sub> to X17H6 and A18H8 and from X17H6–X17CH<sub>3</sub> and X17CH<sub>3</sub>–A16H8.

indicates that the nucleobases are in an *anti* conformation. Furthermore, the NOESY spectra show very weak cross peaks from H3' protons to base protons (data not shown), and only two strong cross peaks appear in the base proton to H2'/H2'' region [(*n*) H6/H8 to (*n*) H2' and (*n*) H6/H8 to (*n* – 1) H2'] at short mixing times. This

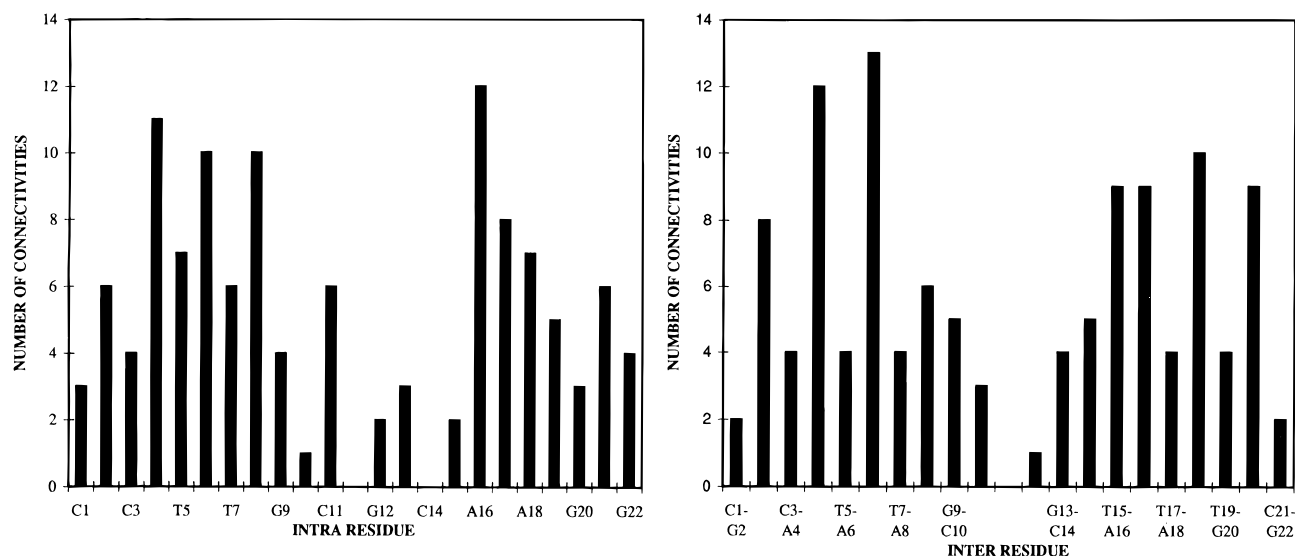


**Figure 3.** Graphical representation of the distribution of restraints for the structure calculations.

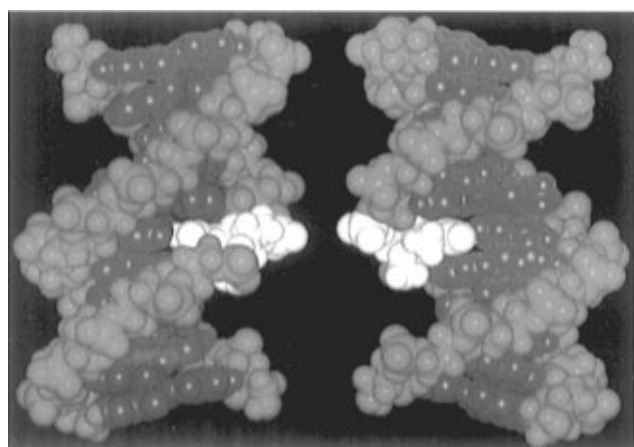


**Figure 4.** Structure of the modified DNA looking into the minor groove (left) and the major groove (right). X17 is shown in yellow, the nucleobases are in blue, and the sugar phosphate backbone is in red.

shows that all of the sugars adopt a C2'-endo (*S*)-type conformation.

**Structure Calculations.** More than 445 NOE cross peaks were observed in the NOESY spectrum obtained with a mixing time of 200 ms. The accuracy of the integration of some cross peaks was hampered by spectral overlap. These cross peaks were therefore not included in the MARDIGRAS calculations. The integrated intensities from 254 NOESY cross peaks for each mixing time were used in the total relaxation matrix analysis. Integrations of the cross peaks in the five NOESY spectra used were done separately for each side of the diagonal. This yielded a total of 10 sets of measured NOE intensities that were converted to distance restraints using the RANDMARDI procedure. Some of the cross peaks used in the structure calculations resulted predominantly from spin diffusion and were consequently not observed in NOESY spectra with shorter mixing times. This is taken into account during the statistical analysis of the results generated by the RANDMARDI procedure (Liu et al., 1995). Cross-peak integrals that corresponded to fixed distances in the DNA were not converted into distance restraints for the use in the RMD simulations. The calculations returned 241 interproton distances that are not covalently fixed in the modified DNA.

The MARDIGRAS calculations have been performed by using a value of the correlation time of  $\tau_c = 2$  ns. This

value was justified from approximate values of  $\tau_c$  calculated from measured spin-lattice relaxation times,  $T_1$ , and spin-spin relaxation times,  $T_2$  (Woessner, 1962). The values of  $T_1$  were obtained from a nonselective inversion recovery experiment, whereas the values of  $T_2$  were calculated from the line widths of corresponding well-separated lines of representative protons, such as methyl group protons, sugar protons, and aromatic protons including adenine H(2)s. Values for  $\tau_c$  were found to be between 1.9 and 2.4 ns. Although this is only an estimated range of values, it justifies the use of  $\tau_c = 2$  ns in the MARDIGRAS calculation. Furthermore, it turns out that the results of the calculations are rather insensitive to the exact values of  $\tau_c$  used. This is similar to what was found earlier (Mujeeb et al., 1993).

The distance bounds used in RMD simulations were determined by combining the results from all of the individual MARDIGRAS calculations performed during the RANDMARDI procedure into one set. They were calculated individually for each proton pair corresponding to a NOESY cross peak included in the RANDMARDI calculations. Each individual restraint was generated from the MARDIGRAS calculations using the calculated average distance  $\pm 1$  standard deviation. Figure 3 gives a summary of the distance restraints used in the RMD calculations. The range of differences between the upper and lower bounds for the 241 NOE-derived restraint bounds used (Figure 3) was between 0.08 and 3.59 Å with an average flat well potential width of 1.02 Å. This range between the upper and lower bounds represents the precision of the restraint calculated from the NOESY data. Weak NOESY cross peaks primarily from spin diffusion had the largest distance between the upper and lower distance bounds.

Evaluation of the spectra obtained in  $\text{H}_2\text{O}$  shows normal Watson-Crick hydrogen bonding, justifying the inclusion of 28 hydrogen bond distance restraints. An additional 10 restraints with loose bounds were derived by inspection of the spectra for a total of 279 interproton restraints and used in the RMD calculations. The distribution of restraints (Figure 3) is anisotropic, with the central part of the duplex being the most well defined.

Twenty final structures were generated for the modified duplex starting either from a B-form or an A-form DNA conformation. All of the structures converged to one family of conformations. The root-mean-square (rms) deviation of the coordinates of the 40 structures was 0.82

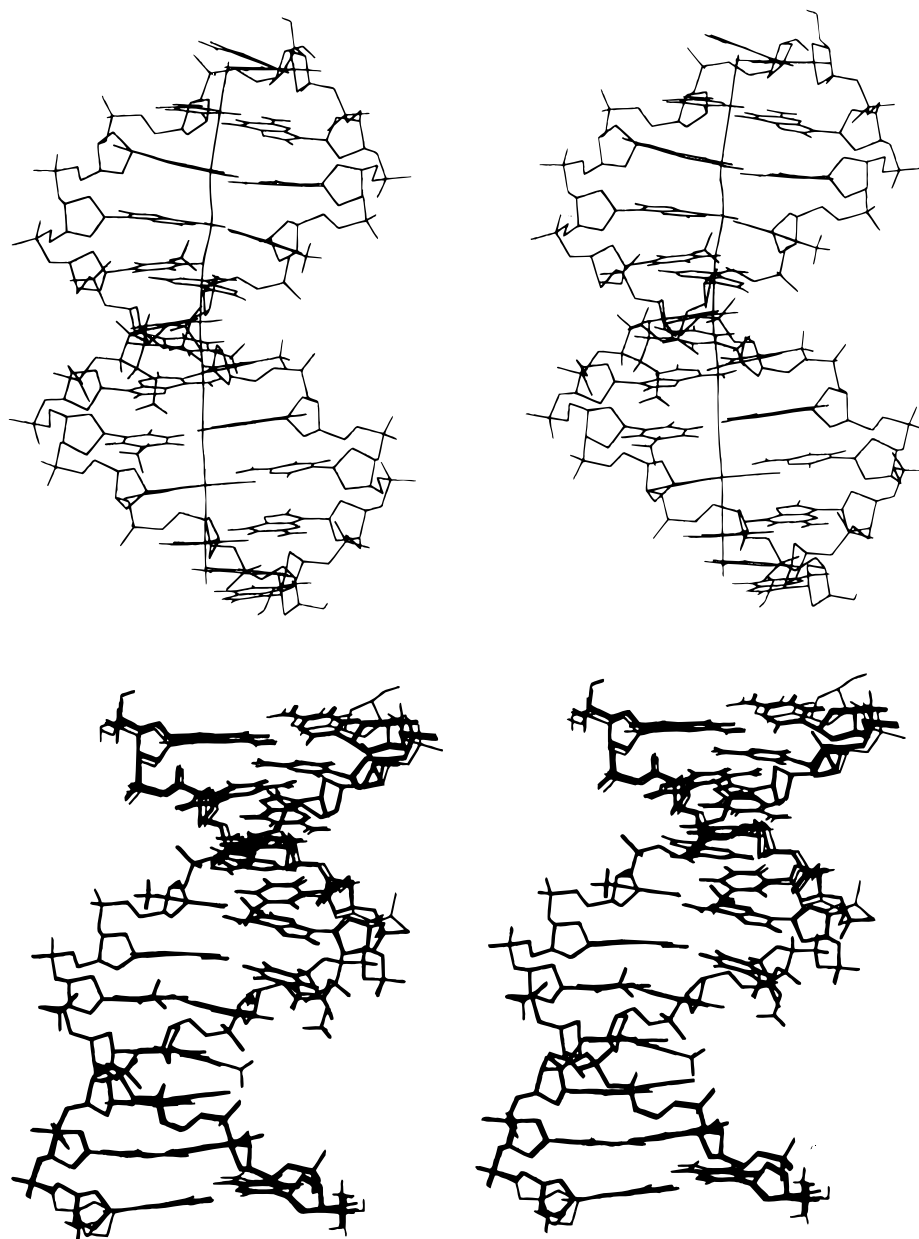


**Figure 5.** Stereoview of the stick plot of the structure of TAXA. Deoxyribose protons have been omitted for clarity except for X17. (Top) One of the calculated structures with an indication of the direction of the helix axis obtained from CURVES. (Bottom) A superposition of 10 of the 40 structures obtained by RMD calculations. The 10 structures are randomly picked from structures calculated from both A- and B-DNA starting structures.

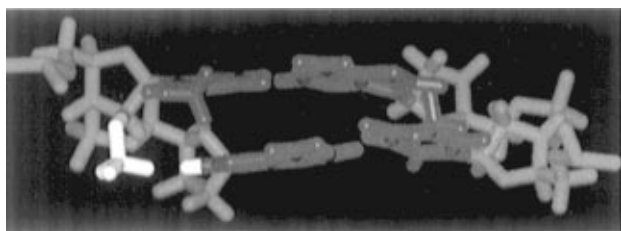


**Figure 6.** View of the  $\text{OCH}_3$  (yellow) in the major groove and the close proximity of it to A18H8 (yellow). The nucleobases are shown in blue and the sugar phosphate backbone is in red.

Å. The sum of residual violations of the distance restraints for the structures shown was 7.6 Å for the B-form starting structure and 7.3 Å for the A-form starting structure. We had five violations  $>0.2$  Å, with no violations  $>0.29$  Å. Different views of the structure are given in Figures 4–6.

Helical parameters for the 40 final structures were calculated with the program CURVES 3.1 (Lavery and

Sklenar, 1988, 1989), and selected parameters are shown in Figure 8. The structural parameters for canonical A-DNA and B-DNA are included in Figure 8 for comparison (Arnott and Hukins, 1972, 1973).

## DISCUSSION

**Description of the Structure.** Qualitative analysis of the NOESY spectra indicates that TAXA adopts a B-form conformation. This is confirmed by the structure calculation. All bases are in an *anti* conformation, and they form normal Watson–Crick base pairs. The structure shows that the  $\text{CH}_3\text{O}$  group is in the major groove. The spectral analysis and the structure calculation give no indications of different families of *O*-methyl rotamers about the  $\text{C}2'\text{--O}2'$  methoxy bond, and there are no mutually inconsistent NOEs observed from the X17  $2'\text{--OCH}_3$  group to the surrounding DNA. There is no sign of exchange peaks to suggest flipping between different positions. The calculations give only one family of

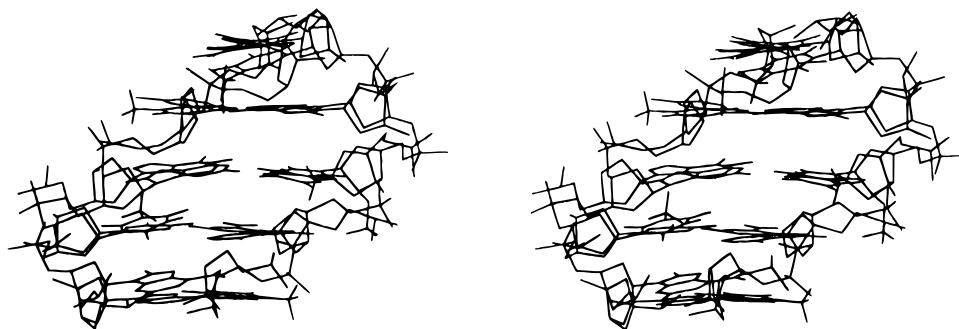


**Figure 7.** Superimposition of the middle ATATA part of TAXA with the ATATA part of d(CG CATATATGCG)<sub>2</sub> taken from the Brookhaven databank. In the figure the deoxyribose protons are removed for clarity.

structures. The X17 2'-OCH<sub>3</sub> is in van der Waals contact with A18 H8 (Figure 6) (2.95–3.28 Å).

Experimentally derived NMR restraints are distributed anisotropically and short range in nature. This largely establishes local structures with some helical parameters better defined by the experimental data than others. We have found a number of NMR-derived restraints in the center of the helix originating from AH2 protons to H1' protons. These NOE-derived distance restraints improved the ability to define the conformation for the central five base pairs of the oligonucleotide (Weisz et al., 1994).

**Helix Parameters.** There are three major categories of helical parameters: axis-base pair, intrabase pair, and interbase pair parameters (Dickerson et al., 1989). Some of these parameters for TAXA are shown in Figure 8. Error bars are not included since we found only very small variations in the calculated values. The global helix axis is bent toward the major groove (Figure 5) with an overall helix axis curvature of 12°. Some of the features of the calculated helix parameters with respect to the calculated global helix axis are discussed below. Values of the pseudorotational angles obtained from CURVES are within the range of 106–178° with the values for X17 at 145°. This is in agreement with the finding that TAXA adopts a B-like DNA conformation.

**Axis-Base Pair Parameters.** The structure around the X-modification in TAXA has inclination values closer to those for B-DNA than for A-DNA. The variation of the inclination follows the width of the minor groove, being tightest around the modification site as compared to the most negative value for the inclination (Yuan et al., 1992). For TAXA the tip angle starts negative and changes to positive, but this is within the interval of ±5° common for canonical A-DNA and B-DNA (Mujeeb et al., 1993).

**Intrabase Pair Parameters.** The calculated values for shear, stretch, stagger, and opening are highly influenced by the restraints used to enforce base pair hydrogen bonding and are therefore not included in Figure 8. The buckle changes smoothly along the helix to a negative value of -16.5° at the modification site from positive values at each end of the helix axis. The large negative value at X shows that the center of this base pair is bent away from the center of the helix (Mujeeb et al., 1993). The propeller twist for all base pairs is negative. This is typical for structures determined in solution by NMR of other oligonucleotides (Baleja et al., 1990; Weisz et al., 1994; Mujeeb et al., 1993; Schmitz et al., 1992). Base pair opening values for TAXA are larger than those for canonical A- and B-form DNAs. This corresponds to a local expansion of the minor groove in the (5'-A4pT5-3'); (5'-A18pT19-3') and (5'-T7pA8-3'); (5'-T15pA16-3') regions. The minor groove is locally tightened in the region between. The width of the minor groove was measured

as the shortest interstrand phosphorus–phosphorus distance ( $i, i + 14$ ) across the groove minus the combined van der Waals radii of the phosphorus atoms (5.8 Å). The tighter minor groove in the (5'-A6pT7pA8-3'); (5'-T15pA16pX17-3') region (5.3–5.9 Å) is reflected in an observable NOE cross peak from A6 H2 and the A18 H1' (Kintanar et al., 1987; Weisz et al., 1994).

**Interbase Pair Parameters.** Values for the rise in the TAXA sequence are midway between those of canonical A-form and B-form. This is also reflected in the overall length of the helix, which is 32.8 Å. The roll parameter exhibits an alternating pattern along the duplex but with a significant positive roll value at the (5'-C3pA4-3'); (5'-T19pG20-3') site and at the (5'-T7pA8-3'); (5'-T15pA16-3') site. Positive rolls open the angle between the base pairs toward the minor groove. As a result, a wider minor groove and bending toward the major groove occurs. In recent *in vitro* and NMR studies (5'-CpA-3'); (5'-TpG-3') steps have been correlated with DNA bending (Beutel and Gold, 1992; Weisz et al., 1994; Mujeeb et al., 1993). Due to local bending from these positive roll values, the global helical curvature is affected. The average twist over the sequence is 36° ± 4°. The variation of the twist is similar to the one observed in d(CG CATATATGCG)<sub>2</sub> (Yoon et al., 1988), showing in particular that the modification does not cause the helix to unwind.

**Comparison with Other Related Structures.** Comparison of the d(5'-A4T5A6T7A8-3'); d(5'-T15A16X17A18-T19-3') part of the TAXA duplex with the central part of the duplex d(CG CATATATGCG)<sub>2</sub> taken from the Brookhaven databank (Yoon et al., 1988; Yuan et al., 1992) shows that the stacking arrangements are almost identical for the central parts (Figure 7). The difference between the two amounts to an rms deviation of 2.63 Å for these five base pairs. However, the alternating pattern for the helical twist and rise through the alternating AT sequence are less pronounced for the TAXA sequence.

Previously, several structures of araC-containing oligonucleotides (with a hydroxyl group in the arabino 2'-position) have been determined (Schweitzer et al., 1994; Gao et al., 1991). These structures showed that the 2'-OH group was positioned in the major groove, equivalent to what we observe in the case of the 2'-OCH<sub>3</sub> in the TAXA oligonucleotide. In the X-ray structure of one of the araC-containing oligonucleotide duplexes it was found that the araC sugar pucker adopts an S-type conformation (Gao et al., 1991). An NMR structure of another araC oligonucleotide duplex shows the sugar pucker of araC to be of the N-type (Schweitzer et al., 1994), but with the overall duplex structure as B-DNA. Partly in agreement with that, the structural observations in the TAXA oligonucleotide imply that a normal B-type helix conformation is present and that the 2'-OCH<sub>3</sub>-araT

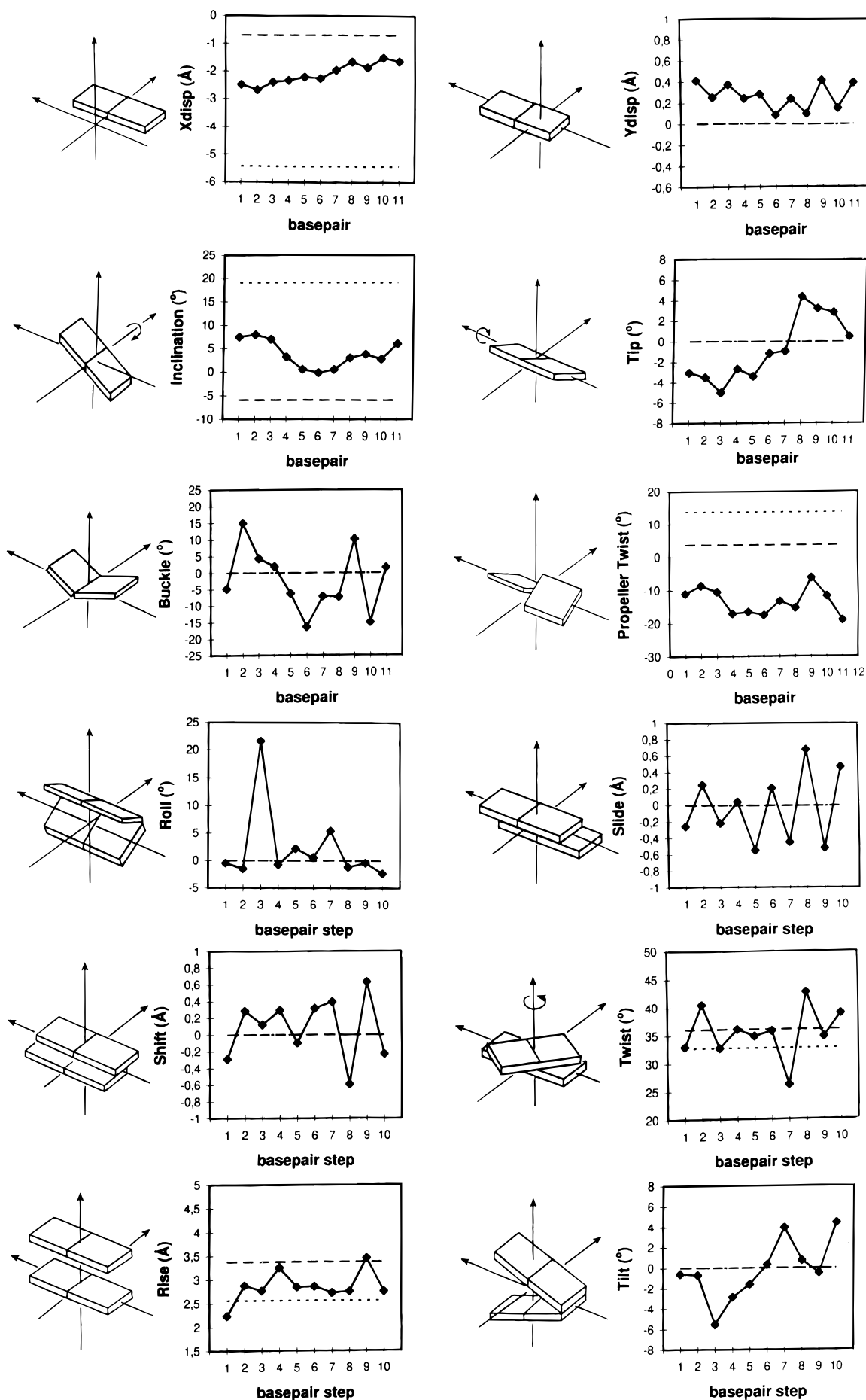


**Figure 8.** Helical parameters for TAXA calculated using CURVES 3.1 and compared with canonical A-DNA (---) and B-DNA (----).



adopts an S-type sugar conformation. A substitution on the 2'-oxygen in the arabino ring with longer alkyl chains would probably maintain the orientation and conformation of the sugar ring systems, retaining a B-form DNA configuration. This constitutes a possible path to create new antisense or antigene agents.

When 2'-OCH<sub>3</sub>-araT is positioned in an oligonucleotide, it favors resistance to the 3'-exonuclease SV PDE, a dual DNA/RNA nuclease (Gotfredsen et al., 1994, 1996). This is an advantage over the normal DNA building blocks, which, when incorporated into a oligonucleotide, are easily degradable. The increased stability of TAXA may be caused by steric interference from the 2'-CH<sub>3</sub>O group in the arabino configuration so that the shape of the polymer does not fit into the active site of the enzyme. It has been reported that a 2'-CH<sub>3</sub>O group on the ribo face of the sugar ring does not interfere with the digestion by SV PDE (Sproat et al., 1989) so that the degradation is as fast as for a normal DNA strand.

**Molecular Design.** The structural study in this work shows that 2'-OCH<sub>3</sub>-araT in the TAXA duplex adopts a B-like helix conformation with only a slightly lower DNA thermal stability and enhanced enzymatic stability toward SV PDE (Gotfredsen et al., 1994, 1996). The 2'-OCH<sub>3</sub> group of X17 is directed toward the major groove with the possibility of tethering a longer alkyl chain to that site without essentially disturbing the B-like conformation.

RNA oligonucleotides modified with CH<sub>3</sub>O in the 2'-position adopt N-type sugar conformations yielding a typical A-DNA structure (Lubini et al., 1994; Blommers et al., 1994). This is contrary to what is seen for TAXA, but in accordance with the suggestion that a strong electronegative group at C2' determines the sugar pucker conformation for various sugar nucleosides (Uesugi et al., 1979; Guschlbauer and Jankowski, 1980). Thus, the 2'-OCH<sub>3</sub>-araT adopts a C2'-endo conformation (S), implying that the electronegative -OCH<sub>3</sub> in an arabino position will favor an S-type sugar conformation, whereas if the OCH<sub>3</sub> is positioned in a 2'-ribo position, it will favor an N-type sugar conformation. Further studies on the ability of a 2'-O-alkylarabinonucleotide to enforce a B-DNA conformation when bound to an RNA strand would be of interest since the 2'-OCH<sub>3</sub>-RNA nucleotides can induce a local A-DNA-type helix when duplexed to a complementary DNA strand (Lubini et al., 1994).

The observations described in this paper indicate that 2'-O-substituted araT-modified nucleotides are promising as alternatives to the normal deoxy nucleotides for use in antisense and antigene strategies. Furthermore, functionalization of the 2'-position of the deoxyribose ring in the arabino configuration with other groups, linker chains, and moieties such as intercalators and groove binders would yield molecules to test many interesting biological questions.

#### ACKNOWLEDGMENT

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#### LITERATURE CITED

Arnott, S., and Hukins, D. W. L. (1972) Optimised Parameters for A-DNA and B-DNA. *Biochem. Biophys. Res. Commun.* 47, 1504-1510.

- Arnott, S., and Hukins, D. W. L. (1973) Refinement of the Structure of B-DNA and Implications for The Analysis of X-Ray Diffraction Data from Fibers of Biopolymers. *J. Mol. Biol.* 81, 93-105.
- Baleja, J. D., Pon, R. T., and Sykes, B. D. (1990) Solution Structure of Phage  $\lambda$  Half-Operator DNA by Use of NMR, Restrained Molecular Dynamics, and NOE-Based Refinement. *Biochemistry* 29, 4828-4839.
- Beutel, B. A., and Gold, L. (1992) *In Vitro* Evolution of Intrinsically Bent DNA. *J. Mol. Biol.* 228, 803-812.
- Blommers, M. J. J., Pieles, U., and De Mesmaeker, A. (1994) An Approach to the Structure Determination of Nucleic Acid Analogues Hybridized to RNA. NMR Studies of a Duplex Between 2'-OMe RNA and an Oligonucleotide Containing a Single Amide Backbone Modification. *Nucleic Acids Res.* 22, 4187-4194.
- Boelens, R., Scheek, R. M., Dijkstra, K., and Kaptein, R. (1985) Sequential Assignment of Imino- and Amino-Proton Resonances in <sup>1</sup>H NMR Spectra of Oligonucleotides by Two-Dimensional NMR Spectroscopy. Application to a *lac* Operator Fragment. *J. Magn. Reson.* 62, 378-386.
- Borgias, B. A., and James, T. L. (1990) MARDIGRAS—A Procedure for Matrix Analysis of Relaxation for Discerning Geometry of an Aqueous Structure. *J. Magn. Reson.* 87, 475-487.
- Borgias, B. A., Gochin, M., Kerwood, D. J., and James, T. L. (1990) Relaxation Matrix Analysis of 2D NMR Data. *Progr. NMR Spectrosc.* 22, 83-100.
- Dickerson, R. E., Bansal, M., Calladine, C. R., Diekmann, S., Hunter, W. N., Kennard, O., von Kitzing, E., Nelson, H. C. M., Lavery, R., Olson, W. K., Saenger, W., Shakked, Z., Soumpasis, D. M., Tung, C.-S., Sklenar, H., Wang, A. H.-J., and Zhurkin, V. B. (1989) Definitions and Nomenclature of Nucleic acid Structure Parameters. *EMBO J.* 8, 1-4.
- Egholm, M., Buchardt, O., Christensen, L., Behrens, C., Freier, S. M., Driver, D. A., Berg, R. H., Kim, S. K., Norden, B., and Nielsen, P. E. (1993) PNA Hybridizes to Complementary Oligonucleotides Obeying the Watson-Crick Hydrogen-Bonding Rules. *Nature* 365, 566-568.
- Gao, Y.-G., Van der Marel, G. A., Van Boom, J. H., and Wang, A. H.-J. (1991) Molecular Structure of a DNA Decamer Containing an Anticancer Nucleoside Arabinosylcytosine: Conformational Perturbation by Arabinosylcytosine in B-DNA. *Biochemistry* 30, 9922-9931.
- Gotfredsen, C. H., Jacobsen, J. P., and Wengel, J. (1994) Novel Oligodeoxynucleotide Analogues Containing a 2'-O-Methyl-arabinonucleoside. *Tetrahedron Lett.* 35, 6941-6944.
- Gotfredsen, C. H., Jacobsen, J. P., and Wengel, J. (1996) Synthesis and Properties of  $\alpha$ - and  $\beta$ -Oligonucleotides Containing  $\alpha$ - and  $\beta$ -1-(2-O-Methyl-D-arabinofuranosyl)thymine. *Bioorg. Med. Chem.* 4, 1217-1225.
- Guéron, M., Plateau, P., and Decroix, M. (1991) Solvent Signal Suppression in NMR. *Progr. NMR Spectrosc.* 23, 135-209.
- Guschlbauer, W., and Jankowski, K. (1980) Nucleoside Conformation is Determined by the Electronegativity of the Sugar Substituent. *Nucleic Acids Res.* 8, 1421-1433.
- Hare, D. R., Wemmer, D. E., Chou, S.-H., Drobny, G., and Reid, B. R. (1983) Assignment of the Non-Exchangeable Proton Resonances of d(C-G-C-G-A-A-T-T-C-G-C-G) Using Two-Dimensional Nuclear Magnetic Resonance Methods. *J. Mol. Biol.* 171, 319-336.
- Hélène, C., and Toulmé, J.-J. (1990) Specific Regulation of Gene Expression by Antisense, Sense and Antigene Nucleic Acids. *Biochim. Biophys. Acta* 1049, 99-125.
- Inoue, H., Hayase, Y., Imura, A., Iwai, S., Miura, K., and Ohtsuka, E. (1987) Synthesis and Hybridization Studies on Two Complementary Nona(2'-O-methyl)ribonucleotides. *Nucleic Acids Res.* 15, 6131-6147.
- Jørgensen, P. N., Stein, P. C., and Wengel, J. (1994) Synthesis of 3'-C-Hydroxymethylthymidine: Introduction of a Novel Class of Deoxynucleosides and Oligodeoxynucleotides. *J. Am. Chem. Soc.* 116, 2231-2232.
- Kintanar, A., Klevit, R. E., and Reid, B. R. (1987) Two-Dimensional NMR Investigation of a Bent DNA Fragment: Assignment of the Proton Resonances and Preliminary Structure Analysis. *Nucleic Acids Res.* 15, 5845-5862.

- Lavery, R., and Sklenar, H. (1988) The Definition of Generalized Helicoidal Parameters and of Axis Curvature for Irregular Nucleic Acids. *J. Biomol. Struct. Dyn.* 6, 63–91.
- Lavery, R., and Sklenar, H. (1989) Defining the Structure of Irregular Nucleic Acids: Conventions and Principles. *J. Biomol. Struct. Dyn.* 7, 655–667.
- Lesnik, E. A., Guinasso, C. J., Kawasaki, A. M., Sasnor, H., Zounes, M., Cummins, L. L., Ecker, D. J., Cook, P. D., and Freier, S. M. (1993) Oligodeoxynucleotides Containing 2'-O-Modified Adenosine: Synthesis and Effects on Stability of DNA:RNA Duplexes. *Biochemistry* 32, 7832–7838.
- Liu, H., Spielmann, H. P., Ulyanov, N. A., Wemmer, D. E., and James, T. L. (1995) Interproton Distances Bounds from 2D-NOE intensities: Effect of Experimental Noise and Peak Integrations Errors. *J. Biomol. NMR* 6, 390–402.
- Lubini, P., Zürcher, W., and Egli, M. (1994) Stabilizing effects of the RNA 2'-substituent: Crystal Structure of an Oligodeoxynucleotide Duplex Containing 2'-O-Methylated Adenosines. *Chem. Biol.* 1, 39–45.
- Mujeeb, A., Kerwin, S. M., Kenyon, G. L., and James, T. L. (1993) Solution Structure of a Conserved DNA Sequence from the HIV-1 Genome: Restrained Molecular Dynamics Simulation with Distance and Torsion Angle Restraints Derived from Two-Dimensional NMR Spectra. *Biochemistry* 32, 13419–13431.
- Scheek, R. M., Russo, N., Boelens, R., and Kaptein, R. (1983) Sequential Resonance Assignments in DNA  $^1\text{H}$  NMR Spectra by Two-Dimensional NOE Spectroscopy. *J. Am. Chem. Soc.* 105, 2914–2916.
- Schmitz, U., and James, T. L. (1996) Nuclear Magnetic Resonance and Nucleic Acids. In *Methods in Enzymology* (T. L. James, Ed.) Vol. 261, pp 3–44, Academic Press, New York.
- Schmitz, U., Sethson, I., Egan, W. M., and James, T. L. (1992) Solution Structure of a DNA Octamer Containing the Pribnow Box via Restrained Molecular Dynamics Simulation with Distance and Torsional Angle Constraints Derived from Two-Dimensional Nuclear Magnetic Resonance Spectral Fitting. *J. Mol. Biol.* 227, 510–531.
- Schweitzer, B. I., Mikita, T., Kellogg, G. W., Gardner, K. H., and Beardsley, G. P. (1994) Solution Structure of a DNA Dodecamer Containing the Anti-Neoplastic Agent Arabinosylcytosine: Combined Use of NMR, Restrained Molecular Dynamics, and Full Relaxation Matrix Refinement. *Biochemistry* 33, 11460–11475.
- Sproat, B. S., Lamond, A. I., Beijer, B., Neuner, P., and Ryder, U. (1989) Highly Efficient Chemical Synthesis of 2'-O-Methyloligoribonucleotides and Tetrabiotinylated Derivatives; Novel Probes that are Resistant to Degradation by RNA or DNA Specific Nucleases. *Nucleic Acids Res.* 17, 3373–3386.
- Uesugi, S., Miki, H., Ikehara, M., Iwahashi, H., and Kyogoku, Y. (1979) A Linear Relationship Between Electronegativity of 2'-Substituents and Conformation of Adenine Nucleosides. *Tetrahedron Lett.* 42, 4073–4076.
- Uhlmann, E., and Peyman, A. (1990) Antisense Oligonucleotides: A New Therapeutic Principle. *Chem. Rev.* 90, 543–584.
- Weisz, K., Shafer, R. H., Egan, W., and James, T. L. (1994) Solution Structure of the Octamer Motif in Immunoglobulin Genes via Restrained Molecular Dynamics Calculations. *Biochemistry* 33, 354–366.
- Woessner, D. E. (1962) Spin Relaxation Processes in a Two-Proton System Undergoing Anisotropic Reorientation. *J. Chem. Phys.* 36, 1–4.
- Wüthrich, K. (1986). *NMR of Proteins and Nucleic Acids*, Wiley, New York.
- Yoon, C., Privé, G. G., Goodsell, D. S., and Dickerson, R. E. (1988) Structure of an Alternating-B DNA Helix and Its Relationship to A-tract DNA. *Proc. Natl. Acad. Sci. U.S.A.* 85, 6332–6336.
- Yuan, H., Quintana, J., and Dickerson, R. E. (1992) Alternative Structures for Alternating Poly(dA-dT) Tracts: The Structure of the B-DNA Decamer C-G-A-T-A-T-A-T-C-G. *Biochemistry* 31, 8009–8021.

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# Antibody Fragments in Tumor Pretargeting. Evaluation of Biotinylated Fab' Colocalization with Recombinant Streptavidin and Avidin

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An evaluation of the use of a biotinylated monoclonal antibody Fab' fragment in tumor pretargeting was conducted. As a model system, tumor colocalization of avidin or recombinant streptavidin (r-streptavidin) and the biotinylated Fab' fragment (Fab'-S-biotin) of A6H, an antirenal cell carcinoma antibody, was evaluated in athymic mice bearing human renal cell carcinoma xenografts. A new water soluble sulfhydryl reactive biotinylation reagent, *N*-(13-*N*-maleimido-4,7,10-trioxatridecanyl)-biotinamide, was synthesized and used for biotinylation of Fab'. A biodistribution of ChT-labeled A6H Fab'-S-biotin was conducted. Data from that distribution indicated that the Fab'-S-biotin localized well (i.e. 28% ID/g at 24 h) to human tumor xenografts in athymic mice. Subsequently, a biodistribution study involving pretargeting radioiodinated A6H Fab'-S-biotin to tumor xenografts, followed by administration of r-streptavidin at 4 or 20 h, was conducted. Specific colocalization of r-streptavidin to tumors containing the A6H Fab'-S-biotin was evident from the data obtained. In a similar biodistribution study, specific colocalization of avidin to tumors pretargeted with A6H Fab'-S-biotin was also observed. The avidin used in the study was radioiodinated with the *N*-hydroxysuccinimidyl ester of *p*-[<sup>125</sup>I]iodobenzoate ([<sup>125</sup>I]PIB-NHS). Very low concentrations (e.g. 0.35% ID/g) of avidin colocalized at the tumor. To further show that specific colocalization within the tumor xenografts had occurred with biotinylated A6H Fab', radioiodinated avidin and r-streptavidin were co-injected into athymic mice bearing tumor xenografts to obtain their distributions without having biotinylated Fab' present. At 20 h postinjection, only small differences in the blood and tumor concentrations of either protein were observed, indicating that the specific tumor colocalization seen in the previous two biodistributions must have been due to the presence of Fab'-S-biotin. Calculations were conducted to estimate how much r-streptavidin (as a molar ratio) was colocalized. From the data obtained it was estimated that 36–61% of the tumor-localized Fab'-S-biotin molecules were bound with r-streptavidin and 4–23% bound with avidin, under the conditions studied.

## INTRODUCTION

An alternative approach to using directly labeled monoclonal antibodies for delivery of radionuclides to tumors, termed "pretargeting", is being investigated by a number of research groups (1–5). In the pretargeting approach, sequestration of radioactivity at a tumor is attained by colocalization of a radiolabeled molecule with an antibody bound to tumor antigens. Colocalization of the radiolabeled molecule is achieved by modifying the antibody in a manner that results in strong binding with the radionuclide-carrying molecule. Thus, the modified antibody is administered, allowed to localize, and clear from nontumor tissues; then in one or two subsequent steps, the radionuclide is delivered attached to a molecule which has a high affinity for binding with another molecule conjugated to the antibody. A schematic representation of tumor delivery by directly labeled antibodies and antibody tumor "pretargeting" is shown in Figure 1. As depicted in Figure 1, the pretargeting approach is more complex than administration of labeled antibodies;

however, pretargeting has the major advantage of being able to separate the *in vivo* clearance rate of radioactivity from the biological half-life of the antibody. Slow clearance rates of radiolabeled antibodies contribute greatly to the nontumor tissue doses (6).

A requirement to obtaining colocalization of the radiolabeled molecule with the pretargeted antibody conjugate at the tumor is that the conjugate have a high affinity for binding with the radiolabeled molecule. One method for obtaining such a conjugate is to prepare bispecific antibodies (7–10) (the pretargeting antibody is modified by conjugation with a second antibody), and tumor targeting of a radionuclide is achieved by infusion of a radiolabeled hapten reactive with the second antibody (11, 12). Alternatively, antibodies can be modified by conjugation of a molecule (other than an antibody) that has a high binding affinity for another (radiolabeled) molecule. Many of the investigations involving the antibody pretargeting method have used conjugates that employ a combination of biotin (13) and the biotin-binding protein avidin (14) or streptavidin (15). These reagents have been used because of the very high affinity for binding of biotin with avidin and streptavidin (i.e.,  $10^{-13}$ – $10^{-15}$  M). This high affinity results in an essentially irreversible binding of biotin to avidin or streptavidin. Biotin-avidin/streptavidin binding and antibody tumor pretargeting can be combined in different ways to achieve tumor targeting of radionuclides, similar to those depicted in Figure 1 (16). For example, several investiga-

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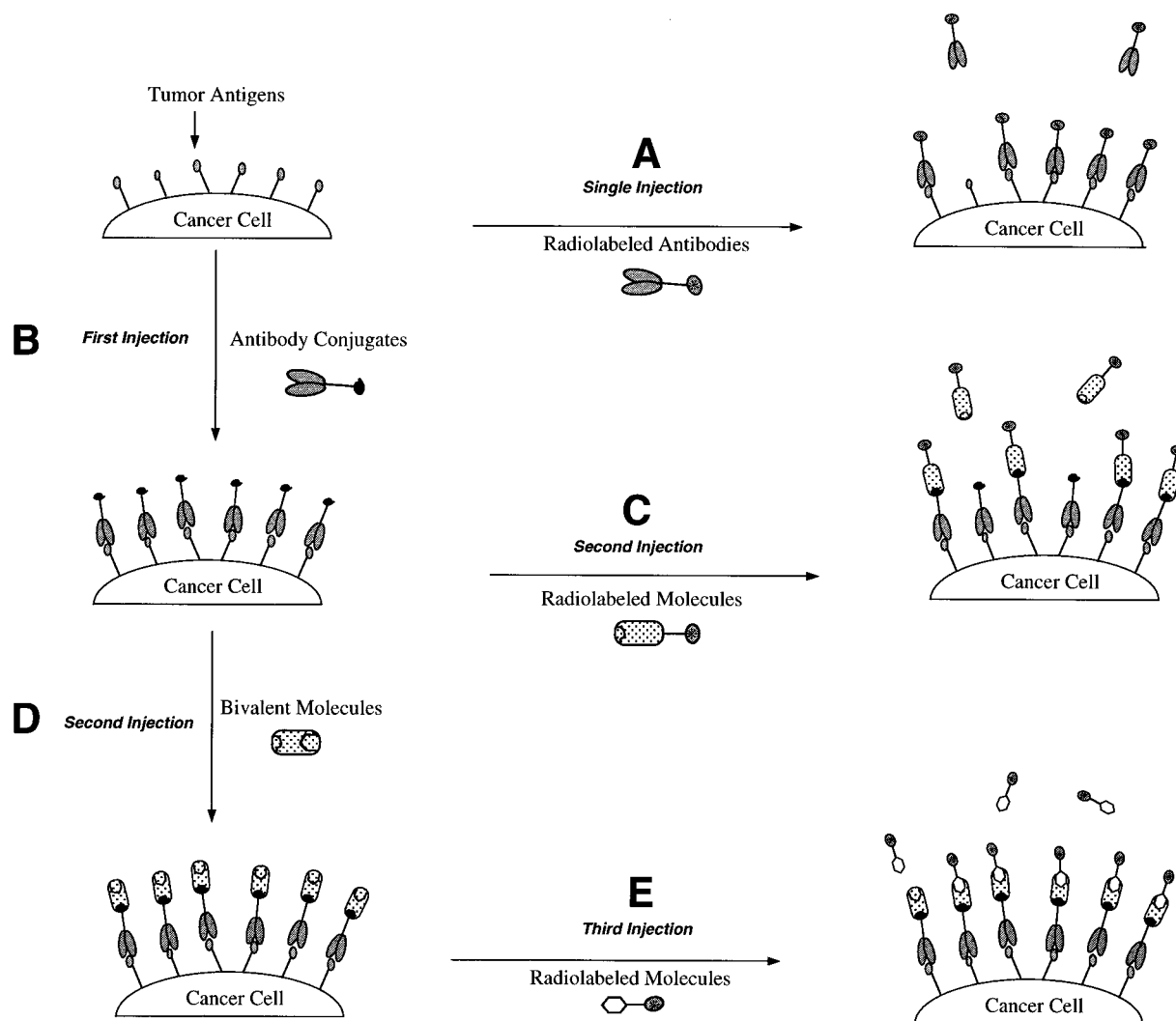


**Figure 1.** Schematic representation for targeting radioactivity to tumors using monoclonal antibodies. Path A represents tumor localization of directly radiolabeled antibodies (i.e., single step—one injection). Path B represents tumor localization of antibody conjugates in “pretargeting” protocols involving two or three steps (i.e. two or three injections). In the pretargeting approach tumor localization of the antibody conjugate is achieved; then the radionuclide can be delivered to the tumor by injection of a radiolabeled molecule that binds with the antibody conjugate (steps B and C—two-step method) or by injection of a bivalent molecule that binds with the antibody and has an affinity for a second molecule, which is administered with a radionuclide attached (steps B, D, E—three-step method).

tions have reported the use of pretargeted biotinylated monoclonal antibodies in a “two-step” tumor localization approach, wherein biotinylated antibody is prelocalized at the tumor and then radiolabeled avidin or streptavidin is administered (17–21). In another version of the two-step approach, the biotin-binding protein is conjugated with the antibody and that conjugate is prelocalized at the tumor and then a radiolabeled derivative of biotin is administered (22–24). Other investigators have described a “three-step” approach, wherein sequential administration of biotinylated antibody, streptavidin, or avidin, and radiolabeled biotin derivative is carried out (16, 21, 25, 26).

Most investigations of tumor pretargeting have employed conjugates prepared with intact antibodies. Unfortunately, use of intact antibody conjugates results in long blood retention times. Retention of the antibody conjugates in blood is problematic as the nonradiolabeled antibody conjugate can combine with the radiolabeled molecule after its administration, resulting in longer blood retention of radioactivity and increased nontumor tissue radiation doses. This fact makes it imperative that there be a waiting period of several days prior to administration of radiolabeled biotin. Similar to the use

of second antibodies to decrease the blood concentrations of radiolabeled antibodies (6, 27), “clearing agents” have been used to decrease the amount of antibody conjugate in the blood (28–34). While clearing agents have been found to be effective, their use introduces an additional reagent administration, which increases the number of steps involved. We thought that this extra step might be alleviated by using conjugates of antibody fragments rather than intact antibodies, as the fragments would clear more rapidly from the tissues (4). Minimizing the number of steps in pretargeting protocols is of particular interest to us as we are investigating the potential for increasing the amount of radioactivity attained on cancer cells by utilizing a multistep antibody/biotin/streptavidin pretargeting approach, as depicted in Figure 2. Another consideration that directed us to investigate the use of antibody fragments in tumor pretargeting was the fact that fragments appear to have decreased immunogenicity in patients (35).

Reported here are the results of preliminary studies designed to evaluate the specific localization of radiolabeled, biotinylated Fab' and subsequent colocalization of a dual ( $^{125}\text{I}$  or  $^{131}\text{I}$ ) radiolabeled recombinant streptavidin (r-streptavidin)<sup>1</sup> or avidin in athymic mice bearing hu-

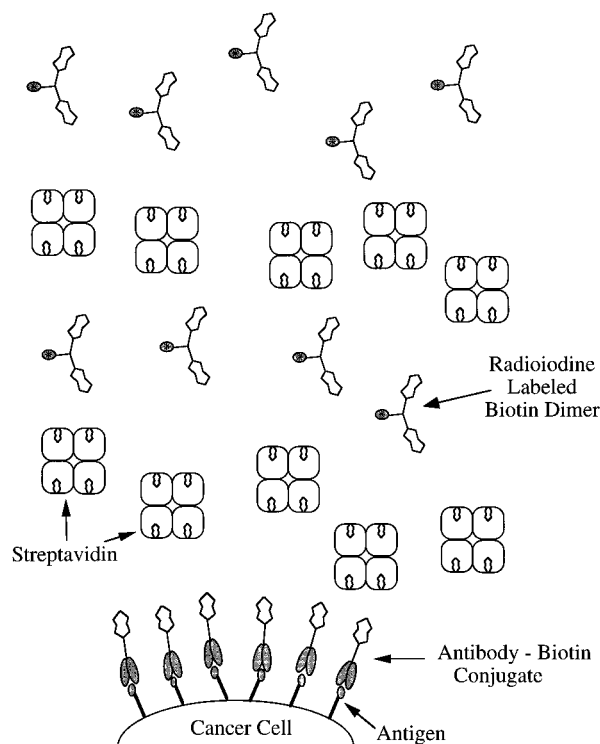


**Figure 2.** Schematic representation of proposed method for amplifying the number of binding sites on a cancer cell. To be successful in tumor targeting, antibodies must be reactive with antigens present in large numbers (e.g.,  $10^5$ – $10^6$ ) on a cancer cell. To provide the maximum bound antibody, a large quantity of an antibody or its fragment (nonradiolabeled) can be pretargeted (bound to antigen). In a subsequent step, streptavidin (nonradiolabeled) can be administered to bind with the exposed biotin on the antibody fragments. In a third step, radiolabeled cross-linking (e.g., dimeric) biotin molecules can be administered to bind with the streptavidin–biotin–antibody fragment. Since only one of the biotin binding sites of the streptavidin is occupied, three molecules of radioiodinated biotin dimer might bind. A second administration of streptavidin will bind to the exposed biotin molecules. The administration of streptavidin and radiolabeled biotin dimer might be repeated several times, potentially increasing the quantity of radioactivity at the tumor by a factor of 2–3 after each administration.

man tumor xenografts. The antirenal cell carcinoma antibody, A6H (36), and a human renal cell carcinoma tumor xenograft, TK-82 (37), were used in the evaluations. In addition to demonstrating colocalization of a biotinylated A6H Fab' and streptavidin or avidin, a primary goal of this study was to quantify the amount of these proteins that colocalized with the biotinylated Fab' in tumor and other tissues. While neither of these reagents would be radiolabeled in a pretargeting protocol, conditions for optimization of the deposition of antibody fragment and colocalization of the biotin-binding protein were deemed to be important. This is particularly true for their application in more complex protocols, such as depicted in Figure 2. Thus, in two biodistribution studies, colocalization and quantification of biotinylated A6H

Fab' and r-streptavidin or avidin were evaluated. Differences in tissue colocalization of radioactivity when radiolabeled streptavidin or avidin was administered at 4 or at 20 h postinjection of the biotinylated Fab' were investigated. Additional studies examined biodistributions of biotinylated A6H Fab', r-streptavidin, and avidin in athymic mice bearing TK-82 tumor xenografts. Dual isotope labeling of the proteins administered (biotinylated A6H Fab', r-streptavidin, avidin) was employed in most biodistributions, such that the distribution of each protein could be followed *in vivo* while using a minimum number of animals in the studies.

## EXPERIMENTAL PROCEDURES

**General.** All chemicals purchased from commercial sources were of analytical grade or better and were used without further purification. *d*-Biotin (**5**) was purchased from Sigma Chemical Co. (St. Louis, MO). 4-Hydroxyazobenzene-2-carboxylic acid (HABA) was purchased from Pierce Chemical Co. (Rockford, IL). *N*-Methoxycarbonylmaleimide (**10**) was obtained from Fluka Chemical Corp. (Ronkonkoma, NY). *N*-Ethylmaleimide (NEM), 4,7,10-trioxa-1,13-tridecanediamine (**8**), 2,3,5,6-tetrafluorophenol, and all other reagents used were obtained from Aldrich Chemical Co. (Milwaukee, WI). 2,3,5,6-Tetrafluorophenyl trifluoroacetate (**6**) was prepared according to the method previously described (38). Recombinant streptavidin was prepared as previously described (39). A6H F(ab')<sub>2</sub> was prepared as previously reported (40). Avidin was purchased from Pierce as a lyophilized powder. Human serum albumin (HSA) was obtained from Miles, Inc. (Elkhart, IN). Pronase was obtained from Boehringer Mannheim (Indianapolis, IN). Prepacked Sephadex G-25M columns (PD-10 and NAP-10) were obtained from Pharmacia Biotech (Piscataway, NJ). Centricon 10 ultracentrifugation filters were obtained from Amicon, Inc. (Beverly, MA). Solvents for HPLC analysis were obtained as HPLC grade and were filtered (0.2  $\mu$ m) prior to use. Silica gel, 70–230 mesh, 60 Å, used in purification of biotin derivatives was obtained from Aldrich. Melting points were obtained in open capillary tubes on a Mel-Temp II apparatus with a Fluke 51 K/J electronic thermometer and are uncorrected. Elemental analyses were obtained from Desert Analytics (Tucson, AZ) or Quantitative Technologies Inc. (Whitehouse, NJ).

Molecular modeling of sulfhydryl reactive biotin derivatives (Figure 3) was conducted to estimate the distance (Å) between the biotin carboxylate carbon and the carbon to which the sulfhydryl is reactive. The modeling used ChemDraw Plus/Chem3D Pro software (CambridgeSoft Corp., Burlington, MA). Structures were drawn (fully extended) in ChemDraw Plus and imported into and minimized for structural parameters and energy in ChemDraw 3D. After minimization, the interatomic distances were obtained directly with ChemDraw 3D by highlighting the two atoms between which the distance was sought. Molecular modeling of proteins was conducted with MacImdad software program (Molecular Applications Group, Palo Alto, CA) using Brookhaven protein data bank files.

Na[<sup>125</sup>I]I and Na[<sup>131</sup>I]I were purchased from NEN/DuPont (Billerica, MA) as high concentration/high specific activity radioiodide in 0.1 N NaOH. Measurement of <sup>125</sup>I and <sup>131</sup>I was accomplished on the Capintec CRC-15R or a Capintec CRC-6A radioisotope calibrator. Tissue samples were counted in an LKB 1282 gamma counter with the following window settings: channels 35–102, 20–90 keV; and channels 165–185, 300–450 keV when <sup>125</sup>I and <sup>131</sup>I were counted together.

<sup>1</sup> Abbreviations: ChT, chloramine-T; cpm, counts per minute; DTT, dithiothreitol; HABA, 4'-hydroxyazobenzene-2-carboxylic acid; HAMA, human anti-mouse antibodies; MAb, monoclonal antibody; NEM, *N*-ethylmaleimide; PBS, phosphate-buffered saline; pi, postinjection; PIB-NHS, *N*-succinimidyl ester of *p*-iodobenzoate; PSNB-NHS, *N*-succinimidyl ester of *p*-tri-*n*-butylstannylbenzoate; RCC, renal cell carcinoma; r-streptavidin, recombinant streptavidin; rt, room temperature; sc, subcutaneous; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; Tris-HCl, 2-amino-2-(hydroxymethyl)-1,3-propanediol hydrochloride.

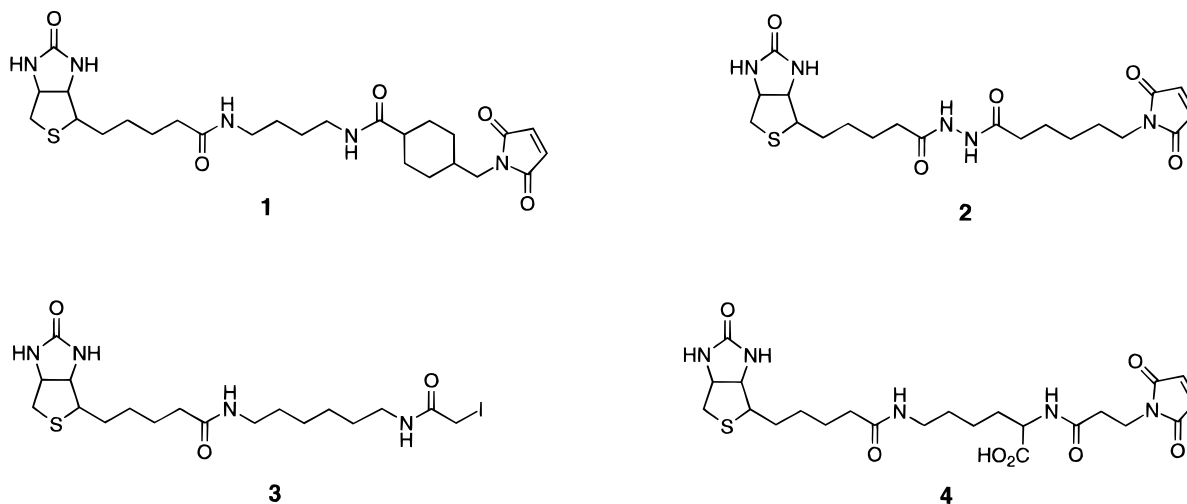


**Figure 3.** Sulfhydryl reactive biotinylation reagents. Biotinylation reagents **1** (1-biotinamido-4-[4'-maleimidomethyl]cyclohexanecarboxamido]butane and **3** (*N*-iodoacetyl-*N*-biotinylhexylenediamine) are available from Pierce. Biotinylation reagent **2** (*N*-biotinyl-*N'*-[6-maleimidohexanoyl]hydrazide) is available from Sigma. Biotinylation reagent **4** (*N*<sup>ε</sup>-biotinyl-*N*<sup>α</sup>-(3-maleimidopropionyl)-*L*-lysine) is available from Fluka.

**Spectroscopic Data.** <sup>1</sup>H NMR spectra were obtained on Bruker AF-300 (300 MHz) instrument. The chemical shifts are expressed as parts per million using tetramethylsilane as an internal standard ( $\delta = 0.0$  ppm). IR data were obtained on a Perkin-Elmer 1420 infrared spectrophotometer. Mass spectral data were obtained on a VG 70SEQ mass spectrometer with 11250J data system. Fast atom bombardment (FAB<sup>+</sup>) mass spectral data were obtained at 8 kV using a matrix of 2-hydroxyethyl sulfide (2HEDS).

**Analytical Chromatography.** *Biotin Derivatives.* HPLC separations of biotin derivatives were obtained on a Hewlett-Packard quaternary 1050 gradient pumping system with a variable wavelength UV detector (254 nm). Analysis of the HPLC data was conducted on Hewlett-Packard HPLC ChemStation software. Biotin derivatives **7**, **9**, and **11** (Scheme 1) were analyzed on a reversed-phase C<sub>18</sub> column (4.6 mm  $\times$  250 mm, 5  $\mu$ m; Altima; Alltech) eluting with a gradient at a flow rate of 1.0 mL/min. The gradient began with 40% MeOH/60% of a 1% aqueous HOAc solution. The initial mixture was held for 2 min, and then the concentration of MeOH was increased to 100% over a 10 min period and held at 100% MeOH for 8 min. Retention times were 10.1 min for **7**, 3.8 min for **9**, and 8.9 min for **11**.

*Proteins.* HPLC separations of (nonradioactive) proteins were conducted using a Hewlett-Packard 1050 multiple wavelength detector, isocratic pump, and TSK-GEL G3000SW column (7.5 cm  $\times$  60 cm, 10  $\mu$ m; Tosoh-Haas, Montgomeryville, PA). The mobile phase consisted of 50 mM potassium phosphate, 300 mM NaCl, 1 mM EDTA, and 1 mM NaN<sub>3</sub> in deionized water. A flow rate of 0.75 mL/min was used. Retention times under these conditions were as follows: A6H F(ab')<sub>2</sub>, 24.5 min; Fab'-SH, 27.4 min; Fab'-S-NEM, 27.0 min, Fab'-S-biotin, 27.7; r-streptavidin, 28.9 min; and avidin, 27.5 min.

*Radioiodination Reagent.* HPLC separation of radioiodinated reagent [<sup>125</sup>I]PIB-NHS was conducted using a gradient system that consisted of two Beckman Model 110B pumps, a Beckman 420 controller, a Beckman Model 153 UV detector (254 nm), and a Beckman Model 170 radioisotope detector. The HPLC conditions for [<sup>125</sup>I]-PIB-NHS were as previously described (41).

*Radiolabeled Proteins.* HPLC separations of radioiodinated proteins were conducted on a system consisting of two Model 306 Gilson pumps with 10SC titanium pump heads, a Gilson Model 118 UV-visible detector

(280 nm), a Beckman Model 170 radioisotope detector, a Gilson System Interface Module, and an IBM 486 with Gilson Unipoint HPLC Controller software. The proteins were separated by size exclusion chromatography on a 300 SW Protein Pak column (8 mm  $\times$  300 mm glass column; 10  $\mu$ m silica particle size; Waters Chromatography, Milford, MA), eluting with a solvent mixture of 25 mM Tris-HCl, 75 mM NaCl, and 1 mM EDTA, pH 7.2, at a flow rate of 0.70 mL/min. Retention times were as follows: intact A6H, 10.6 min; A6H F(ab')<sub>2</sub>, 11.3 min; Fab'-S-NEM, 16.3 min; Fab'-S-biotin, 16.3 min; r-streptavidin, 14.8 min; and avidin, 17.2 min.

**Handling of Radioactive Materials.** All reactions involving radioactive materials were conducted in a charcoal-filtered Plexiglas enclosure within a fume hood. All radioactive materials were opened, handled, and stored in the Plexiglas enclosure until used in studies or removed as radiation waste. Radioiodination reactions were conducted in 1 mL vials with Teflon-coated septa. The reaction mixtures were vented through a 10 mL charcoal-filled syringe, and addition of reagents or removal of materials was conducted by passing a syringe needle through the septum. Standard radiation safety procedures for handling radioiodine, including double gloves, were used. Radiation monitoring followed approved procedures.

**Synthesis of Biotin Tetrafluorophenyl Ester, **7**.** Biotin (**5**; 10 g, 40.9 mmol) was dissolved in 200 mL of warm (70 °C) DMF under argon atmosphere. The solution was allowed to cool to ambient temperature, and 10 mL (82 mmol) of triethylamine was added, followed by the addition of 16 g (61 mmol) of 2,3,5,6-tetrafluorophenyl trifluoroacetate (**6**). The reaction was stirred at room temperature for 30 min, and solvent was removed under vacuum. The product was triturated in 100 mL of ether and was filtered. The isolated product was dried under vacuum to yield 14 g (83%) of biotin TFP ester (**7**) as a colorless solid: mp 185–187 °C; <sup>1</sup>H NMR (DMSO-*d*<sub>6</sub>,  $\delta$ ) 1.4–1.8 (m, 6H), 2.5 (m, 1H), 2.6–2.9 (m, 3H), 3.1 (m, 1H), 4.2 (m, 1H), 6.4 (d, 2H), 7.9 (m, 1 H); IR (KBr, cm<sup>-1</sup>) 3250, 2915, 1790, 1710, 1520, 1480, 1090. Anal. Calcd for C<sub>16</sub>H<sub>16</sub>F<sub>4</sub>N<sub>2</sub>O<sub>3</sub>S: C, 48.98; H, 4.11; N, 7.14. Found: C, 48.90; H, 4.14; N, 6.86.

**Synthesis of *N*-(13-Amino-4,7,10-trioxatridecan-yl)biotinamide (**9**).** Biotin TFP ester (**7**; 5 g, 12.8 mmol) was added to a dry flask containing 200 mL of anhydrous DMF. In another dry flask containing 28 g (128 mmol)

of 4,7,10-trioxa-1,13-tridecanediamine (**8**) was added 4 mL of triethylamine. Both flasks were cooled to 0–5 °C with ice–water baths. The TFP ester of biotin was added dropwise to the trioxatridecanediamine solution over the period of 1 h. The reaction was stirred at room temperature for 30 min, and solvent was removed under vacuum. The resulting oil was triturated in 500 mL of ether and was stirred for 30 min. The solid was filtered, then dissolved in methanol/ethyl acetate (4:1), and loaded onto a silica column (2.5 cm × 35 cm). The column was eluted with the same solvent mixture. Fractions containing product were collected, and solvent was removed under vacuum. The isolated product was dried under vacuum to yield 4.5 g (79%) of **9** as a colorless solid: mp 104–106 °C; <sup>1</sup>H NMR (MeOH, δ) 1.46 (m, 2H), 1.6–1.8 (m, 9H), 2.2 (t, 2H), 2.7 (d, 1H), 2.75–2.9 (m, 3H), 3.2–3.3 (m, 5H), 3.5–3.6 (m, 14H), 4.3 (m, 1H), 4.5 (m, 1H); IR (KBr, cm<sup>-1</sup>) 3280, 2910, 2850, 1690, 1640, 1110, 940. Anal. Calcd for C<sub>20</sub>H<sub>38</sub>N<sub>4</sub>O<sub>5</sub>·H<sub>2</sub>O: C, 51.70; H, 8.68; N, 12.06. Found: C, 51.95; H, 7.98; N, 11.65.

**Synthesis of N-(13-N-Maleimido-4,7,10-trioxatridecanyl)biotinamide (11).** Biotin-amine (**9**; 1.0 g, 2.2 mmol) was dissolved in 12 mL of saturated aqueous sodium bicarbonate and was cooled with ice–water. N-Methoxycarbonylmaleimide (**10**; 4.5 mmol, 0.696 g) was added, and the reaction was stirred at 0 °C for 10 min. A 50 mL quantity of water was added to the reaction, and the stirring was continued at room temperature for an additional 15 min. The solution was extracted with (4 × 100 mL) chloroform. The combined chloroform extracts were washed with (2 × 50 mL) water and dried over anhydrous sodium sulfate, and chloroform was removed under vacuum. The product was triturated in 100 mL of ether and filtered. The isolated product was dried under vacuum to yield 0.57 g (49%) of **11** as a colorless solid: mp 112–114 °C; <sup>1</sup>H NMR (MeOH, δ) 1.46 (m, 2H), 1.6–1.8 (m, 9H), 2.2 (t, 2H), 2.7 (d, 1H), 2.9 (dd, 1H), 3.2–3.3 (m, 4H), 3.5–3.6 (m, 15H), 4.3 (m, 1H), 4.5 (m, 1H), 6.8 (s, 2H); IR (KBr, cm<sup>-1</sup>) 3280, 2910, 2850, 1760, 1690, 1640, 1110, 940; HRMS calcd for C<sub>24</sub>H<sub>38</sub>N<sub>4</sub>O<sub>7</sub>S (M + H) 527.2539, found 527.2526. Anal. Calcd for C<sub>24</sub>H<sub>38</sub>N<sub>4</sub>O<sub>7</sub>S: C, 54.75; H, 7.22; N, 10.65. Found: C, 54.47; H, 7.03; N, 10.59.

**Preparation of A6H Fab'.** To 150 μL of a 17 mg/mL solution of A6H F(ab')<sub>2</sub> was added 6 μL of 100 mM DTT in 20 mM sodium phosphate, pH 6.8. The solution was mixed periodically over 1 h at rt. The reaction progress was followed by size exclusion HPLC. After 1 h, the solution was placed on a Sephadex G-25 (PD-10) column that was pre-equilibrated with 20 mM sodium phosphate, pH 6.8, containing 1 mM EDTA. The column was eluted with the same buffer, collecting 1 mL fractions. The Fab' was obtained in fractions 3 and 4. The concentration of A6H Fab' in the (combined) collected fractions was determined by UV at 280 nm (0.1% solution gives an estimated absorbance of 1.15). A protein recovery of 95% was obtained.

**Preparation of Fab'–S-NEM.** To 2 mL of a 0.25 mg/mL solution of Fab' was added 100 μL (50 equiv, 0.64 mg/mL) of a solution containing NEM in 20 mM sodium phosphate, pH 6.8. The reaction was allowed to proceed at room temperature for 30 min before the mixture was split into two equal volume fractions and placed in separate Centricon 10 ultracentrifugation filters that were prewashed with 1 mL of 20 mM sodium phosphate, pH 6.8. The solutions were centrifuged at 3200 rpm for 15 min to concentrate the solutions to 100 μL volume. Each Centricon retentate was rinsed four times with 500 μL of the phosphate buffer, reducing the volume to 100 μL by centrifugation during each rinse. The final con-

centration of A6H Fab'–S-NEM was determined in the manner as described for A6H Fab'. A protein recovery of 81% was obtained.

**Preparation of Fab'–S-Biotin.** Biotinylation of Fab' sulfhydryl groups was accomplished by reaction with biotin–maleimide using the same conditions as described for the NEM sulfhydryl capping. Briefly, 100 μL (50 equiv, 8.6 mg/mL) of a solution containing **11** in 20 mM sodium phosphate, pH 6.8, was added to 1.8 mL of a 0.9 mg/mL solution of Fab'. The final concentration of A6H Fab'–S-biotin was determined in the manner as described for A6H Fab'. A protein recovery of 91% was obtained.

**Determination of Number of Biotin Molecules Conjugated to Fab'.** The spectrophotometric method of HABA displacement by biotin was used to evaluate the number of biotin moieties conjugated with A6H Fab'–SH (42, 43). To begin, a standard curve for displacement of bound HABA was obtained as follows. To 1 mL of a 0.5 mg/mL solution of avidin in 50 mM sodium phosphate and 0.9% NaCl, pH 6.0, was added 25 μL of 10 mM HABA in 10 mM NaOH. The absorbance at 500 nm was measured, and then 2 μL of a 0.167 mM biotin in 50 mM sodium phosphate buffer, 0.9% NaCl, pH 6.0, was added (0.33 × 10<sup>-6</sup> mmol of biotin/aliquot). The absorbance at 500 nm was measured again. The addition/UV measurement sequence was repeated for 10 additions, and the change in absorbance obtained after each addition was plotted (KaleidaGraph; Synergy Software, Reading, PA) vs biotin added (millimoles × 10<sup>-6</sup>). A least-squares fit of the data for the standard curve indicated that the data had a correlation coefficient of 0.989, and the data fit the equation  $Y = -0.003342 + 0.035157X$ .

Two methods of analyzing the number of biotins on the Fab'–S-biotin were conducted. In the first evaluation, 15 μL of a 2.9 mg/mL solution of the biotinylated Fab' was added to 1 mL of avidin–HABA solution, and the absorbance at 500 nm was measured. In the second method, 100 μL of a 2.6 mg/mL of biotinylated Fab' was heated to 56 °C for 10 min, and then 10 μL of a 1% Pronase solution in distilled H<sub>2</sub>O was added. The mixture was allowed to digest at room temperature for 16 h, and then a 10 μL aliquot of the digested biotinylated Fab' solution was added to a mixture of avidin and HABA. The absorbance at 500 nm was measured. The quantity of biotin (millimoles) was obtained from the standard curve. The number of biotin molecules (average) per molecule of Fab' was determined by dividing the millimoles per milliliter of biotin by the millimoles per milliliter of Fab' used in the assay.

#### **Radioiodination of A6H Fab'–S-NEM Using ChT.**

**Preparation A.** To a vial containing a mixture of 20 μL of 0.5 M sodium phosphate, pH 7.4, and 0.5 μL of Na-[<sup>125</sup>I]I (700 μCi) in 0.1 N NaOH was added 200 μg of Fab'–S-NEM in 100 μL of 20 mM sodium phosphate and 1 mM EDTA at pH 6.2. To that mixture was added 22 μL of a 1.0 mg/mL solution of chloramine-T (ChT) in H<sub>2</sub>O. After 5 min at rt, the reaction was quenched with the addition of 1.4 μL of a 10 mg/mL solution of sodium metabisulfite. A 652 μCi quantity of the iodinated Fab' was placed on a Sephadex G-25 column (NAP-10), eluted with 0.9% saline, to give 556 μCi (85%) of the radiolabeled Fab'. A specific activity of 2.36 μCi/μg was obtained. HPLC indicated that the isolated product had 100% radiochemical purity. An immunoreactive binding fraction of 14% [30% relative to F(ab')<sub>2</sub>] was measured.

**Preparation B.** This preparation was conducted as described for preparation A (above). In the radiolabeling 470 μg of Fab'–S-NEM was radioiodinated with 445 μCi Na[<sup>125</sup>I]I using 24 μg of ChT. An 87% radiochemical yield

of [ $^{125}\text{I}$ ]Fab'-S-NEM was obtained. This material had 100% radiochemical purity by HPLC. The specific activity of the radioiodinated Fab' was 0.98  $\mu\text{Ci}/\mu\text{g}$ . A relative immunoreactive binding fraction of 11% [23% relative to  $\text{F}(\text{ab}')_2$ ] was measured.

#### **Radioiodination of A6H Fab'-S-Biotin Using ChT.**

**Preparation A.** A 457  $\mu\text{g}$  quantity of A6H Fab-S-Biotin in 150  $\mu\text{L}$  of PBS was added to a vial containing a mixture of 25  $\mu\text{L}$  of 0.5 M sodium phosphate, pH 7.4, and 1  $\mu\text{L}$  (554  $\mu\text{Ci}$ ) of  $\text{Na}[^{125}\text{I}]\text{I}$  in 0.1 N NaOH. To that mixture was added 48  $\mu\text{L}$  of a 1.0 mg/mL solution of ChT in  $\text{H}_2\text{O}$ . After 5 min at rt, 4.8  $\mu\text{L}$  of a 10 mg/mL sodium metabisulfite solution was added to quench the reaction. A 527  $\mu\text{Ci}$  aliquot of radioiodinated Fab'-S-biotin was purified by elution on a NAP-10 column with 0.9% saline to give 494  $\mu\text{Ci}$  (94%). The specific activity of this preparation was 1.11  $\mu\text{Ci}/\mu\text{g}$ , and it had a 100% radiochemical purity by HPLC analysis. An immunoreactive binding fraction of 12% (26% relative to  $\text{F}(\text{ab}')_2$ ) was measured.

**Preparation B.** A 621  $\mu\text{g}$  quantity of A6H Fab'-S-biotin in 180  $\mu\text{L}$  of PBS was radioiodinated with 702  $\mu\text{Ci}$  of  $\text{Na}[^{125}\text{I}]\text{I}$  and 48  $\mu\text{g}$  of ChT as described for preparation A. Purification of the radioiodinated Fab' by elution on a NAP-10 column provided 636  $\mu\text{Ci}$  (91%) of radiolabeled Fab', which had a specific activity of 1.06  $\mu\text{Ci}/\mu\text{g}$  and a 100% radiochemical purity by HPLC analysis. An immunoreactive binding fraction of 14% [30% relative to  $\text{F}(\text{ab}')_2$ ] was measured.

**Preparation C.** A 609  $\mu\text{g}$  quantity of A6H Fab'-S-biotin in 70  $\mu\text{L}$  of PBS was radioiodinated with 651  $\mu\text{Ci}$  of  $\text{Na}[^{131}\text{I}]\text{I}$  and 17  $\mu\text{g}$  of ChT as described for preparation A. Purification of the radioiodinated Fab' by elution on a NAP-10 column yielded 522  $\mu\text{Ci}$  (80%), which had a specific activity of 1.41  $\mu\text{Ci}/\mu\text{g}$  and a 100% radiochemical purity by HPLC analysis. An immunoreactive binding fraction of 49% [57% relative to  $\text{F}(\text{ab}')_2$ ] was measured.

#### **Radioiodination of A6H $\text{F}(\text{ab}')_2$ Using ChT.**

**Preparation A.** A 100  $\mu\text{g}$  quantity of A6H  $\text{F}(\text{ab}')_2$  in 59  $\mu\text{L}$  of PBS was added to a mixture of 25  $\mu\text{L}$  of 0.5 M sodium phosphate and 0.5  $\mu\text{L}$  of  $\text{Na}[^{125}\text{I}]\text{I}$  (166  $\mu\text{Ci}$ ) in 0.1 N NaOH. To that solution was added 16  $\mu\text{L}$  of a 1.0 mg/mL solution of ChT in  $\text{H}_2\text{O}$ . After 5 min at rt, 1.6  $\mu\text{L}$  of a 10 mg/mL solution of sodium metabisulfite in  $\text{H}_2\text{O}$  was added to quench the reaction. A 137  $\mu\text{Ci}$  quantity of labeled  $\text{F}(\text{ab}')_2$  was purified by elution on a NAP-10 column with 0.9% saline to yield 114  $\mu\text{Ci}$  (83%). A specific activity of 1.2  $\mu\text{Ci}/\mu\text{g}$  and a radiochemical purity (by HPLC) of 100% were obtained. An immunoreactive binding fraction of 47% was measured.

**Preparation B.** The radioiodination was conducted as described for preparation A. A 204  $\mu\text{g}$  quantity of A6H  $\text{F}(\text{ab}')_2$  was reacted with 377  $\mu\text{Ci}$  of  $\text{Na}[^{125}\text{I}]\text{I}$ /ChT to give 253  $\mu\text{Ci}$  (67%) of labeled product. An immunoreactive binding fraction of 86% was measured.

#### **Radioiodination of r-Streptavidin Using ChT.**

**Preparation A.** A 1.75 mg quantity of r-streptavidin in 125  $\mu\text{L}$  of a 50 mM Tris/50 mM NaCl solution, pH 8, was added to a mixture of 25  $\mu\text{L}$  of 0.5 M sodium phosphate, pH 7.4, and 1  $\mu\text{L}$  (0.98 mCi) of  $\text{Na}[^{131}\text{I}]\text{I}$  in 0.1 N NaOH. To that solution was added 30  $\mu\text{L}$  of a 1 mg/mL solution of ChT in  $\text{H}_2\text{O}$ . After 5 min at rt, 3  $\mu\text{L}$  of a 10 mg/mL solution of sodium metabisulfite in  $\text{H}_2\text{O}$  was added to quench the reaction. A 941  $\mu\text{Ci}$  quantity of radioiodinated protein solution was eluted on a NAP-10 column with 0.9% saline to give 797  $\mu\text{Ci}$  (85%) of radiolabeled r-streptavidin. The labeled r-streptavidin had a specific activity of 0.65  $\mu\text{Ci}/\mu\text{g}$  and a radiochemical purity of 100% (by HPLC).

**Preparation B.** The radioiodination was conducted as described for preparation A. In the labeling, 700  $\mu\text{g}$  of r-streptavidin in 50  $\mu\text{L}$  of solution was radioiodinated with 955  $\mu\text{Ci}$  of  $\text{Na}[^{131}\text{I}]\text{I}$  using 14  $\mu\text{g}$  of ChT. A yield of 867  $\mu\text{Ci}$  (91%) of labeled r-streptavidin was obtained. The [ $^{131}\text{I}$ ]-r-streptavidin had a specific activity of 1.43  $\mu\text{Ci}/\mu\text{g}$  and a radiochemical purity of 100% (HPLC).

#### **Radioiodination of Avidin Using [ $^{125}\text{I}$ ]PIB-NHS.**

Synthesis of *N*-succinimidyl 4-(tri-*n*-butylstannyl)benzoate (PSnB-NHS) was accomplished as previously reported (41). The radioiodination was conducted as follows. A 1.0 mCi quantity of  $\text{Na}[^{125}\text{I}]\text{I}$  in 3  $\mu\text{L}$  0.1 N NaOH was added to a mixture of 50  $\mu\text{g}$  of PSnB-NHS in 50  $\mu\text{L}$  of a 1% HOAc solution in MeOH. To that solution was added 10  $\mu\text{L}$  of a 1 mg/mL solution of NCS in MeOH. After 5 min at rt, 1  $\mu\text{L}$  of a 10 mg/mL solution of sodium metabisulfite was added to quench the reaction. The solvent was removed under a stream of argon (through a charcoal filter), and the crude residue containing [ $^{125}\text{I}$ ]PIB-NHS was used directly in the radiolabeling reaction.

To the vial containing [ $^{125}\text{I}$ ]PIB-NHS was added a mixture containing 100  $\mu\text{L}$  of a 7.0 mg/mL solution of avidin in PBS and 100  $\mu\text{L}$  of a 0.5 M sodium borate buffer, pH 9.5. The reaction was allowed to react for 15 min, and then it was passed over a NAP-10 column, eluting with 0.9% saline. A total of 524  $\mu\text{Ci}$  (52%) of PIB-labeled avidin, specific activity of 0.82  $\mu\text{Ci}/\mu\text{g}$ , which had a 100% radiochemical purity by HPLC analysis, was obtained.

**Antibody Cell Binding Assay.** Tumor cell binding immunoreactivity assays of the radioiodinated antibody fragments were conducted as previously described (44). Briefly, the assay was conducted by addition of approximately 200 000 counts per minute (cpm) of intact A6H, A6H  $\text{F}(\text{ab}')_2$ , A6H Fab'-S-NEM, or A6H Fab'-S-biotin preparation (contained in 0.1 mL of PBS) to 2 000 000 trypsinized 7860 renal cell carcinoma cells suspended in 1.0 mL of PBS containing 1% BSA. The actual number of cpm added to each tube was determined by quantification in a gamma counter. Cells were incubated at rt for 1 h with constant rotary inversion mixing, pelleted by centrifugation at 5000g for 3 min, decanted to remove supernatant, and resuspended in 1 mL of PBS. The cell suspension was then transferred to a new tube and recounted. The immunoreactivity estimation was taken as the counts remaining divided by initial total counts times 100. The cell binding values (average of 2) obtained are listed with each of the experimental descriptions for radiolabeled/modified antibody fragments above, except for intact A6H (radiolabeling not described) which had an immunoreactivity of 54%.

**In Vivo Studies.** All animal uses and procedures were approved by the University of Washington's Animal Care Committee. Animal care and use were conducted in accordance with the NIH guidelines.<sup>2</sup> Male athymic mice (*nu/nu*), 4-5 weeks of age, were obtained from Simonsen Laboratories (Gilroy, CA). All mice were housed for 1 week in the animal facility prior to beginning the study. Tumor pieces (5-10 mg) of TK-82 renal cell carcinoma (37) were implanted sc over the right shoulder of the mice. The average animal weight in each group of a biodistribution, and average tumor weight for each group involving mice bearing tumor xenografts, are provided in the footnotes for Tables 1-4.

In the biodistribution studies, radiolabeled antibodies were administered to mice in a total volume of approximately 100  $\mu\text{L}$  via the lateral tail vein. The actual

<sup>2</sup> NIH guidelines are described in NIH Publication 86-23: "Guide for the Care and Use of Laboratory Animals".



**Table 1. Distribution of Radioactivity for A6H Fab'-S-Biotin Radiolabeled with Na<sup>125</sup>I]/ChT in Athymic Mice Bearing Human Tumor Xenografts<sup>a,b</sup>**

| tissue             | 1 h <sup>c</sup> | 4 h <sup>c</sup> | 24 h <sup>c</sup> |
|--------------------|------------------|------------------|-------------------|
| blood              | 32.21 ± 9.20     | 17.26 ± 1.63     | 2.70 ± 0.26       |
| tumor <sup>d</sup> | 14.53 ± 3.90     | 31.04 ± 8.56     | 27.57 ± 5.84      |
| muscle             | 0.86 ± 0.19      | 1.24 ± 0.26      | 0.43 ± 0.05       |
| lung               | 16.02 ± 4.14     | 9.79 ± 1.87      | 2.57 ± 0.44       |
| kidney             | 17.80 ± 6.59     | 14.42 ± 2.36     | 1.75 ± 0.22       |
| spleen             | 10.61 ± 7.91     | 4.28 ± 0.89      | 0.86 ± 0.13       |
| liver              | 7.55 ± 3.78      | 5.46 ± 0.77      | 0.89 ± 0.08       |
| intestine          | 4.94 ± 3.11      | 3.04 ± 0.65      | 0.63 ± 0.07       |
| neck               | 8.07 ± 4.63      | 7.61 ± 1.70      | 10.86 ± 9.34      |
| stomach            | 3.22 ± 1.58      | 5.46 ± 1.43      | 3.59 ± 0.80       |
| urine <sup>e</sup> | 23.84 ± 13.91    | 76.52 ± 17.43    | 33.13 ± 10.91     |

<sup>a</sup> Values shown are average % injected dose/g ± SD. <sup>b</sup> Data were obtained for *n* = 6 mice at each time point; average animal weight, 28.32 ± 2.01 g; injectate for each animal had 11 μCi/10 μg of A6H Fab'-S-biotin labeled with Na<sup>125</sup>I]/ChT in approximately 100 μL of 0.9% sterile saline. <sup>c</sup> Time of sacrifice from injection of radiolabeled Fab'. <sup>d</sup> Tumor xenografts were TK-82 renal cell carcinoma; average tumor weight for each group: 1 h, 0.17 ± 0.12 g; 4 h, 0.15 ± 0.03 g; 24 h, 0.25 ± 0.10 g. <sup>e</sup> Urine was collected by syringe bladder tap after sacrifice.

amount of injectate administered to each animal was determined by weighing the administering syringe pre- and postinjection. Injections and sacrifice of the animals were done at predetermined times. The tissues excised were tumor, muscle, lung, kidney, spleen, liver, intestine, neck (including thyroid), and stomach. Blood samples were obtained by cardiac puncture immediately before sacrifice. Urine samples were obtained by syringe bladder tap at the time the tissues were excised. Excised tissues were blotted free of blood, weighed, and counted. Calculation of the percent injected dose (%ID) and percent injected dose per gram (% ID/g) in the tissues was based on internal standards containing 1 μL of the injected dose. The counts obtained were compensated for decay, and when dual radionuclide counting was required, the <sup>125</sup>I counts were compensated (13.5%) for spillover counts from the <sup>131</sup>I. Total blood volume was estimated to be 8% of body weight for the calculations (4). A spread sheet program in Excel (Microsoft Corp.,

Redmond, WA) was used to perform the calculations. Data on the number of animals per time point, specific activity of each preparation, and quantities administered are provided in the footnotes of Tables 1–4.

## RESULTS

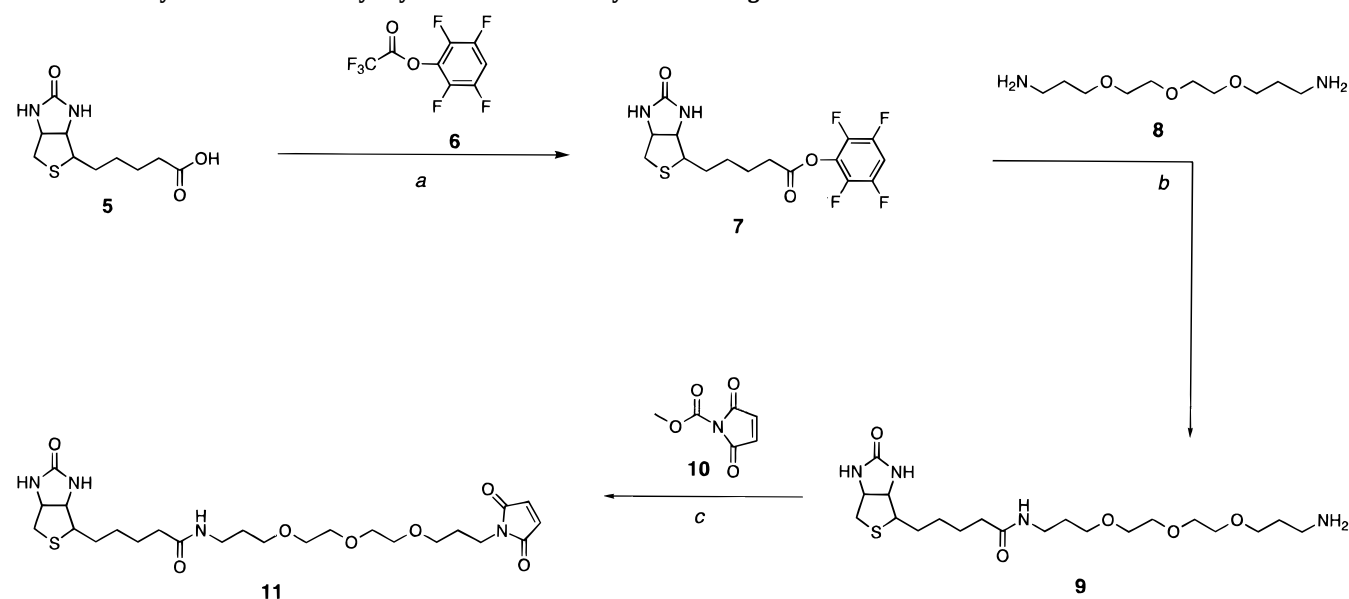
**Synthesis of Maleimido-Biotin (11).** The synthetic steps to prepare the water-solubilized biotin-maleimide (11) are shown in Scheme 1. In the initial step, the tetrafluorophenyl ester of biotin (7) was prepared by reaction with tetrafluorophenyl trifluoroacetate (6). The use of 6 for preparation of tetrafluorophenyl esters of other carboxylates has been previously described (38). The esterification reaction gave an 83% yield of 7. Reaction of the biotin-TFP ester with 4,7,10-trioxo-1,13-tridecanediamine (8) provided the adduct 9 in 79% yield. The final synthetic step, reaction of 9 with *N*-methoxycarbonylmaleimide (10), was facile, although only a 49% yield of 11 was obtained. The overall yield from biotin was 32%.

**Reactions of Maleimido Compounds with Fab' Sulfhydryl Groups.** The Fab' fragment of the antirenal cell carcinoma antibody A6H (36) was prepared by treatment of A6H F(ab')<sub>2</sub> with 4 mM dithiothreitol (DTT) for 1 h at rt. After the excess DTT was removed on a size exclusion column, the Fab'-SH was kept at rt for a minimum of 30 min prior to reaction with sulfhydryl reagents. This time was allotted because it was thought that this would permit re-formation of intra- and inter-chain reduced disulfide bonds. The reaction mixture and column elution solvent contained 1 mM EDTA as it had been previously noted that the reoxidation of Fab' to F(ab')<sub>2</sub> was slowed considerably with this reagent present. In initial evaluations of biotinylation of A6H Fab', 20 equiv of 11 was used; however, after setting at room temperature for 1 h, a small amount (<10%) of F(ab')<sub>2</sub> was present by HPLC analysis.<sup>3</sup> This indicated that some of the sulfhydryl groups had not reacted with 11. Therefore, the amount of 11 employed in the reaction was increased to 50 equiv. This resulted in no F(ab')<sub>2</sub> being formed after setting at room temperature for 18 h. To mimic the conditions of biotinylation in the NEM-reacted

**Table 2. Distribution of Radioactivity for [<sup>125</sup>I]A6H Fab'-S-Biotin and [<sup>131</sup>I]-r-Streptavidin Radiolabeled with Na<sup>125,131</sup>I]/ChT in Athymic Mice with Human Tumor Xenografts<sup>a</sup>**

| tissue             | [ <sup>125</sup> I]A6H Fab' distribution |                                      |                             | [ <sup>131</sup> I]streptavidin distribution |                                      |                             |
|--------------------|------------------------------------------|--------------------------------------|-----------------------------|----------------------------------------------|--------------------------------------|-----------------------------|
|                    | A6H Fab'-S-biotin <sup>b</sup>           |                                      | A6H Fab'-S-NEM <sup>c</sup> | A6H Fab'-S-biotin <sup>b</sup>               |                                      | A6H Fab'-S-NEM <sup>c</sup> |
|                    | + r-streptavidin (4 h) <sup>d</sup>      | + r-streptavidin (20 h) <sup>e</sup> |                             | + r-streptavidin (4 h) <sup>d</sup>          | + r-streptavidin (20 h) <sup>e</sup> |                             |
| blood              | 8.68 ± 1.00                              | 3.47 ± 0.30                          | 1.90 ± 1.05                 | 3.00 ± 0.23                                  | 8.12 ± 0.61                          | 2.15 ± 0.34                 |
| tumor <sup>f</sup> | 24.71 ± 7.50                             | 29.20 ± 16.20                        | 23.45 ± 8.85                | 6.29 ± 1.47                                  | 6.59 ± 2.59                          | 3.20 ± 0.74                 |
| muscle             | 0.82 ± 0.17                              | 0.44 ± 0.10                          | 0.38 ± 0.14                 | 0.72 ± 0.09                                  | 0.93 ± 0.12                          | 0.73 ± 0.23                 |
| lung               | 5.19 ± 0.86                              | 4.27 ± 1.36                          | 1.64 ± 0.17                 | 2.42 ± 0.30                                  | 4.95 ± 0.63                          | 2.23 ± 0.39                 |
| kidney             | 0.44 ± 0.30                              | 0.52 ± 0.27                          | 0.24 ± 0.22                 | 25.63 ± 3.84                                 | 20.93 ± 2.01                         | 27.54 ± 5.00                |
| spleen             | 1.91 ± 0.26                              | 1.08 ± 0.19                          | 0.64 ± 0.11                 | 2.78 ± 0.54                                  | 2.64 ± 0.40                          | 2.37 ± 0.39                 |
| liver              | 1.97 ± 1.37                              | 0.83 ± 0.13                          | 0.51 ± 0.09                 | 3.24 ± 1.91                                  | 2.49 ± 0.36                          | 2.52 ± 0.41                 |
| intestine          | 1.07 ± 0.17                              | 0.52 ± 0.07                          | 0.41 ± 0.10                 | 0.89 ± 0.11                                  | 1.54 ± 0.18                          | 0.82 ± 0.11                 |
| neck               | 8.77 ± 7.37                              | 5.95 ± 3.84                          | 5.44 ± 4.35                 | 2.76 ± 0.48                                  | 3.25 ± 0.47                          | 2.47 ± 0.56                 |
| stomach            | 5.22 ± 2.29                              | 6.21 ± 2.88                          | 2.92 ± 1.11                 | 1.24 ± 0.34                                  | 1.67 ± 0.19                          | 1.16 ± 0.30                 |
| urine <sup>g</sup> | 20.69 ± 21.60 <sup>h</sup>               | 76.48 ± 185.17 <sup>h</sup>          | 15.25 ± 14.22 <sup>i</sup>  | 5.33 ± 4.31 <sup>h</sup>                     | 36.94 ± 85.65 <sup>h</sup>           | 7.14 ± 6.45 <sup>i</sup>    |

<sup>a</sup> Values shown are % injected dose/g ± SD; all injections were approximately 100 μL volume of radioiodinated protein in 0.9% sterile saline; all mice were sacrificed 24 h from injection of radiolabeled Fab'-S-biotin. <sup>b</sup> Data were obtained for *n* = 8 mice in two groups (16 mice total); average animal weight, 27.85 ± 2.97 g (group 1; 4 h streptavidin injection) and 27.80 ± 1.61 g (group 2; 20 h streptavidin injection); first injectate for each animal had 11 μCi/10 μg of [<sup>125</sup>I]Fab'-S-biotin labeled with Na<sup>125</sup>I]/ChT; second injectate had 19 μCi/30 μg of [<sup>131</sup>I]streptavidin labeled with Na<sup>131</sup>I]/ChT administered at 4 h (group 1) or 20 h (group 2) after the first injection. <sup>c</sup> Data were obtained for *n* = 6 mice; average mouse weight 27.85 ± 2.21 g; first injectate for each animal had 9.8 μCi/10 μg of [<sup>125</sup>I]Fab'-S-NEM; second injectate had 19 μCi/30 μg of [<sup>131</sup>I]streptavidin administered 4 h after the first injection. <sup>d</sup> r-Streptavidin was present in blood for 20 h before sacrifice. <sup>e</sup> r-Streptavidin was present in blood for 4 h before sacrifice. <sup>f</sup> Tumor xenografts were TK-82 renal cell carcinoma; average tumor weights for each group were 0.33 ± 0.16, 0.24 ± 0.16, and 0.19 ± 0.11 g, respectively. <sup>g</sup> Urine was collected by syringe bladder tap after sacrifice. <sup>h</sup> Urine was collected from six of eight animals. <sup>i</sup> Urine was collected from four of six animals.

**Scheme 1. Synthesis of Sulfhydryl Reactive Biotinylation Reagent 11**

<sup>a</sup> **6**, DMF, 70 °C, TEA, 30 min, 83%. <sup>b</sup> **8**, DMF, TEA, 0 °C–rt, 1.5 h, 79%. <sup>c</sup> **10**, saturated NaHCO<sub>3</sub>, 0 °C–rt, 30 min, 49%.

**Table 3. Distribution of Radioactivity for A6H Fab'-S-Biotin Radiolabeled with Na[<sup>131</sup>I]/ChT and Subsequently Administered Avidin Labeled with [<sup>125</sup>I]PIB-NHS in Athymic Mice Bearing Human Tumor Xenografts<sup>a-c</sup>**

| tissue             | 4 h avidin injection <sup>d</sup> |                | 20 h avidin injection <sup>e</sup> |                |
|--------------------|-----------------------------------|----------------|------------------------------------|----------------|
|                    | I-131 (Fab')                      | I-125 (avidin) | I-131 (Fab')                       | I-125 (avidin) |
| blood              | 1.68 ± 0.41                       | 0.06 ± 0.02    | 1.79 ± 0.28                        | 0.45 ± 0.20    |
| tumor <sup>f</sup> | 25.26 ± 5.34                      | 0.35 ± 0.21    | 28.87 ± 5.94                       | 0.93 ± 0.22    |
| muscle             | 0.28 ± 0.07                       | 0.06 ± 0.10    | 0.35 ± 0.10                        | 0.15 ± 0.13    |
| lung               | 1.43 ± 0.33                       | 0.12 ± 0.06    | 1.47 ± 0.24                        | 0.78 ± 0.36    |
| kidney             | 1.37 ± 0.23                       | 2.91 ± 1.64    | 1.45 ± 0.37                        | 17.36 ± 3.30   |
| spleen             | 1.45 ± 0.48                       | 12.35 ± 6.38   | 1.45 ± 0.25                        | 18.54 ± 2.72   |
| liver              | 0.52 ± 0.19                       | 5.42 ± 2.99    | 0.34 ± 0.08                        | 12.22 ± 2.51   |
| intestine          | 0.44 ± 0.16                       | 1.47 ± 0.87    | 0.44 ± 0.12                        | 5.53 ± 3.30    |
| neck               | 12.56 ± 13.96                     | 0.11 ± 0.09    | 6.25 ± 4.61                        | 0.41 ± 0.15    |
| stomach            | 1.79 ± 1.05                       | 0.30 ± 0.25    | 4.13 ± 1.79                        | 0.50 ± 0.30    |
| urine <sup>g</sup> | 18.18 ± 4.44                      | 16.67 ± 11.14  | 20.22 ± 12.53                      | 74.25 ± 48.73  |

<sup>a</sup> Values shown are % injected dose/g ± SD. <sup>b</sup> All mice were sacrificed at 24 h from injection of radiolabeled A6H Fab'-S-biotin. <sup>c</sup> Data were obtained for *n* = 5 mice at each time point; average animal weight, 26.83 ± 2.25 g; injectate for each animal had 14 μCi/10 μg of A6H Fab'-S-biotin labeled with Na[<sup>131</sup>I]/ChT, and 25 μCi/30 μg of avidin labeled with [<sup>125</sup>I]PIB-NHS in approximately 100 μL of 0.9% sterile saline. <sup>d</sup> Avidin was present in blood for 20 h before sacrifice. <sup>e</sup> Avidin was present in blood for 4 h before sacrifice. <sup>f</sup> Tumor xenografts were TK-82 renal cell carcinoma; average tumor weight for each group: 4 h avidin, 0.20 ± 0.09 g; 20 h avidin, 0.17 ± 0.15 g. <sup>g</sup> Urine was collected by syringe bladder tap after sacrifice.

control, 50 equiv of that reagent was also used in those conjugation reactions.

Measurement of the number of sulfhydryl groups present after DTT treatment is difficult (45). Rather than expending an effort on determining the number of sulfhydryls present (46), it seemed that the important issue was to determine how many biotin moieties were present after conjugation. Initially, it was thought that the disulfides connecting the light and heavy chains of the Fab' would not be cleaved under our DTT treatment or would recombine, making them nonreactive in the conjugations. Also, it was assumed that pepsin digestion of A6H to form the F(ab')<sub>2</sub> fragment gave a product which had only one disulfide remaining in the hinge region (40), which might not be the case as mouse IgG<sub>1</sub> antibodies

**Table 4. Distribution of Radioactivity for Co-injected Avidin Radiolabeled with [<sup>125</sup>I]PIB-NHS and [<sup>131</sup>I]-r-Streptavidin Radiolabeled by ChT Method in Athymic Mice Bearing Human Tumor Xenografts<sup>a,b</sup>**

| tissue             | 4 h <sup>c</sup> |                        | 20 h <sup>c</sup> |                        |
|--------------------|------------------|------------------------|-------------------|------------------------|
|                    | I-125 (avidin)   | I-131 (r-streptavidin) | I-125 (avidin)    | I-131 (r-streptavidin) |
| blood              | 1.06 ± 0.36      | 11.13 ± 0.34           | 0.02 ± 0.00       | 2.49 ± 0.23            |
| tumor <sup>d</sup> | 0.72 ± 0.18      | 5.23 ± 0.68            | 0.03 ± 0.01       | 2.85 ± 1.15            |
| muscle             | 0.20 ± 0.05      | 1.16 ± 0.11            | 0.01 ± 0.01       | 0.82 ± 0.32            |
| lung               | 0.99 ± 0.37      | 6.28 ± 0.61            | 0.04 ± 0.01       | 2.21 ± 0.07            |
| kidney             | 14.19 ± 3.29     | 27.29 ± 2.41           | 0.32 ± 0.38       | 34.84 ± 4.63           |
| spleen             | 13.84 ± 1.18     | 2.79 ± 0.22            | 2.19 ± 0.90       | 2.20 ± 0.21            |
| liver              | 10.38 ± 1.61     | 1.33 ± 0.25            | 0.93 ± 0.33       | 2.12 ± 0.12            |
| intestine          | 9.25 ± 3.13      | 1.72 ± 0.11            | 0.37 ± 0.11       | 0.85 ± 0.06            |
| neck               | 0.70 ± 0.20      | 4.62 ± 0.35            | 0.02 ± 0.01       | 2.86 ± 0.25            |
| stomach            | 0.84 ± 0.30      | 2.11 ± 0.31            | 0.18 ± 0.06       | 1.11 ± 0.18            |
| urine <sup>e</sup> | 112.93 ± 58.40   | 16.41 ± 9.17           | 5.48 ± 1.08       | 7.12 ± 0.85            |

<sup>a</sup> Values shown are % injected dose/g ± SD. <sup>b</sup> Data were obtained for *n* = 5 mice at each time point; average animal weight, 28.69 ± 1.66 g; injectate for each animal had 12 μCi/15 μg of avidin labeled with [<sup>125</sup>I]PIB-NHS and 21 μCi/15 μg of r-streptavidin labeled with Na[<sup>131</sup>I]/ChT in approximately 100 μL of 0.9% sterile saline. <sup>c</sup> Time of sacrifice from injection of radiolabeled proteins. <sup>d</sup> Tumor xenografts were TK-82 renal cell carcinoma; average tumor weight for each group: 4 h, 0.21 ± 0.12 g; 20 h, 0.17 ± 0.14 g. <sup>e</sup> Urine was collected by syringe bladder tap after sacrifice.

are known to have three disulfide bonds in the hinge region (46). A simple experiment indicated that more than one biotin was present on the biotinylated Fab'. In that experiment, reaction of biotinylated Fab' with an excess of r-streptavidin resulted in a polymeric material being obtained, rather than the 1:1 adduct of Fab' and r-streptavidin that would have been prepared if only one biotin were present. To determine the number of biotin molecules on the biotinylated Fab', the method of HABA dye displacement on avidin by biotin was conducted (42, 43). A value of 1.9 biotin molecules/Fab' was obtained when the Fab'-S-biotin was measured directly, and values of 1.9 and 2.0 biotin molecules/Fab' were obtained in duplicate tests when the Fab'-S-biotin was completely digested with the enzyme pronase.

**Protein Radioiodination.** Several protein radioiodination methods were used in the evaluations. When possible, radioiodinations were conducted by direct labeling using ChT and Na[<sup>125/131</sup>I]. Radioiodinations via the ChT method were accomplished on the biotinylated and

<sup>3</sup> Provided in Supporting Information.

NEM-reacted Fab' to give 80–94% radiochemical yield. Similar yields were obtained in several other experiments (unreported data). Radioiodinations of r-streptavidin using the ChT method were accomplished in 85–91% yield.

Direct radioiodination of avidin by the ChT method, or similar methods, is not readily achieved as the tetrameric protein has only one tyrosine per subunit, and that tyrosine is buried within the protein structure (47, 48). Investigators have reported directly labeling avidin (48), but that method resulted in an unstable radiolabel. Other investigators have used the Bolton–Hunter reagent (49) to radioiodinate avidin (50). In our studies, radioiodination of avidin was accomplished by conjugation with the *N*-hydroxysuccinimidyl ester of *p*-[<sup>125</sup>I]-iodobenzoate ([<sup>125</sup>I]PIB-NHS). The PIB-NHS reagent is readily prepared (41), and when reasonably high concentrations of protein are used (i.e., 5–20 mg/mL) in the conjugation reactions, good radiochemical yields (40–65%) are obtained. In the labeling conducted for this investigation a 52% radiochemical yield was obtained.

**Antibody Immunoreactivity Assessment.** The effect of antibody modification on binding to cells expressing the antigen was obtained from a cell binding assay. In that assay, the fraction of radiolabeled antibody that bound to 7860 RCC cells<sup>4</sup> was measured, and that value was used as a qualitative estimate of change in immunoreactivity. Since it has been our experience that appreciable variations in immunoreactivity values are obtained in the day-to-day analysis of antibody fragments (even the same antibody preparation), the binding fraction of radioiodinated (ChT method) F(ab')<sub>2</sub> fragment was measured along with the radioiodinated Fab' fragments under study. The measured binding fraction of radioiodinated A6H F(ab')<sub>2</sub> was set to 100%, and measured binding fractions of the Fab' preparations were standardized to it. Thus, relative immunoreactive binding fractions were estimated to be as follows: A6H F(ab')<sub>2</sub>, (preparation A) 100%, (preparation B) 100%; A6H Fab'–S-NEM, (preparation A) 30%, (preparation B) 23%; and A6H Fab'–S-biotin, (preparation A) 26%, (preparation B) 30%, and (preparation C) 57%.

In a separate cell binding assay, [<sup>125</sup>I]PIB-labeled avidin and [<sup>131</sup>I]-r-streptavidin were included to determine if either of these proteins bound with the 7860 RCC cells. Radioiodinated avidin was found to have 4% binding, whereas radioiodinated r-streptavidin had only 1.2% binding.

**In Vivo Studies.** A number of biodistribution studies were conducted to answer specific questions about biotinylation and use of a Fab' fragment in pretargeting of cancer in a model tumor system. Athymic mice were used in all biodistributions so that the data could be correlated between studies. The neck (including thyroid gland) and stomach tissue were examined since the concentrations of radioiodine in those tissues generally reflect the amount of free radioiodide *in vivo*. The concentration (%ID/g) of radioiodine in the thyroid and stomach was expected to be high for those proteins labeled according to the ChT method and low for avidin labeled with PIB-NHS. Values obtained for urine are not representative of total material excreted via that route since the volume can change dramatically at various times but are included so that the ratio of isotopes obtained at a particular time point can be assessed.

**A6H Fab'–S-Biotin Labeled According to the ChT Method.** A biodistribution of the A6H Fab'–S-biotin labeled with Na[<sup>125</sup>I]I/ChT was conducted. The distribution was evaluated at 1, 4, and 24 h after injection to gain pharmacokinetic data. Data on the injected [<sup>125</sup>I]Fab' preparation and concentration of radioactivity (%ID/g) in tissues, blood, and urine obtained at 1, 4, and 24 h pi are provided in Table 1. Importantly, the distribution and tumor localization were found to be very similar to that of radioiodinated nonbiotinylated A6H Fab' evaluated in the same animal/tumor model.<sup>3</sup>

**A6H Fab'–S-Biotin/A6H Fab'–S-NEM Colocalization with r-Streptavidin.** The primary goal of this investigation was to determine whether a specific colocalization of r-streptavidin with biotinylated Fab' could be obtained within a tumor xenograft following pretargeting of the Fab'–S-biotin. To conduct that biodistribution, [<sup>125</sup>I]-Fab'–S-biotin was injected first, and at 4 and 20 h after the initial injection, [<sup>131</sup>I]-r-streptavidin was administered. Administration at 4 h pi of [<sup>125</sup>I]Fab–S-biotin provided information on the distribution of radiolabeled proteins when a substantial amount of [<sup>125</sup>I]Fab–S-biotin was present to combine with the injected [<sup>131</sup>I]-r-streptavidin in the blood. Administration at 20 h pi of [<sup>125</sup>I]Fab–S-biotin provided information on the radiolabeled protein distributions when most of the [<sup>125</sup>I]Fab–S-biotin had cleared from the blood and was not present to combine with the injected [<sup>131</sup>I]-r-streptavidin. Data on the injected [<sup>125</sup>I]Fab'–S-biotin administered, [<sup>131</sup>I]-r-streptavidin administered, and concentration of radioactivity (%ID/g) in tissues, blood, and urine obtained at 24 h pi are provided in Table 2. To show that a specific localization had occurred, a control group (Fab'–S-NEM) in which the Fab' could not specifically bind with streptavidin was also studied.

A critical factor in the development of our approach to tumor binding site amplification is tumor localization of biotinylated antibody fragments. Tumor localization of A6H Fab'–S-biotin was similar at 24 h pi without addition of r-streptavidin (Table 1) to that obtained when r-streptavidin was added at 4 or 20 h pi of the Fab'–S-biotin (Table 2). In the mice that were administered r-streptavidin 4 h pi of the Fab'–S-biotin, it was hypothesized that the biotinylated Fab' in blood would combine with the r-streptavidin, resulting in formation of a protein conjugate with a higher molecular weight. Therefore, it was expected that blood clearance of the Fab'–biotin–streptavidin conjugate would be slower than that of Fab'–S-biotin, increasing the blood concentration at 24 h appreciably. The data support this, with the blood activity increasing from  $2.7 \pm 0.26$  (Table 1, 24 h) to  $8.68 \pm 1.00\%$  ID/g (Table 2). When r-streptavidin was administered at 20 h pi of the Fab'–S-biotin, it was expected that only a small difference in blood concentration would be seen. Again, the data support this, with the blood activity increasing from  $2.7 \pm 0.26$  to  $3.47 \pm 0.30\%$  ID/g. In the control, the Fab'–S-NEM did not contain a biotin, so specific binding of the r-streptavidin could not occur. No increase in the blood concentration was expected, and the data support this. The increased blood concentration of Fab'–S-biotin brought about by administration of r-streptavidin resulted in decreased tumor/blood ratios. The tumor/blood ratio for the Fab–S-NEM control was 10:1, as was observed in the experiment that did not have r-streptavidin added, but the tumor/blood ratio for the Fab–S-biotin preparations was 2.8 when r-streptavidin was administered at 4 h and 8.4 when r-streptavidin was administered at 20 h.

<sup>4</sup> The TK-82 human RCC cells used as xenografts in athymic mice do not grow in tissue culture. Therefore, binding of radioiodinated preparations is measured in another cell line, 7870, which expresses the same antigen.

The data for the distribution of [ $^{131}\text{I}$ ]-r-streptavidin administered 4 and 20 h pi of the Fab' are included in Table 2. The distribution of r-streptavidin administered after the control Fab'-S-NEM is similar to that obtained in studies without Fab'-S-biotin present (Table 4); thus, it can be considered as a control for the r-streptavidin. A 30  $\mu\text{g}$  quantity of r-streptavidin was used in the investigation. This quantity is 3 times the molar quantity required for combining 1:1 with the Fab'-S-biotin administered (i.e., MW for Fab'  $\approx$  50 000; r-streptavidin = 53 080). We conducted the biodistribution studies with an excess of r-streptavidin as it was thought that this would help to maximize the amount that colocalized at the tumor. In the distribution in which r-streptavidin was injected 4 h after Fab'-S-biotin, less than one-third of the streptavidin would be expected to combine with circulating antibody such that the majority of the injected r-streptavidin would be expected clear from the blood. The data in Table 2 support this, as the blood concentration is only increased from  $2.15 \pm 0.34$  to  $3.00 \pm 0.23\%$  ID/g. When r-streptavidin was administered 20 h after the Fab'-S-biotin, a significantly different blood concentration of [ $^{131}\text{I}$ ]-r-streptavidin was expected due to the fact that it had circulated for only 4 h prior to sacrifice. It is interesting to note that the concentration of streptavidin in the tumor is roughly the same irrespective of the blood concentration, suggesting that the available biotin might be completely saturated. Importantly, the concentration of streptavidin in the tumor wherein biotinylated Fab'-S-biotin had been administered is twice that of the control Fab'-S-NEM.

**A6H Fab'-S-Biotin Colocalization with Avidin.** The specific colocalization of avidin in athymic mice with preadministered biotinylated A6H Fab' was also investigated. It was of particular interest to determine the effect on tumor concentration with the rapid clearance of avidin from blood (48). The experiment was designed to correlate with the study of r-streptavidin (above); thus, avidin administrations (30  $\mu\text{g}$ ) were made at 4 or 20 h postadministration of (10  $\mu\text{g}$ ) A6H Fab'-S-biotin. Data on the injected [ $^{131}\text{I}$ ]Fab' administered, [ $^{125}\text{I}$ ]avidin administered, and concentration of radioactivity (%ID/g) in tissues, blood, and urine obtained at 24 h post Fab' injection are provided in Table 3. Administration of avidin decreased the amount of Fab'-S-biotin remaining in the blood at 24 h, resulting in higher tumor-to-blood ratios (i.e., 15–16:1 tumor/blood) for [ $^{131}\text{I}$ ]Fab'-S-biotin than obtained with r-streptavidin administration. However, much lower concentrations of avidin were found in the tumor xenografts in comparison to that obtained with r-streptavidin. Interestingly, even though the concentration of avidin in the tumor was low, tumor-to-blood ratios of nearly 6:1 and 2:1 were observed, demonstrating that a specific localization was obtained.

**r-Streptavidin and Avidin in Mice Bearing Tumor Xenografts.** A biodistribution was conducted wherein [ $^{125}\text{I}$ ]avidin and [ $^{131}\text{I}$ ]-r-streptavidin were co-injected into athymic mice bearing TK-82 tumor xenografts. The primary purpose of the biodistribution was to determine if either of these proteins was selectively retained within the tumor xenograft employed. This was particularly relevant as a tumor/blood ratio of 1.5:1 was obtained when r-streptavidin was administered 4 h after administration of A6H Fab'-S-NEM (Table 2). No specific tumor retention had been expected, and low nonspecific uptake might be expected due to the low RCC cell binding of r-streptavidin (i.e., 1.2%) noted *in vitro*. Data on the injected [ $^{125}\text{I}$ ]avidin and [ $^{131}\text{I}$ ]-r-streptavidin administered and concentration of radioactivity (%ID/g) in tissues, blood, and urine obtained at 4 and 20 h pi are provided

in Table 4. The data clearly show that no selective accumulation of either protein occurs within the human tumor xenografts.

## DISCUSSION

The method of tumor "pretargeting" using monoclonal antibodies and biotin with streptavidin or avidin has been investigated in both preclinical and clinical studies and appears to hold promise for improved diagnosis and therapy of cancer (1). In contrast to previous studies, the studies described here are an initial part of an investigation aimed at improving antibody directed radiotherapy of cancer by augmenting the quantity of radioactivity retained at a tumor. The general approach for increasing the amount of radioactivity retained at a tumor is to use antibody pretargeting with radiolabeled biotin cross-linking molecules (51) and streptavidin or avidin to augment the number of binding sites for the radiolabeled molecules on tumor cells, as depicted in Figure 2. The concept for increasing the amount of radioactivity at a tumor by utilizing antibody pretargeting with biotin/(strept)avidin technology (52–54) is not unique to us. Indeed, many investigators have mentioned the possibility of obtaining some "amplification" or "augmentation" in their studies. In a recently published paper, Kassiss et al. described a method of "target signal augmentation" using biotinylated monoclonal antibodies and avidin or streptavidin (55). Similar to the approach presented in Figure 2, they propose obtaining signal amplification through "repeated and successive administration" of avidin or streptavidin and polybiotin derivatives (e.g., polybiotinylated human serum albumin).

Many parameters must ultimately be examined to achieve the goal of tumor binding site augmentation. One important parameter is that of blood and nontumor clearance of the radioactivity. The clearance of radiolabeled species is related to the concentration of antibody conjugates, such as biotinylated antibody or antibody-streptavidin/avidin conjugates, in the tissues. Most of the previous studies have employed conjugates of intact antibodies that have long retention times in blood. To improve the clearance rate from blood and nontumor tissues, "clearing agents" that bind with the antibody conjugates have been used (28–34). In a multistep approach, minimizing the number of administrations of agents is of paramount importance. Therefore, it is best if the pharmacokinetics of antibody conjugate clearance from blood did not require the administration of a clearing agent. In this study, the focus of the experimentation was aimed at determining the extent of colocalization of r-streptavidin or avidin in a human tumor xenograft model when a more rapidly clearing biotinylated antibody Fab' fragment was used. One can hypothesize that other investigators have not evaluated Fab' fragments in tumor pretargeting due to the fact that Fab' fragments do not, in general, localize to tumors in as high concentration (i.e., %ID/g) as divalent intact antibodies or their  $\text{F(ab')}_2$  fragments. Ultimately, we plan to compare results obtained from pretargeting with Fab' to divalent antibody fragments such as genetically engineered antibody single-chain Fv dimers ( $\text{scFv}_2$ ). These small bivalent antibody fragments have been shown to have pharmacokinetics similar to that of Fab' but to bind to tumor cells in a manner similar to that of  $\text{F(ab')}_2$  fragments (56).

An important aspect of the research undertaken was biotinylation of the Fab'. In contrast to intact antibodies or  $\text{F(ab')}_2$  fragments, it can be expected that smaller fragments are more sensitive to conjugation with biotin.

To minimize the effect of conjugation on fragments, a site-specific attachment of biotin was desired. Importantly, in Fab' the sulfhydryl generated by reduction of  $F(ab')_2$  can be used for site-specific conjugation. Therefore, we chose to employ a sulfhydryl reactive maleimido-biotin reagent for biotinylation of the Fab'. Several sulfhydryl reactive biotinylation reagents, such as biotin-BMCC (**1**), biotin-maleimide (**2**), iodoacetyl-LC-biotin (**3**), and *N*-(3-maleimidopropionyl)biocytin (**4**) (Figure 3) are available from commercial sources, and the syntheses of these and several similar reagents are described in the literature (57, 58). Virtually all of the reagents previously described have limited water solubility and must be mixed with organic solvents (e.g., DMSO or DMF) prior to addition to aqueous solutions of protein. We felt that more favorable conjugation conditions could be achieved if a water soluble maleimido-biotin were prepared. Since modification of biotin was known to decrease its binding with avidin and streptavidin (14), and modification of a maleimide could not be easily achieved, use of a water-solubilizing linker molecule between biotin and the maleimide moiety appeared to be the best synthetic approach. Fortunately, a good candidate as a water-solubilizing linker, the ether-containing linker 4,7,10-trioxa-1,13-tridecanediamine (**8**), was commercially available. Further, the increased distance between the biotin carboxylate and the maleimide (20.4 Å) that the linking trioxadamine, **8**, provides was considered to be an advantage over the distances provided by the linkers in **1** (16.3 Å), **2** (13.3 Å), **3** (12.4 Å), or **4** (15.9 Å).<sup>5</sup> The ether-containing linker was also attractive as it appeared that solubilization of the linking arm might permit the biotin moiety to be more extended from the protein, thus making it more available for binding with avidin or streptavidin.

The Fab' fragment of A6H was successfully biotinylated with the new water soluble sulfhydryl reactive reagent, **11**, without significantly altering its immunoreactivity or tumor-targeting characteristics. Evaluation of the number of biotin molecules on the Fab' showed that it had two biotin moieties attached. This fact indicated that conjugation reactions occurred with more than the one sulfhydryl, or one of the conjugation reactions occurred with an amine on the protein. The conjugations were conducted at pH 6.8, at which conjugation with a terminal  $\alpha$ -amine might occur (59). However, reaction with an  $\alpha$ -amine would be expected to be much slower than reaction with free sulfhydryl groups. Cleavage of the disulfide bond(s) in the hinge region of A6H  $F(ab')_2$  was accomplished with DTT. Although mild conditions were used in the reduction, it is possible that cleavage of disulfides on the Fab' molecule may have occurred. Molecular modeling of a mouse IgG<sub>1</sub> Fab' fragment<sup>6</sup> shows that four intrachain disulfides are buried in the molecule and are not likely to be reduced by DTT, whereas the single interchain disulfide is readily accessible. Thus, it is possible that the additional biotin molecule is conjugated with a sulfhydryl generated from the reduced interchain disulfide. However, it seems likely that three biotin molecules might be conjugated if

this were the case. Additional studies are planned to evaluate the number of biotin moieties conjugated as a function of pH. The results obtained should help determine whether the second biotin is conjugated with the terminal  $\alpha$ -amine or with another sulfhydryl group.

A goal of these studies was to determine the amount of biotin binding proteins at the tumor relative to the amount of Fab'-S-biotin prelocalized there. Due to the difference in molar quantities of streptavidin and avidin administered relative to Fab'-S-biotin, estimates of the amount of these proteins colocalized cannot be gained by simply examining the concentrations in the tumor. A 3-fold excess of r-streptavidin or avidin over the molar quantity of Fab' was administered as it was thought that such an excess might be needed to saturate the Fab-S-biotin at the tumor. Estimates of the quantities of r-streptavidin or avidin colocalized at the tumor with A6H Fab'-S-biotin were made by calculation using the data in Tables 2-4. The amount of each protein in a particular tissue, on a per gram basis, can be readily calculated from the quantity injected multiplied by the percent injected dose per gram.<sup>3</sup> The amount of r-streptavidin bound to the biotinylated Fab' in the tumor was estimated as the quantity of r-streptavidin retained in the tumor minus the quantity in the blood volume of the tumor. The per gram concentration of streptavidin contributed by the blood volume of the tumor should be somewhere between the concentration in whole blood and in muscle. Therefore, calculations to estimate the specific localization were obtained as a range that encompassed the measured concentration in tumor minus the concentration in blood or muscle on a per gram basis. An estimated 36-61% of the Fab'-S-biotin molecules at the tumor were bound with r-streptavidin. A smaller percentage (4-23%) of the Fab'-S-biotin molecules at the tumor were bound with avidin when that protein was evaluated.

Although the amount of tumor-localized r-streptavidin was lower than the amount of Fab'-S-biotin bound at the tumor, the amplification of binding sites provided by the tetrameric nature of streptavidin may improve the total number of sites available for binding radiolabeled biotin. Binding of streptavidin to Fab'-S-biotin could provide 2-3 times the number of biotin binding sites relative to the number of bound Fab'-S-biotin molecules. Therefore, the estimated 36-61% molar quantity for r-streptavidin bound with Fab'-S-biotin may actually provide 50-200% of the quantity of Fab' bound in binding sites for radiolabeled biotin.

In the consideration of the results of these studies, it became apparent that two important biological factors had not been included in the design of the experiments. One very important biological factor is the amount of endogenous biotin present in mice. Indeed, mammals are known to have fairly high concentrations of endogenous biotin in their serum (60, 61). The endogenous biotin can combine with the injected r-streptavidin (or avidin), and, in the portion of r-streptavidin in which the binding sites became saturated, the r-streptavidin would not bind with the biotinylated Fab'-S-biotin at the tumor. To correct this problem, Beaumier et al. (62) have indicated that they place the mice to be used in a study involving streptavidin and biotin on biotin-deficient diets for 5 days prior to the experiment. A second biological factor that may have affected the results obtained is the high concentration of the enzyme biotinidase in serum (63). This enzyme binds with biotin and cleaves biotin carboxamide bonds. If biotin were cleaved from the Fab'-S-biotin, specific localization of r-streptavidin or avidin could not occur. Fortunately, investigators have shown

<sup>5</sup> Distances are measured from the carboxyl carbon of biotin to the sulfhydryl reactive atom, when the atom chain linking the two carbons is in its fully extended form. The distances were estimated by molecular modeling as described under Experimental Procedures. The Pierce catalog lists the "spacer arm" of **1** as being 32.6 Å and that of **3** as 27.1 Å. There is no indication of how those values were obtained.

<sup>6</sup> Molecular modeling was conducted with the data for a mouse IgG<sub>1</sub> Fab' fragment of B1312 submitted by R. L. Stanfield, March 21, 1991, Brookhaven protein data bank file 2IGF.

that the biotinidase amide cleavage can be blocked by chemical modification (64). Studies are planned to evaluate the affect of these biological factors on pretargeting with antibody fragments.

#### SUMMARY

We have described preliminary studies conducted to determine the extent of colocalization of r-streptavidin or avidin with biotinylated A6H Fab' within a RCC tumor xenograft in athymic mice. Due to the expectation that site-specific conjugation of the biotinylation reagent would be needed with antibody fragments, and a desire to eliminate the use of organic solvents in the conjugation reactions, a new water soluble biotinylation reagent was developed. A6H Fab', which was produced by reduction of F(ab')<sub>2</sub> with DTT, was readily conjugated with the new biotinylation reagent. Four biodistribution studies were conducted to assess the specific colocalization of streptavidin or avidin with Fab'-S-biotin in the RCC tumor xenograft. It is apparent from the data in Table 2 that appreciable colocalization at the tumor was obtained for r-streptavidin, binding an estimated 36-61% molar quantity of r-streptavidin relative to tumor-localized Fab'-S-biotin. A specific colocalization of avidin was also obtained. The lower quantities of avidin localized at the tumor appear to preclude its use in therapeutic applications, except for its use as a reagent for clearing biotin-containing molecules from the blood. An important finding of this study was that similar amounts of r-streptavidin were localized at the tumor whether it was injected 4 or 20 h after injection of the Fab'-S-biotin. This suggests that administration of the streptavidin can be done after most of the biotinylated Fab' has cleared, which may make it unnecessary to administer a clearing agent to effectively colocalize the streptavidin.

Continued studies of antibody fragments in tumor pretargeting will include investigating other antibody fragments and will address clearance of r-streptavidin from blood. Other studies will be directed at determining the availability of the streptavidin localized in kidney to bind with biotin and the possibility of blocking streptavidin binding in the kidney. New biotin derivatives are being prepared that should block the amide-cleaving action of biotinidase, and mice involved in studies will be put on biotin-deficient diets prior to a study. We believe that altering these factors will provide improvements on the results described herein. Once these factors are addressed, the concept of increasing the number of biotin binding sites at a tumor can be evaluated.

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**Supporting Information Available:** Biodistribution (Table A) of nonbiotinylated A6H Fab' which had been radioiodinated directly with ChT/Na[<sup>125</sup>I] or radioiodinated by conjugation with sulfhydryl groups with N-(4-[<sup>131</sup>I]iodophenethyl)maleimide ([<sup>131</sup>I]IPEM); HPLC chromatograms of biotinylated Fab' obtained when 20 and 50 equiv of **11** were used; calculations used to quantify

the amounts of A6H Fab'-S-biotin, r-streptavidin, or avidin in tumor xenografts (4 pages). Ordering information is given on any current masthead page.

#### LITERATURE CITED

- (1) Goodwin, D. A. (1995) Tumor Pretargeting: Almost the Bottom Line. *J. Nucl. Med.* 36, 876-879.
- (2) Sung, C., and van Osdol, W. W. (1995) Pharmacokinetic Comparison of Direct Antibody Targeting with Pretargeting Protocols Based on Streptavidin-Biotin Binding. *J. Nucl. Med.* 36, 867-876.
- (3) Buchsbaum, D. J. (1995) Experimental Approaches to Increase Radiolabeled Antibody Localization in Tumors. *Cancer Res.* 55, 5729-5732.
- (4) Fritzberg, A. R., Beaumier, P. L., Bottino, B. J., and Reno, J. M. (1994) Approaches to improved antibody- and peptide-mediated targeting for imaging and therapy of cancer. *J. Controlled Release* 28, 167-173.
- (5) Maraveyas, A., and Epenetos, A. A. (1991) An overview of radioimmunotherapy. *Cancer Immunol. Immunother.* 34, 71-73.
- (6) Sharkey, R. M., Blumenthal, R. D., Hansen, H. J., and Goldenberg, D. M. (1990) Biological Considerations for Radioimmunotherapy. *Cancer Res. (Suppl.)* 50, 964s-969s.
- (7) Goodwin, D. A., Meares, C. F., McTigue, M., and David, G. S. (1986) Monoclonal antibody hapten radiopharmaceutical delivery. *Nucl. Med. Commun.* 7, 569-580.
- (8) Stickney, D. R., Anderson, L. D., Slater, J. B., Ahlem, C. N., Kirk, G. A., Schweighardt, S. A., and Frinke, J. M. (1991) Bifunctional Antibody: A Binary Radiopharmaceutical Delivery System for Imaging Colorectal Carcinoma. *Cancer Res.* 51, 6650-6655.
- (9) Kranenborg, M. H. G. C., Boerman, O. C., Oosterwijk-Wakka, J. C., de Weijert, M. C. A., Corstens, F. H. M., and Oosterwijk, E. (1995) Development and Characterization of Anti-Renal Cell Carcinoma X Antichelate Bispecific Monoclonal Antibody for Two-Phase Targeting of Renal Cell Carcinoma. *Cancer Res.* 55, 5864s-5867s.
- (10) Santos, O., Payne, J. K., Domitrowsky, J. B., Berkeley, C., and Mackensen, D. G. (1995) A Two Step Delivery System Utilizing a Bi-Specific Monoclonal Antibody for Radioimmunotherapy. *Antibody, Immunoconjugates, Radiopharm.* 8, 93-109.
- (11) Le Doussal, J.-M., Martin, M., Gautherot, E., Delaage, M., and Barbet, J. (1989) In Vitro and In Vivo Targeting of Radiolabeled Monovalent and Divalent Haptens with Dual Specificity Monoclonal Antibody Conjugates: Enhanced Divalent Hapten Affinity for Cell-Bound Antibody Conjugate. *J. Nucl. Med.* 30, 1358-1366.
- (12) Goodwin, D. A., Meares, C. F., Watanabe, N., McTigue, M., Chaovapong, W., Ransone, C. McK., Renn, O., Greiner, D. P., Kukis, D. L., and Kronenberger, S. I. (1994) Pharmacokinetics of Pretargeted Monoclonal Antibody 2D12.5 and <sup>88</sup>Y-Janus-2-(p-Nitrobenzyl)-1,4,7,10-tetraazacyclododecanetetraacetic Acid (DOTA) in BALB/c Mice with KHJJ Mouse Adenocarcinoma: A Model for <sup>90</sup>Y Radioimmunotherapy. *Cancer Res.* 54, 5937-5946.
- (13) Sebrell, W. H., and R. S. Harris, R. S., Eds. (1968) Biotin. *The Vitamins: Chemistry, Physiology, Pathology, Methods*, 2nd ed., Vol. II, pp 261-285, Academic Press, New York.
- (14) Green, N. M. (1975) Avidin. *Adv. Protein Chem.* 29, 85-133.
- (15) Green, N. M. (1990) Avidin and Streptavidin. *Methods Enzymol.* 184, 51-67.
- (16) Paganelli, G., Malcovati, M., and Fazio, F. (1991) Monoclonal Antibody pretargeting techniques for tumour localization: the avidin-biotin system. *Nucl. Med. Commun.* 12, 211-234.
- (17) Pimm, M. V., Fells, H. F., Perkins, A. C., and Baldwin, R. W. (1988) Iodine-131 and indium-111 labelled avidin and streptavidin for pre-targeted immunoscintigraphy with biotinylated anti-tumour monoclonal antibody. *Nucl. Med. Commun.* 9, 931-941.
- (18) Paganelli, G., Riva, P., Deleide, G., Clivio, A., Chiolerio, F., Scassellati, G. A., Malcovati, M., and Siccardi, A. G. (1988) In Vivo Labelling of Biotinylated Monoclonal Antibodies by

- Radioactive Avidin: A Strategy to Increase Tumor Radiolocalization. *Int. J. Cancer* 2, 121–125.
- (19) Khawli, L. A., Alauddin, M. M., Miller, G. K., and Epstein, A. L. (1993) Improved Immunotargeting of Tumors with Biotinylated Monoclonal Antibodies and Radiolabeled Streptavidin. *Antibody, Immunoconjugates, Radiopharm.* 6, 13–27.
- (20) Saga, T., Weinstein, J. N., Jeong, J. M., Heya, T., Lee, J. T., Le, N., Paik, C. H., Sung, C., and Neumann, R. D. (1994) Two-Step Targeting of Experimental Lung Metastases with Biotinylated Antibody and Radiolabeled Streptavidin. *Cancer Res.* 54, 2160–2165.
- (21) Paganelli, G., Pervez, S., Siccardi, A. G., Rowlinson, G., Deleide, G., Chiolerio, F., Malcovati, M., Scasellati, G. A., and Epenetos, A. A. (1990) Intraperitoneal Radio-Localization of Tumors Pre-Targeted by Biotinylated Monoclonal Antibodies. *Int. J. Cancer* 45, 1184–1189.
- (22) van Osdol, W. W., Sung, C., Dedrick, R. L., and Weinstein, J. N. (1993) A Distributed Pharmacokinetic Model of Two-Step Imaging and Treatment Protocols: Application to Streptavidin-Conjugated Monoclonal Antibodies and Radiolabeled Biotin. *J. Nucl. Med.* 34, 1552–1564.
- (23) Rowlinson-Busza, G., Hnatowich, D. J., Ruszkowski, M., Snook, D., and Epenetos, A. A. (1993) Xenograft Localization Using Pretargeted Streptavidin-conjugated Monoclonal Antibody and <sup>111</sup>In-Labeled Biotin. *Antibody, Immunoconjugates, Radiopharm.* 6, 97–109.
- (24) Kalofonos, H. P., Ruszkowski, M., Siebecker, D. A., Sivolapenko, G. B., Snook, D., Lavender, J. P., Epenetos, A. A., and Hnatowich, D. J. (1990) Imaging of Tumor in Patients with Indium-111-Labeled Biotin and Streptavidin-Conjugated Antibodies: Preliminary Communication. *J. Nucl. Med.* 31, 1791–1796.
- (25) Paganelli, G., Magnani, P., Zito, F., Villa, E., Sudati, F., Lopalco, L., Rossetti, C., Malcovati, M., Chiolerio, F., Seccamani, E., Siccardi, A. G., and Fazio, F. (1991) Three-Step Monoclonal Antibody Tumor Targeting in Carcinoembryonic Antigen-positive Patients. *Cancer Res.* 51, 5960–5966.
- (26) Modorati, G., Barnacle, R., Paganelli, G., Magnate, P., Paving, R., and Fazio, F. (1994) Immunoscintigraphy with three step monoclonal pretargeting technique in diagnosis of uveal melanoma: preliminary results. *Br. J. Ophthalmol.* 78, 19–23.
- (27) Ullen, A., Ahlstrom, K. R., Hietala, S.-O., Nilsson, B., Arlestig, L., and Stigbrand, T. (1996) Secondary Antibodies as Tools to Improve Tumor to Non Tumor Ratio at Radioimmunolocalization and Radioimmunotherapy. *Acta Oncol.* 35, 281–285.
- (28) Kobayashi, H., Sakahara, H., Endo, K., Hosono, M., Yao, Z., Toyama, S., and Konishi, J. (1995) Comparison of the Chase Effects of Avidin, Streptavidin, Neutravidin, and Avidin-Ferritin on a Radiolabeled Biotinylated Anti-tumor Monoclonal Antibody. *Jpn. J. Cancer Res.* 85, 310–314.
- (29) Marshall, D., Pedley, R. B., Melton, R. G., Boden, J. A., Boden, R., and Bergent, R. H. J. (1995) Galactosylated streptavidin for improved clearance of biotinylated intact and F(ab')<sub>2</sub> fragments of an anti-tumour antibody. *Br. J. Cancer* 71, 18–24.
- (30) Kobayashi, H., Sakahara, H., Hosono, M., Yao, Z., Toyama, S., Endo, K., and Konishi, J. (1994) Improved Clearance of Radiolabeled Biotinylated Monoclonal Antibody Following the Infusion of Avidin as a "Chase" without Decreased Accumulation in the Target Tumor. *J. Nucl. Med.* 35, 1677–1684.
- (31) Yao, Z., Zhang, M., Kobayashi, H., Sakahara, H., Nakada, H., Yamashina, I., and Konishi, J. (1995) Improved Targeting of Radiolabeled Streptavidin in Tumors Pretargeted with Biotinylated Monoclonal Antibodies through an Avidin Chase. *J. Nucl. Med.* 36, 837–841.
- (32) Kobayashi, H., Sakahara, H., Endo, K., Yao, Z.-s., Toyama, S., and Konishi, J. (1995) Repeating the Avidin "Chase" Markedly Improved the Biodistribution of Radiolabeled Biotinylated Antibodies and Promoted the Excretion of Additional Background Radioactivity. *Eur. J. Cancer* 31A, 1689–1696.
- (33) Klivanov, A. L., Martynov, A. V., Slinkin, M. A., Sakharov, I. Y., Smirnov, M. D., Muzykantov, V. R., Danilov, S. M., and Torchilin, V. P. (1988) Blood Clearance of Radiolabeled Antibody: Enhancement by Lactosamination and Treatment with Biotin-Avidin or Anti-Mouse IgG Antibodies. *J. Nucl. Med.* 29, 1951–1956.
- (34) Ogiwara-Umeda, I., Sasaki, T., Toyama, H., Oda, K., Senda, M., and Nishigori, H. (1994) Rapid Tumor Imaging by active Background Reduction Using Biotin-bearing Liposomes and Avidin. *Cancer Res.* 54, 463–467.
- (35) Gulec, S. (1996) Antibody Imaging: Tc-99m Anti-CEA Fab' Imaging (CEAscan). *Proceedings of Nuclear Oncology: Advances in Diagnostic and Therapeutic Applications*, Key Biscayne, FL, February 1996, University of Miami School of Medicine, Miami.
- (36) Vessella, R. L., Palme, D. F., Elson, M. K., Wessels, B. W., Chiou, R. K., and Lange, P. H. (1988) Radioiodinated monoclonal antibodies in the imaging and treatment of human renal cell carcinoma xenografts in nude mice. In *Targeted Diagnosis and Therapy* (John Rodwell et al., Eds.) Chapter 9, pp 245–282, Dekker, New York.
- (37) Vessella, R. L., Alvarez, V., Chiou, R.-K., Rodwell, J., Elson, M., Palme, D., Shafer, R., and Lange, P. (1987) Radioimmunosintigraphy and Radioimmunotherapy of Renal Cell Carcinoma Xenografts. *NCI Monogr.* 3, 159–167.
- (38) Gamper, H. B., Reed, M. W., Cox, T., Viroso, J. S., Adams, A. D., Gall, A. A., Scholler, J. K., and Meyer, R. B. (1993) Facile preparation of nuclease resistant 3' modified oligodeoxynucleotides. *Nucleic Acids Res.* 21, 145–150.
- (39) Chilkoti, A., Tan, P. H., and Stayton P. S. (1995) Site Directed Mutagenesis Studies of the High-Affinity Streptavidin-Biotin Complex: Contributions of Tryptophan Residues 79, 108 and 120. *Proc. Natl. Acad. Sci. U.S.A.* 92, 1754–1758.
- (40) Wilbur D. S., Stray, J. E., Hamlin, D. K., Curtis, D. K., and Vessella, R. L. (1994) Monoclonal Antibody Fab' Fragment Cross-Linking Using Equilibrium Transfer Alkylation Reagents. Strategy for Site-Specific Conjugation of Diagnostic and Therapeutic Agents with F(ab')<sub>2</sub> Fragments. *Bioconjugate Chem.* 5, 220–235.
- (41) Wilbur, D. S., Hadley, S. W., Hylarides, M. D., Abrams, P. G., Beaumier, P. L., Morgan, A. C., Reno, J., and Fritzberg, A. R. (1989) Development of a Stable Radioiodinating Reagent to Label Monoclonal Antibodies For Radiotherapy of Cancer. *J. Nucl. Med.* 30, 216–226.
- (42) Savage, M. D., Mattson, G., Desai, S., Nielander, G. W., Morgensen, S., and Conklin, E. J. (1992) Assays for Biotin and Avidin. *Avidin-Biotin Chemistry: A Handbook*, 2nd ed., pp 14–21, Pierce Chemical Co., Rockford, IL.
- (43) Green, N. M. (1965) A Spectrophotometric Assay for Avidin and Biotin Based on Binding of Dyes by Avidin. *Biochem. J.* 94, 23–24.
- (44) Wilbur, D. S., Vessella, R. L., Stray, J. E., Goffe, D. K., Blouke, K. A., and Atcher, R. W. (1993) Preparation and Evaluation of para-[<sup>211</sup>At]Astatobenzoyl Labeled Anti-Renal Cell Carcinoma Antibody A6H F(ab')<sub>2</sub>. *In Vivo Distribution Comparison with para-[<sup>125</sup>I]Iodobenzoyl Labeled A6H F(ab')<sub>2</sub>*. *Nucl. Med. Biol.* 20, 917–927.
- (45) Thakur, M. L., and DeFulvio, J. D. (1990) Determination of Reduced Disulfide Groups in Monoclonal Antibodies. *BioTechniques* 8, 512–515.
- (46) Day, E. D. (1990) The Secondary, Tertiary, and Quaternary Structures of Assembled Immunoglobulins. In *Advanced Immunochimistry*, 2nd ed., Chapter 3, pp 107–181, Wiley-Liss, New York.
- (47) Gitlin, G., Bayer, E. A., and Wilchek, M. (1990) Studies on the biotin-binding sites of avidin and streptavidin. Tyrosine residues are involved in the binding site. *Biochem. J.* 269, 527–530.
- (48) Rosebrough, S. F. (1993) Pharmacokinetics and Biodistribution of Radiolabeled Avidin, Streptavidin and Biotin. *Nucl. Med. Biol.* 20, 663–668.
- (49) Bolton A. E., and Hunter W. M. (1973) The Labeling of Proteins to High Specific Radioactivities by Conjugation to a I-125 Containing Acylating Agent. *Biochem. J.* 133, 529–539.
- (50) Schechter, B., Silberman, R., Arnon, R., and Wilchek, M. (1990) Tissue distribution of avidin and streptavidin injected to mice. *Eur. J. Biochem.* 189, 327–331.
- (51) Wilbur D. S., Weerawarna, S. A., Hamlin, D. K., Stray, J. E., Pathare, P. M., Buhler, K. R., Vessella, R. L., Stayton, P., Tan, P., and Klumb, L. (1996) Synthesis and Evaluation of Biotin Dimers and Trimers Designed for Clearance of Biotin

- Binding Proteins from Blood. *Proceedings of Nuclear Oncology; Advances in Diagnostic and Therapeutic Applications*, Key Biscayne, FL, February 11–15, 1996, University of Miami School of Medicine, Miami.
- (52) Diamandis, E. P., and Christopoulos, T. K. (1991) The Biotin-(Strept)Avidin System: Principles and Applications in Biotechnology. *Clin. Chem.* 37, 625–636.
- (53) Wilchek, M., and Bayer, E. A. (1990) Introduction to Avidin-Biotin Technology. *Methods Enzymol.* 184, 5–45.
- (54) Wilchek, M., and Bayer, E. A. (1988) The Avidin-Biotin Complex in Bioanalytical Applications. *Anal. Biochem.* 171, 1–32.
- (55) Kassis, A. I., Jones, P. L., Matalka, K. Z., and Adelstein, S. J. (1996) Antibody-Dependent Signal Amplification in Tumor Xenografts after Pretreatment with Biotinylated Monoclonal Antibody and Avidin or Streptavidin. *J. Nucl. Med.* 37, 343–352.
- (56) Haunschild, J., Faro, H.-P., Pack, P., and Pluckthun, A. (1995) Pharmacokinetic Properties of Bivalent Miniantibodies and Comparison to Other Immunoglobulin Forms. *Antibody, Immunoconjugate, Radiopharm.* 8, 111–128.
- (57) Bayer, A. B., Zalis, M. G., and Wilchek, M. (1985) 3-(N-Maleimido-propionyl)Biocytin: A Versatile Thiol-Specific Biotinylation Reagent. *Anal. Biochem.* 149, 529–536.
- (58) Wilchek, M., and Bayer, E. A. (1990) Biotin-Containing Reagents. *Methods Enzymol.* 184, 123–138.
- (59) Atassi, M. Z. (1977) Chemical Modification and Cleavage of Proteins and Chemical Strategy in Immunochemical Studies of Proteins. In *Immunochemistry of Proteins* (M. Z. Atassi, Ed.) Vol. 1, pp 1–161, Plenum Press, New York.
- (60) Whitehead, C. C. (1985) Assessment of Biotin Deficiency in Animals. *Ann. N. Y. Acad. Sci.* 447, 86–96.
- (61) Guilarte, T. R. (1985) Radiometric-Microbiological Assay of Biotin in Human Plasma. *Ann. N. Y. Acad. Sci.* 447, 398–399.
- (62) Beaumier, P. L., Axworthy, D. B., Fritzberg, A. R., Hylarides, M. D., Mallett, R. W., Theodore, L. J., Gustavson, L. M., Su, F.-M., and Reno, J. M. (1995) The Pharmacology of Pretargeting Components: Optimizing Therapeutic Targeting. *Q. J. Nucl. Med.* 39, 20.
- (63) Wolf, B., Hymes, J., and Heard, G. S. (1990) Biotinidase. *Methods Enzymol.* 184, 103–111.
- (64) Gustavson, L. M., Su, F.-M., Reno, J. M., Axworthy, D. B., Lyen, L. J., Fritzberg, A. R., and Theodore, L. J. (1995) Design and Synthesis of Metabolically Stable Chelate-Biotin Conjugates for Pretargeted Tumor Radioimmunotherapy. *Proceedings of 209th American Chemical Society Meeting*, Anaheim, CA, Med. Chem. Abstract 010, American Chemical Society, Washington, DC.

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# Supporting Information: Distribution of non-biotinylated A6H Fab'

(note: when comparing tumor concentrations of Fab' the amount of antibody injected must be compared)

**Table A: Distribution of Radioactivity for Co-injected A6H Fab' Radiolabeled with Na<sup>[125I]</sup>I/ChT and [<sup>131I</sup>]IPEM\* in Athymic Mice bearing Human Tumor Xenografts.<sup>a</sup>**

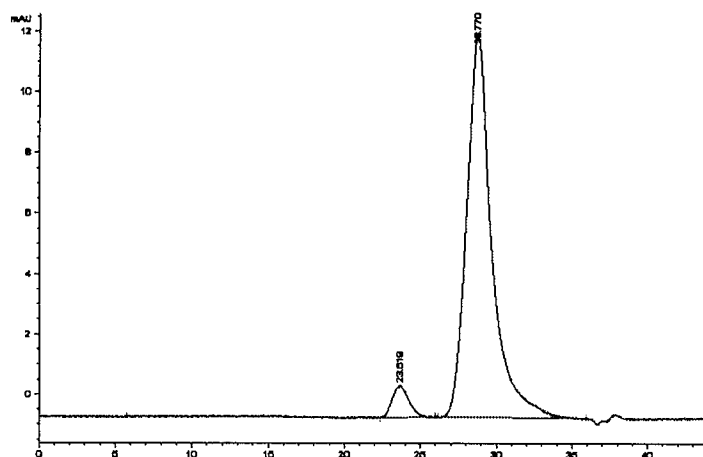
| Tissues            | <u>1 h<sup>b,c</sup></u> |              |                            | <u>4 h</u>                 |                           |                           | <u>24 h</u> |              |             |
|--------------------|--------------------------|--------------|----------------------------|----------------------------|---------------------------|---------------------------|-------------|--------------|-------------|
|                    | I-125 (ChT)              | I-131 (IPEM) | I-125 (ChT)                | I-131 (IPEM)               | I-125 (ChT)               | I-131 (IPEM)              | I-125 (ChT) | I-131 (IPEM) | I-125 (ChT) |
| blood              | 14.09 ± 0.83             | 15.09 ± 0.75 | 9.66 ± 1.05                | 8.87 ± 0.96                | 2.04 ± 0.31               | 1.48 ± 0.26               |             |              |             |
| tumor <sup>d</sup> | 11.91 ± 4.68             | 13.06 ± 5.46 | 16.40 ± 5.32               | 16.49 ± 6.34               | 15.15 ± 3.24              | 11.79 ± 3.20              |             |              |             |
| muscle             | 0.57 ± 0.09              | 0.63 ± 0.09  | 0.91 ± 0.11                | 0.77 ± 0.09                | 0.33 ± 0.02               | 0.20 ± 0.02               |             |              |             |
| lung               | 7.46 ± 0.70              | 8.39 ± 0.66  | 6.27 ± 0.60                | 5.80 ± 1.02                | 2.18 ± 0.97               | 2.17 ± 1.65               |             |              |             |
| kidney             | 14.36 ± 1.94             | 11.84 ± 1.23 | 10.95 ± 2.06               | 9.34 ± 0.84                | 1.60 ± 0.27               | 2.65 ± 0.50               |             |              |             |
| spleen             | 3.75 ± 0.39              | 3.84 ± 0.43  | 2.78 ± 0.39                | 1.89 ± 0.31                | 0.77 ± 0.11               | 0.45 ± 0.08               |             |              |             |
| liver              | 3.72 ± 0.57              | 4.71 ± 0.89  | 2.76 ± 0.17                | 4.34 ± 0.90                | 0.65 ± 0.11               | 0.97 ± 0.14               |             |              |             |
| intestine          | 1.86 ± 0.49              | 2.74 ± 1.04  | 1.77 ± 0.35                | 5.53 ± 0.83                | 0.51 ± 0.10               | 1.28 ± 0.44               |             |              |             |
| neck               | 3.97 ± 0.87              | 4.15 ± 0.89  | 5.66 ± 2.51                | 2.67 ± 0.31                | 6.78 ± 7.65               | 0.62 ± 0.12               |             |              |             |
| stomach            | 1.26 ± 0.22              | 1.46 ± 0.23  | 3.74 ± 1.08                | 2.44 ± 0.94                | 2.67 ± 0.99               | 0.79 ± 0.33               |             |              |             |
| urine <sup>e</sup> | 12.00 ± 4.66             | 12.41 ± 7.12 | 25.06 ± 10.76 <sup>f</sup> | 80.53 ± 40.38 <sup>f</sup> | 28.74 ± 7.15 <sup>g</sup> | 24.41 ± 8.77 <sup>g</sup> |             |              |             |

<sup>a</sup>Values shown are % injected dose / gram ± standard deviation. <sup>b</sup>Time of sacrifice from injection of radiolabeled Fab'. <sup>c</sup>Data were obtained for  $n = 6$  mice at 1 h and  $n = 7$  mice at 4 h and 24 h; average animal weight, 30.17 ± 2.23 g; Injectate for each animal had 14 µCi / 5.9 µg of A6H Fab' labeled with Na<sup>[125I]</sup>I/ChT and 10 µCi / 5.9 µg of A6H Fab' labeled with [<sup>131I</sup>]IPEM in approximately 100 µL of 0.9% sterile saline. <sup>d</sup>Tumor xenografts were TK-82 renal cell carcinoma; average tumor weight for each group, 1 h: 0.22 ± 0.06 g, 4 h: 0.20 ± 0.04 g, 24 h: 0.24 ± 0.14 g. <sup>e</sup>Urine was collected by syringe bladder tap after sacrifice. <sup>f</sup>Urine was collected from 4 of 7 animals. <sup>g</sup>Urine was collected from 6 of 7 animals.

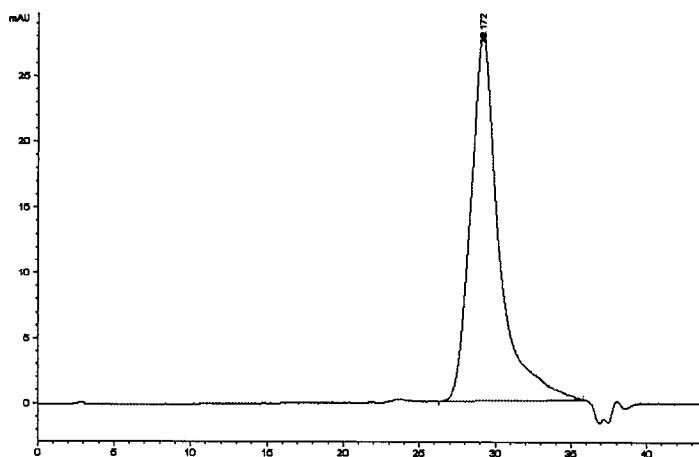
<sup>\*</sup>[<sup>131I</sup>]IPEM refers to the radioiodination reagent N-(4-[<sup>131I</sup>]iodophenethyl)maleimide. Synthesis and radiolabeling conditions are described in *Bioconjugate Chem.* 2, 435-440 (1991).

**Supporting Information** Size exclusion HPLC chromatograms of the biotinylated and NEM capped Fab' preparations are provided below:

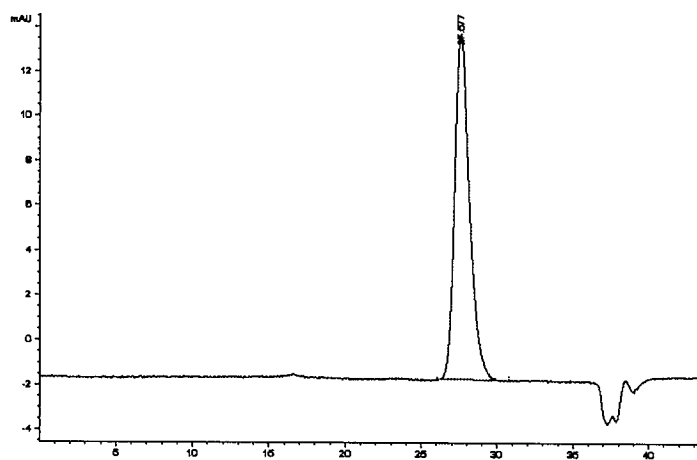
**A. Fab' + 10 equiv. biotinylation reagent**



**B. Fab' + 50 equiv. biotinylation reagent**



**C. Fab' + 50 equiv. N-ethylmaleimide**



## **Supporting Information: Calculation of the Amount of Co-Localization of r-Streptavidin and Avidin with Prelocalized A6H Fab'-S-biotin.**

### **A. Streptavidin Co-Localization**

To obtain an estimate of the co-localization of r-streptavidin at the tumor, the quantity of A6H Fab' specifically localized at the tumor had to be estimated first. Thus, the concentration of A6H Fab'-S-biotin in the tumor was measured to be 29.2 %ID/g (Table 2; +r-Strept, 20 h *pi*). To obtain a more accurate value, the concentration in blood (3.47 %ID/g) was subtracted. Thus, an estimated 26% ID/g of Fab'-S-biotin was specifically localized at the tumor. Accordingly, it was estimated that injection of 10 µg Fab'-S-biotin resulted in a tumor concentration of 2.6 µg / g, which can be converted to  $5.2 \times 10^{-5}$  µmol Fab'/g tumor by division by the approximate mass per mole of Fab' (i.e. 50,000 µg/µmol).

The amount of r-streptavidin bound to the biotinylated Fab' in the tumor was estimated as the quantity of r-streptavidin retained in the tumor minus the quantity in the blood volume of the tumor. The per gram concentration of streptavidin contributed by the blood volume of the tumor should be somewhere between the concentration in whole blood and in muscle. Therefore, calculations to estimate the specific localization were obtained as a range which encompassed the measured concentration in tumor minus the concentration in blood or muscle on a per gram basis.

Assuming a 1:1 binding of Fab'-S-biotin and r-streptavidin at the tumor, an expected quantity of streptavidin was calculated from the estimated quantity of A6H Fab' at the tumor. Thus, for a 1:1 binding ratio, there was  $5.2 \times 10^{-5}$  µmol r-streptavidin / g tumor. To estimate the quantity of r-streptavidin at the tumor,  $5.2 \times 10^{-5}$  µmol was multiplied by the mass per mole of r-streptavidin (e.g. 53,000 µg / µmol). This calculation provided an estimate of 2.8 µg r-streptavidin / g tumor. To obtain a lower limit on the amount of co-localization, the amount present in the blood was subtracted from the tumor concentration. However, this calculation was complicated by the fact that in this example (Table 2, 20 h), the %ID/g r-streptavidin in blood was higher than in the tumor. Since the tumor concentration of r-streptavidin in the 20 h group was nearly the same as in the 4 h group (i.e. 6.59 %ID/g vs 6.29 %ID/g, respectively), one might assume that a similar portion of the concentration in the 20 h group was specifically retained. Therefore, the amount of specifically retained r-streptavidin was estimated using the data from the 4 h group. The difference between tumor concentration and blood concentration in the 4 h group was 3.3 %ID/g (i.e. 6.29 %ID/g - 3.00 %ID/g). If 3.3% ID/g r-streptavidin was bound at the tumor when 30 µg were injected, then approximately 1.0 µg / g of r-streptavidin was localized there. This value indicates that only 36% ( $1 \mu\text{g} / \text{g} + 2.8 \mu\text{g} / \text{g}$ ) of the Fab'-S-biotin molecules at the tumor were bound to r-streptavidin. The upper limit for the r-streptavidin concentration in tumor was obtained by conducting the same evaluation, except the muscle concentration was subtracted rather than the blood concentration. This

calculation results in an estimated 61% of Fab'-S-biotin at the tumor being bound with r-streptavidin. Therefore, an estimated 36-61% of the Fab'-S-biotin molecules at the tumor were bound to r-streptavidin.

#### B. Avidin Co-Localization

The percent of co-localization of avidin can be estimated in the same manner as done for r-streptavidin. The quantity of A6H Fab' specifically localized at the tumor was estimated as follows. The concentration of A6H Fab'-S-biotin in the tumor was measured to be 28.9 %ID/g (Table 3, 20 h *pi*). To obtain a more accurate value, the concentration in blood (1.8 %ID/g) was subtracted to give an estimated 27% ID/g of Fab'-S-biotin was specifically localized at the tumor. Thus, it was estimated that injection of 10 µg Fab'-S-biotin resulted in a tumor concentration of 2.7 µg / g, which can be converted to  $5.4 \times 10^{-5}$  µmol Fab'/g tumor by division by the approximate mass per mole of Fab' (i.e. 50,000 µg/µmol).

For a 1:1 binding ratio, there was  $5.4 \times 10^{-5}$  µmol avidin / g tumor. This quantity was multiplied by the mass per mole of r-streptavidin (e.g. 68,000 µg / µmol) to provide an estimate of 3.7 µg avidin / g tumor. To obtain a lower limit on the amount of co-localization, the amount present in the blood (0.45%) was subtracted from the tumor concentration (0.93%) to yield 0.48% ID/g as the amount of specifically retained avidin. If 0.48% ID/g avidin was bound at the tumor when 30 µg were injected, then approximately 0.14 µg / g of r-streptavidin was localized there. This value indicates that only 4% ( $0.14 \mu\text{g} / \text{g} \div 3.7 \mu\text{g} / \text{g}$ ) of the Fab'-S-biotin molecules at the tumor were bound to avidin. The upper limit for the avidin concentration in tumor was obtained by conducting the same evaluation, except the muscle concentration (0.15 %ID/g) was subtracted rather than the blood concentration. This calculation results in an estimated 23% of Fab'-S-biotin at the tumor being bound with avidin. Therefore, an estimated 4-23% of the Fab'-S-biotin molecules at the tumor were bound to avidin.

# In Vitro Gene Delivery by Degraded Polyamidoamine Dendrimers

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Transfection of cultured cells has been reported using complexes between DNA and spherical cationic polyamidoamine polymers (Starburst dendrimers) that consist of primary amines on the surface and tertiary amines in the interior. The transfection activity of the dendrimers is dramatically enhanced (>50-fold) by heat treatment in a variety of solvolytic solvents, e.g., water or butanol. Such treatment induces significant degradation of the dendrimer at the amide linkage, resulting in a heterodisperse population of compounds with molecular weights ranging from the very low (<1500 Da) to several tens of kilodaltons. The compound facilitating transfection is the high molecular weight component of the degraded product and is denoted as a "fractured" dendrimer. Transfection activity is related both to the initial size of the dendrimer and its degree of degradation. Fractured dendrimers exhibit an increased apparent volume change as measured by an increase in the reduced viscosity upon protonation of the terminal amines as pH is reduced from 10.5 to 7.2 whereas intact dendrimers do not. Dendrimers with defective branching have been synthesized and also have improved transfection activity compared to that of the intact dendrimers. For a series of heat-treated dendrimers we observe a correlation between transfection activity and the degree of flexibility, computed with a random cleavage simulation of the degradation process. We suggest that the increased transfection after the heating process is principally due to the increase in flexibility that enables the fractured dendrimer to be compact when complexed with DNA and swell when released from DNA.

## INTRODUCTION

Advances in determining the genetic basis of diseases has made the concept of gene replacement to treat afflicted tissues increasingly attractive. Viral-based gene delivery is currently the most effective way to transfer genes to cells (1); however, concerns with immunogenic and long-term oncologic effects have prompted the concurrent development of synthetic gene delivery systems (2, 3). Nonviral delivery systems generally consist of linear cationic polymers, such as polylysine and its conjugates (4–6), poly(*N*-ethyl-4-vinylpyridinium bromide) and its derivatives (7), branched polymers such as polyamidoamines (8), and polyethyleneimine (9), or cationic lipids (10), that bind electrostatically with the negatively charged DNA to form a complex that can be taken up by target cells.

Cationic polyamidoamine (PAMAM)<sup>1</sup> cascade polymers mediate unexpectedly high levels of transfection in a wide variety of cells in culture (8) with significantly less toxicity than seen for comparably effective lipid-based systems (unpublished observations). On further study, we find that stringently synthesized and purified, monodisperse PAMAM dendrimers exhibit only low levels of transfection. However, high transfection levels can be obtained using PAMAM dendrimers if they are partially degraded by solvolysis. Here, we characterize the prop-

erties of these dendrimer-like polymers that determine their transfection activity. Namely, these degraded dendrimers are less sterically constrained than when intact; increased transfection activity generated by partial solvolysis of the dendrimer can also be obtained by incorporation of defects in the dendrimer during their synthesis. A random degradation model suggests that transfection activity is dependent upon the positive charge of the polymer and its flexibility, and less so upon its molecular weight.

## EXPERIMENTAL PROCEDURES

**Cationic Polymers.** PAMAM dendrimers based on different initiator core molecules were obtained: (1) ammonia (Polysciences, Inc.), (2) ethylenediamine (EDA) (Aldrich Biochemicals), and (3) tris(2-aminoethyl)amine (TAEA) (in-house synthesis). Ammonia and TAEA core molecules both yield dendrimers with 3-fold core geometry and similar molecular weight. The EDA core molecule yields dendrimers with a 4-fold core geometry and, thus, 33% higher molecular weight than 3-fold core dendrimers of the corresponding generation. Polyethyleneimine (Aldrich, MW = 25 kD) was diluted and dialyzed extensively in water, lyophilized, then redissolved in water before use.

**Synthesis of TAEA Core PAMAM Dendrimers.** Synthesis involves the stepwise, exhaustive addition of two monomers, methacrylate and ethylenediamine. Two methacrylate monomers add to each bifunctional ethylenediamine, leading to increasingly branched structures with each cycle, or generation (11, 12). The general synthesis scheme, theoretical molecular weights and charges are shown in Scheme 1. A single macromolecule that is highly homogeneous with respect to molecular weight, composition, and branching will result provided two potential problems are kept in check.

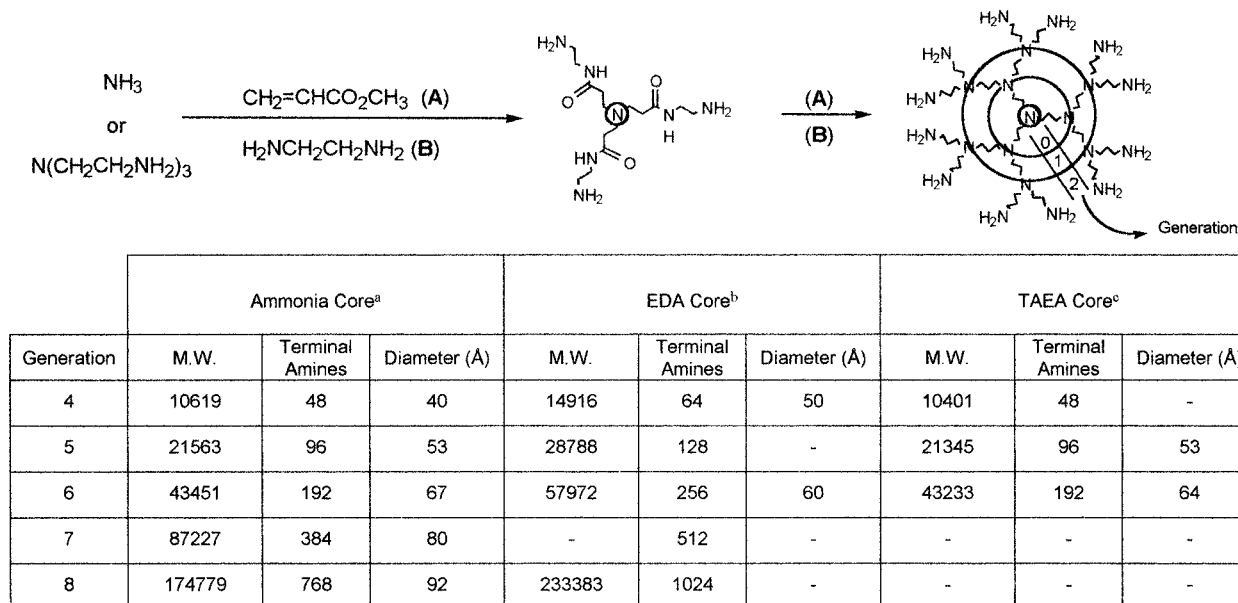
First, unreacted ethylenediamine must be completely removed at each step to prevent the initiation of cores that will lead to lower generation EDA dendrimers. For polyamides of generation 3 and above, vacuum evapora-

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<sup>1</sup> Abbreviations used: DLS (dynamic light scattering), DME (Dulbecco's Modified Eagle's medium), DOTAP (*N*-[2,3-(dioleoyloxy)propyl]-*N,N,N*-trimethylammonium methyl sulfate), EDA (ethylenediamine), EM (electron microscopy), HBS (HEPES buffered saline), MOPS (*N*-morpholinosulfonic acid), NDMA (*N,N*-dimethylacrylamide), ONPG (*o*-nitrophenol galactoside), PAMAM polyamidoamine, SEC (size exclusion chromatography), TAEA (tris(2-aminoethyl)amine).

**Scheme 1. General Synthesis Scheme Following Tomalia (11, 12) and Physical Parameters (molecular weight, number of terminal amines, and hydrodynamic diameter) of Selected PAMAM Dendrimers<sup>d</sup>**

<sup>a</sup> From ref 11. <sup>b</sup> MW, from Aldrich specifications; terminal amines, from ref 11; diameters, from SEC (see the Experimental Procedures). <sup>c</sup> MW and terminal amines, from theoretical calculations; diameters, from SEC (see the Experimental Procedures).

<sup>d</sup> Both the ammonia and TAEA core dendrimers are based on a 3-fold core symmetry. The EDA core dendrimers are based on a 4-fold core symmetry and, so, have larger molecular weights than the ammonia and TAEA core dendrimers of the corresponding generation.

tion followed by extended dialysis against deionized water was sufficient. Polyamides were then lyophilized and dried over phosphorus pentoxide. For the polyamides of generations 1 and 2, vacuum evaporation was followed by, for removal of ethylenediamine, extensive drying in a vacuum desiccator over concentrated sulfuric acid. This is in contrast to the previously published procedures in which wiped film distillation in 1-butanol was used to remove excess ethylenediamine (11, 12). Excess methacrylate at each generation was removed by vacuum evaporation followed by extensive drying over activated 13X molecular sieves. The second potential problem is the reaction of single ethylenediamine molecule with adjacent branches within a single dendrimer, leading to internal bridging or, with two different dendrimers, leading to dimerization. This was minimized by high dilution of the higher generation polymer in the reaction mixture containing methanol, a high excess of ethylenediamine used (in amounts as recommended in ref 11), and cooling the solution to -25 °C during addition of reactants to ensure complete mixing before reaction.

Defective dendrimers based on the TAEA core were synthesized by starting from the first generation by adding to the methacrylate monomer feed at each subsequent generation a specific proportion of *N,N*-dimethylacrylamide (NDMA), which, like methacrylate, reacts with the amine but prohibits further addition along the branch.

**Plasmids.** The reporter gene plasmids, pLUC4 (13) encoding firefly luciferase and pCMV- $\beta$ -Gal (14) encoding  $\beta$ -galactosidase, were generous gifts from Dr. M. Cotten (Institute of Molecular Pathology, Vienna, Austria) and Dr. G. McGregor (Howard Hughes Medical Institute, Houston, TX), respectively. Plasmids were amplified in *Escherichia coli*, extracted by alkaline lysis, and purified either by two successive CsCl gradients or by column separation (Qiagen). Plasmid integrity was confirmed by gel electrophoresis in 0.8% agarose. DNA concentrations were determined by absorbance at 260 nm (50  $\mu$ g/mL/

absorbance unit)). Endotoxin levels were less than 20 endotoxin units/mg of DNA (BioWhittaker endotoxin assay).

High-purity plasmid DNA used in all biophysical characterization work was a generous gift from Dr. Eric Eastman, GeneMedicine, Houston, TX. Prior to use, the DNA was dialyzed in five stages in 4 M, 2 M, 1 M, and 0.5 M NaCl and NaCl-free solutions containing 10 mM Tris/1 mM EDTA at pH 8.0. Step dialysis was done to remove trace amounts of small multivalent cations. Subsequent gel electrophoresis indicated that at least 95% of the plasmid was intact and supercoiled.

**Transfection Protocol.** For the  $\beta$ -galactosidase reporter gene assay, CV-1 cells (obtained from the UCSF Cell Culture Facility) were plated in 96-well trays at a density of 20 000 cells per well in 100  $\mu$ L of growth medium consisting of DME-H21 with 10% fetal calf serum and antibiotics (100 units/mL of penicillin and 100  $\mu$ g/mL of streptomycin). Cells were then incubated for 19 h at 37 °C. Growth medium was aspirated and then 150  $\mu$ L serum-free medium added.

The DNA/dendrimer complexes were made up by adding a solution of DNA in water to an equal volume of dendrimer in water in 96-well trays. Complex (50  $\mu$ L) containing 0.6  $\mu$ g of DNA and varying proportions of dendrimer were added to the serum-free medium. Cells were incubated at 37 °C for another 5 h before the complex and serum-free medium were aspirated and 150  $\mu$ L of growth medium was reapplied. After an additional incubation period of 1–2 days, cells were lysed and assayed.  $\beta$ -Galactosidase activity was detected spectrophotometrically using *o*-nitrophenol galactoside (ONPG, CalBiochem) as the substrate and purified  $\beta$ -galactosidase enzyme (EIA grade, CalBiochem) as the primary reference standard. The monodisperse fifth-generation ammonia core dendrimer (Polysciences, Lot #425292), which exhibits a baseline level of about 0.003 activity units against the enzyme standard, was used as a relative reference for all transfections. DOTAP (Avanti Polar Lipids) liposomes at a 3:1 charge ratio of lipid to DNA

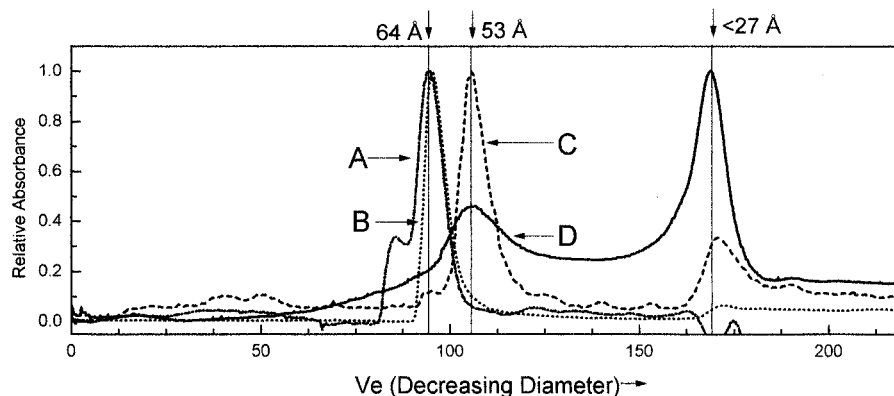


**Figure 1.** Size exclusion chromatography traces: (A) sixth-generation TAEA dendrimer prior to size fractionation; (B) sixth-generation TAEA dendrimer following size purification; (C and D) two different vials of the Polysciences sixth-generation ammonia core dendrimer. In the previous work (8), this Polysciences dendrimer was found to have the highest transfection activity. The Polysciences samples shown above have only moderate activity.

(with a baseline level of 0.012 activity units) was also used as a reference in most transfections. Relative concentrations of cation and DNA are given as weight ratios (cationic polymer/DNA) or, if the primary amine content is known, as charge ratios (cationic polymer primary amines/DNA phosphates).

**Polymer Sizing.** Size exclusion chromatography (SEC) analysis and purification were performed on a Sephacryl S100HR (Pharmacia) column with dimensions of 1.5 cm  $\times$  120 cm. The column was calibrated for hydrodynamic diameters using a standard kit (Sigma MW-GF-70), assuming that the proteins therein had a specific volume of 0.73 cm<sup>3</sup>/g and a degree of hydration of 0.3 mL/g. For analytical determination, compounds were eluted in 100 mM NaCl/100 mM phosphate at pH 7.2 at a flow rate of 0.2 mL/min and monitored for absorbance at 206 nm. To obtain sized fractions, the degraded dendrimer was eluted in 150 mM NaCl/10 mM MOPS/1 mM EDTA at pH 7.2.

Equilibrium sedimentation experiments were run in an analytical ultracentrifuge (Beckman Optima XL-A). Solutions of dendrimer in deuterium oxide or water were made up in 10 mM NaCl/1 mM HEPES at pH 7.2. The relative molecular weight ratio of deuterated to nondeuterated intact PAMAM dendrimer was calculated to be 1.0222. Polymer volumes were estimated by dilute solution viscosity measurements in water as performed in a Schott automated viscometer at 37 °C (15).

**Other Analyses.** Terminal amine contents were determined by absorbance at 570 nm using a ninhydrin reagent (16). The extent of complex formation was determined by gel retention and light scattering. For gel retention studies, complexes containing 0.1  $\mu$ g of DNA with varying amounts of dendrimer in HEPES-buffered saline (HBS) were electrophoresed through a 0.8% agarose gel using Tris-acetate-EDTA buffer and stained afterward using ethidium bromide. Dynamic light scattering of polymer/DNA complexes in HBS was performed on the Coulter N4.  $\zeta$  potentials of complexes diluted in 10 mM NaCl/1 mM MOPS/0.1 mM EDTA, pH 7.2, were determined using the Malvern Zetasizer IV. For both dynamic light scattering and  $\zeta$  potential measurement, the final concentration of DNA was 12  $\mu$ g/mL.

**Electron Microscopy.** Complexes were made up to a final DNA concentration of 12  $\mu$ g/mL at 4:1 weight ratio of dendrimer to DNA in 150 mM ammonium acetate and allowed to incubate for 10 min before grid preparation. Carbon-coated grids pretreated by glow discharge for 60 s were floated on a 50  $\mu$ L drop of solution containing the complex placed on parafilm. After 15 min, the grids were dipped briefly in water, touched to filter paper to remove

excess liquid, then air-dried under a heat lamp. Grids were rotary shadowed at 7° using platinum/tungsten and analyzed under the Phillips 300 transmission electron microscope.

## RESULTS

### Characterization of Synthesized Dendrimers.

Dendrimers synthesized as described in the Experimental Procedures were characterized by proton NMR. Polyesters were dissolved in CDCl<sub>3</sub> and exhibited at each generation characteristic triplet peaks at  $\delta$ 's of 2.4, 2.5, and 2.8 and a strong singlet peak due to the methoxy groups at 3.7. Polyamides were dissolved in D<sub>2</sub>O and at each generation exhibited peaks at  $\delta$ 's of 2.4, 2.6, 2.8, and 3.3. The absence of the methoxy singlet apparent in the polyesters indicated that ethylenediamine addition had gone to completion in the polyamides. Mass spectroscopy data could be obtained only for the first-generation polyester and polyamide and were found to be within 0.1% of the values expected.

The presence of lower order generation dendrimers and dimers was assayed by SEC. In general, no more than 10% of the total observed absorbance could be attributed to these impurities (Figure 1, trace A). Using SEC, these impurities were removed from a sixth-generation TAEA dendrimer to finally yield a single product with a narrow distribution and hydrodynamic diameter of  $64 \pm 1$  Å (Figure 1, trace B). The molecular weight of this purified fraction as determined by sedimentation equilibrium was within 3% of the theoretical value of 43.2 kD. The hydrodynamic diameters of other dendrimers are listed in Scheme 1.

As described in the Experimental Procedures, dendrimers with 8%, 17%, and 33% defective polyester branches were produced by introducing the corresponding amount of NDMA into the ester monomer feed at each generation of the synthesis. SEC analysis of these dendrimers showed narrow peaks eluting to yield hydrodynamic diameters that became progressively smaller with increasing NDMA added (Table 3). Each trace exhibited a small, high molecular weight, shoulder indicating the presence of dimerized product (data not shown), the amount of which was estimated to be no more than 10% of the total.

**Transfection Activity of Intact Dendrimers.** Inability to reproduce the high transfection activity levels previously reported with the sixth-generation 3-fold ammonia core dendrimer (Polysciences) (8) led to suspicions the polymer had degraded. SEC analysis of the dendrimers in stock confirmed considerable degradation

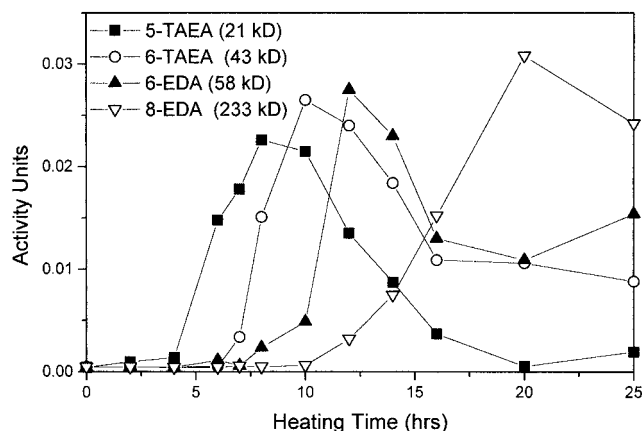


**Figure 2.** Transfection activity of a series of heat-treated dendrimers of varying initial size. Dendrimers were prepared at a concentration of 4 mg/mL in water and refluxed for the times indicated. The dendrimers treated were the sixth- and eighth-generation EDA core (6EDA, 8EDA) and the fifth- and sixth-generation TAEA core dendrimers (5TAEA, 6TAEA). The dendrimers were then used at a 4:1 weight ratio (equivalent to 6:1 charge ratio for the intact dendrimer) to transfect cells as described in the text. Values reported are the mean and standard deviation of triplicate wells in one run.

**Table 1. Selected Physical and Chemical Properties of Intact and Fractured Dendrimers and Their Complexes at a 4:1 Weight Ratio (Dendrimer/DNA)<sup>a</sup>**

| property                                                            | intact                           | fractured                         |
|---------------------------------------------------------------------|----------------------------------|-----------------------------------|
| primary amine content                                               | 4.4 $\mu$ mol/mg $\pm$ 3%        | 3.5 $\mu$ mol/mg $\pm$ 6%         |
| diameter of complex (by dynamic light scattering)                   | 2500 $\pm$ 2000 nm               | 130 $\pm$ 15 nm                   |
| diameter of toroids (by EM)                                         | 48 $\pm$ 16 nm ( <i>n</i> = 101) | 57 $\pm$ 16 nm ( <i>n</i> = 189)  |
| diameter of spheres (by EM)                                         | 61 $\pm$ 11 nm ( <i>n</i> = 184) | 70 $\pm$ 19 nm ( <i>n</i> = 162)  |
| $\zeta$ potential                                                   | 36 $\pm$ 3 mV                    | 44 $\pm$ 3 mV                     |
| transfection activity (-galactosidase) e.u.                         | 0.0003 $\pm$ 0.0003              | 0.016 $\pm$ 0.005                 |
| transfection activity (Luciferase) 10 <sup>7</sup> LU/mg of protein | 10 <sup>7</sup> LU/mg of protein | 10 <sup>10</sup> LU/mg of protein |
| 100% retention of DNA                                               | > <i>R</i> = 1                   | > <i>R</i> = 1                    |

<sup>a</sup> The sixth-generation TAEA core intact dendrimer is compared with its fractured counterpart (refluxed for 43 h in anhydrous 1-butanol.) Analytical methods are as described in the Experimental Procedures.

as can be seen by the extended tail and large salt volume signal in the traces (Figure 1, traces C and D). There was also a significant downward shift in the hydrodynamic particle diameter to 53 Å, which is substantially less than the manufacturer's specified value of 68 Å for this polymer. Moreover, although these two vials had the same manufacturer's lot number, different extents of degradation are evident in these traces.

Dendrimers with the 3-fold TAEA core were synthesized (see above), and those based on the 4-fold EDA core were purchased. These dendrimers were monodisperse, of the appropriate hydrodynamic diameter as monitored by SEC, and showed no signs of degradation. The sixth-generation TAEA core dendrimer was synthesized to be equivalent to the sixth-generation ammonia core dendrimer described in our previous work; however, transfection activity by this and the other intact dendrimers, as measured by  $\beta$ -galactosidase activity, was still 100-fold lower than previously characterized. In the initial work (8), luciferase activity values of greater than  $1 \times 10^{10}$  light units/mg of protein were obtained for trans-

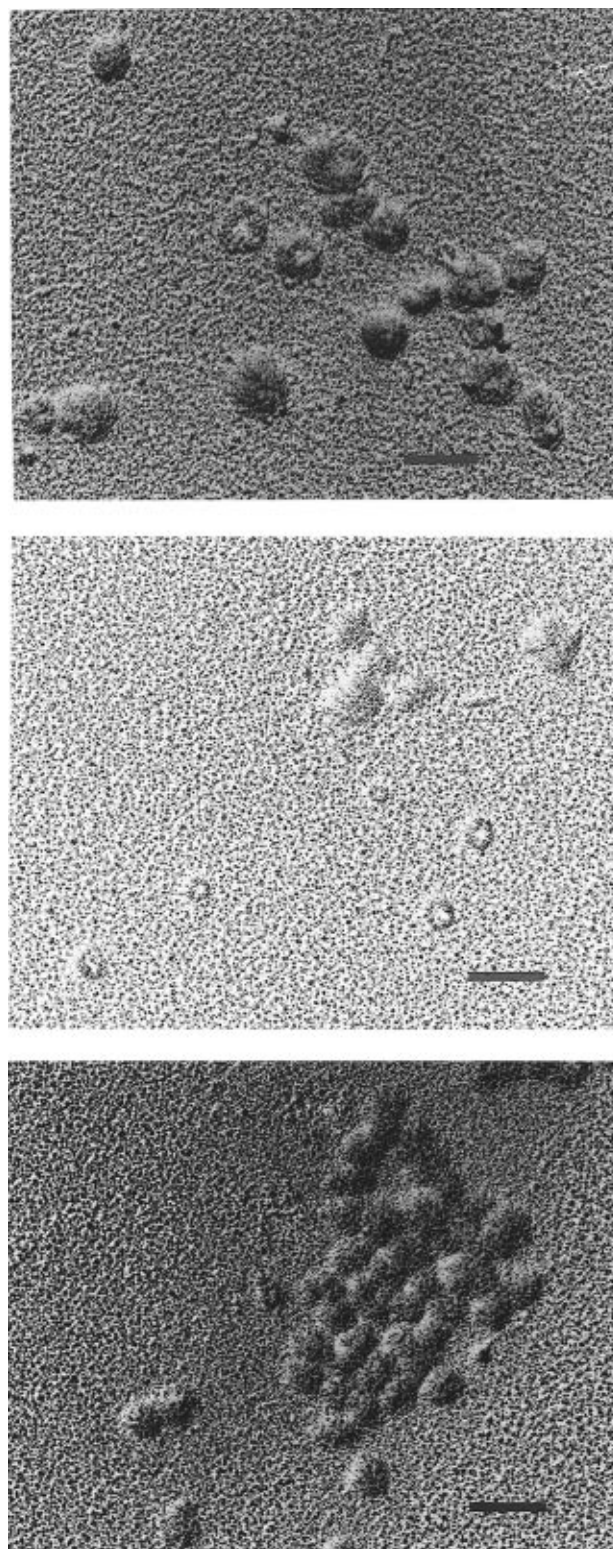


**Figure 3.** Electron micrographs of dendrimer complexed with DNA: (a, top) fractured dendrimer/DNA complexes at a 4:1 weight ratio; (b, center) intact dendrimer/DNA complexes at a 4:1 weight ratio (6:1 charge ratio); (c, bottom) aggregates of intact dendrimer/DNA complexes at a 4:1 weight ratio. The bar indicates 100 nm.

fusions of CV-1 cells by the pLUC4 plasmid, whereas activity levels of these pure dendrimers were 1000-fold lower. These results suggested that a degradation product may actually mediate transfection.

**Heating Treatment of Dendrimers.** Heating of dendrimers in water increases transfection activity up to a maximum level (Figure 2). Continued heating after



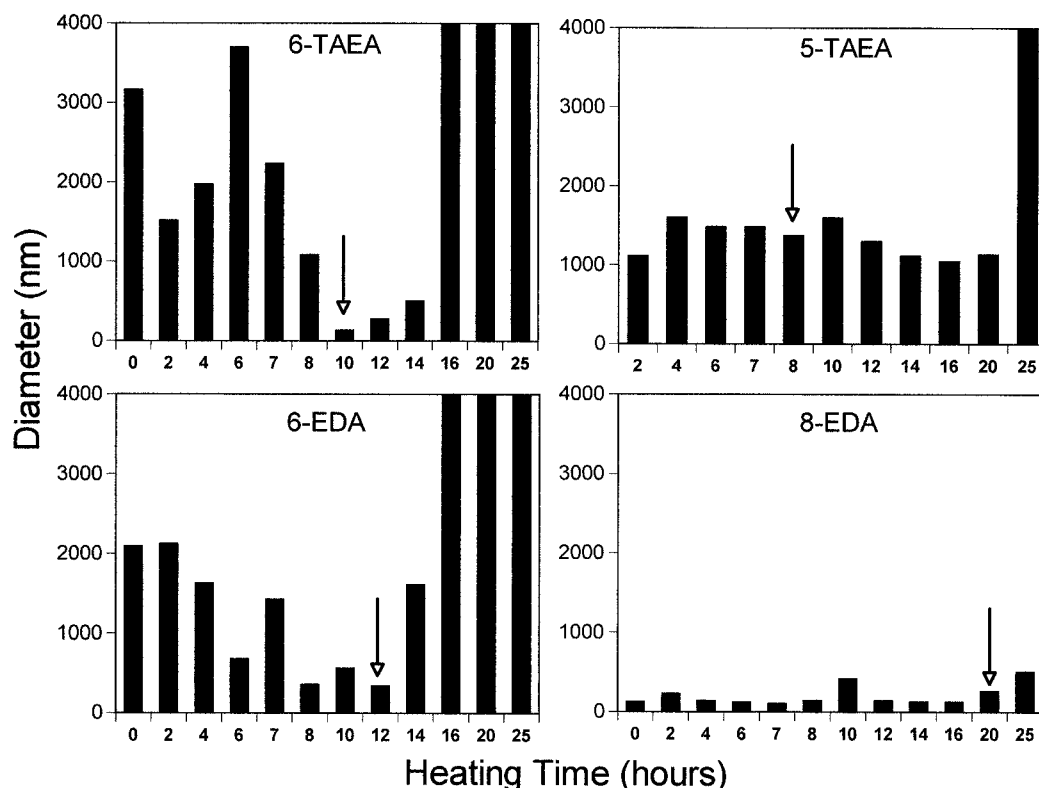


**Figure 4.** Diameters of DNA complexes of different dendrimers with various heating times. The dendrimers were heat treated as described in Figure 2 and then complexed with DNA in HBS at a 4:1 weight ratio (equivalent to a 6:1 charge ratio for the intact dendrimer). Arrows indicate the heating times at which maximum transfection activity was observed. The values reported are the weight average mean as determined by dynamic light scattering. Values greater than 1000 nm tend to be comprised of unstable aggregates and have large variability.

this point leads to a decrease in activity which may correspond to excessive degradation of the dendrimer. Lower generation dendrimers appear to require less heating time to reach peak transfection activity, although their maximum transfection activity levels are slightly lower than that of the higher generations.

Heat activation can be done in any solvolytic solvent, although heating time to attain maximum activity will vary with the solvent used. Water and solvents such as 1-butanol, 2-butanol, 2-ethoxyethanol, and mixtures thereof, induce activity at rates related to their degree of solvolytic behavior. Heat treatment in nonsolvolytic solvents, such as DMF, did not induce activity at all, thus suggesting that the degradation process occurs by solvolysis of peptide bonds in the dendrimer.

**Characterization of 6-TAEA Intact and Fractured Dendrimers and Their DNA Complexes.** Additional biophysical characterization was performed with the 6-TAEA intact and optimally fractured (heated for 42 h in 1-butanol) dendrimers and their DNA complexes which were selected to represent the low and high limits of transfection, respectively. The results are tabulated in Table 1. The fractured dendrimer/DNA complex, as measured by dynamic light scattering, is very small and very stable, remaining the same diameter over several days. In contrast, DNA complexes of the intact dendrimer become very large after 15 min of incubation and continue to increase in size over several hours. When examined under EM, the DNA complexes of either dendrimer appear as compact structures, with the same toroidal and spheroidal morphology frequently observed for DNA complexes of small multivalent cations (17,18) (Figure 3a,b). The fractured dendrimer formed DNA complexes that were uniformly dispersed throughout the grid. The intact dendrimer/DNA complexes, however, tend to be found as clusters and aggregates and only

occasionally as single particles on the EM grid (Figure 3c). Thus, the discrepancy between the large complexes measured by DLS for the intact dendrimers (Table 1) and the small particle size observed under EM (Figure 3) is most likely due to the sensitivity of DLS to aggregates of these unit particles.

Toroids and spheres of the fractured dendrimer/DNA complexes are slightly larger than those of the intact dendrimer/DNA complexes (Table 1). Spherical particles are slightly, but significantly, larger than toroids for both dendrimers. Many of the toroids are indistinguishable in size and general appearance from spherical particles but for a slight dimple (Figure 3a,b). Fractured dendrimer/DNA complexes generally appear as mono-toroid or mono-spheroid units, although dimers and occasionally trimers of the unit structures are observed. The particle size of the fractured dendrimer/DNA complexes as measured by DLS is 2-fold greater than observed under EM. This discrepancy could be attributed to the assumption, in the DLS determination, that the particle is perfectly spherical, since a smaller diameter toroid will yield the same friction factor and, therefore, the same apparent particle size as a larger sphere (19).

Both dendrimer/DNA complexes are retarded equally well at a 1:1 charge ratio when electrophoresed through an agarose gel, indicating that complexation between the polymer and DNA is complete. The  $\zeta$  potentials of the dendrimer complexes are strongly positive and differ only slightly. Most importantly, transfection activity as measured by both  $\beta$ -galactosidase and luciferase assays is significantly improved for the fractured dendrimer (Table 1).

**The Diameter of DNA Complexed Heat-Activated Dendrimers.** In Figure 4, the diameter of the complexes between DNA and the various dendrimers that were heated treated in Figure 2 are shown; the arrows indicate

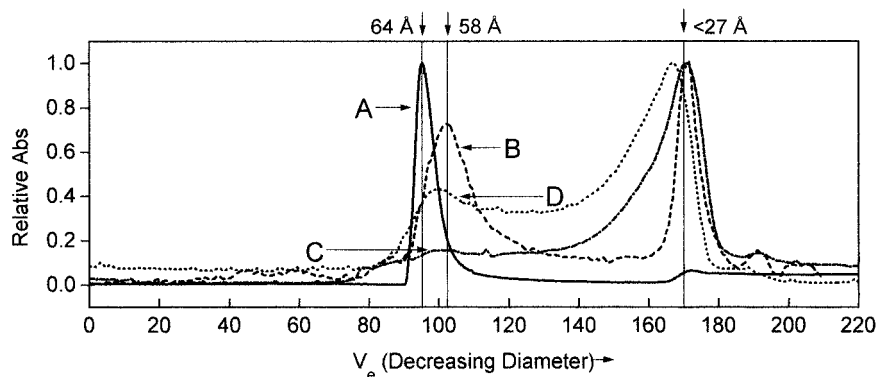


**Figure 5.** SEC elution traces of the intact 6-TAEA and several heat-activated dendrimers. Note the considerable tailing, downward shift in peak location, and enlarged signal in the salt fraction with heated samples: (A) intact 6-TAEA; (B) 6-EDA heated for 10 h in water; (C) 6-TAEA heated for 16 h in 2-ethoxyethanol; (D) 8-EDA heated for 20 h in water.

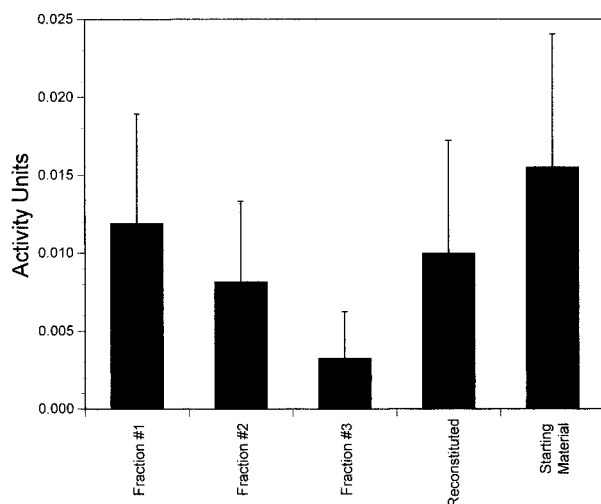


**Figure 6.** Transfection activity of heat-activated dendrimers that have been separated by size. Using SEC, heat-activated (for 42 h in 1-butanol) sixth-generation TAEA core dendrimer was separated into three size fractions: fraction 1, with a hydrodynamic diameter of about 60–70 Å; fraction 2, 44–60 Å; fraction 3, 27–44 Å. The fourth fraction, containing salts and small molecular weight fragments, was disregarded. The fractions were dialyzed extensively, lyophilized, and brought up in water. Concentrations were estimated using absorbance at 204 nm. These size fractionated dendrimers were then used to transfect pCMV- $\beta$ Gal plasmid into CV-1 cells as described in the Experimental Procedures. Mean and standard deviation of activity levels at a 4:1 weight ratio (6:1 charge ratio for intact product) for duplicate wells in three separate runs are reported.

the heating time at which maximum transfection activity was observed. As shown in Figure 4, the DNA complexes of 6-TAEA and 6-EDA fractured dendrimers show highest activity at which their complexes are smallest in diameter, as measured by DLS. Their intact counterparts (at zero heating time) exhibit not only large particle size as measured by DLS but also visible aggregates which appear several hours after mixing. The observation based on these two dendrimers would appear to support the hypothesis that smaller complex size facilitates transfection. However, the DNA complexes of the 5-TAEA fractured dendrimer show highest activity at 8 h of heating and little or no activity at 20 h although all the complexes are the same rather large (about 1000 nm) size. In contrast, the 8-EDA complexes show highest activity at 20 h of heating and very low activity at 8 h, yet both complexes are very small. Thus, the diameter of the dendrimer/DNA complex, as opposed to the diameter of the individual dendrimer, does not appear to be the most important factor in obtaining high transfection activity.

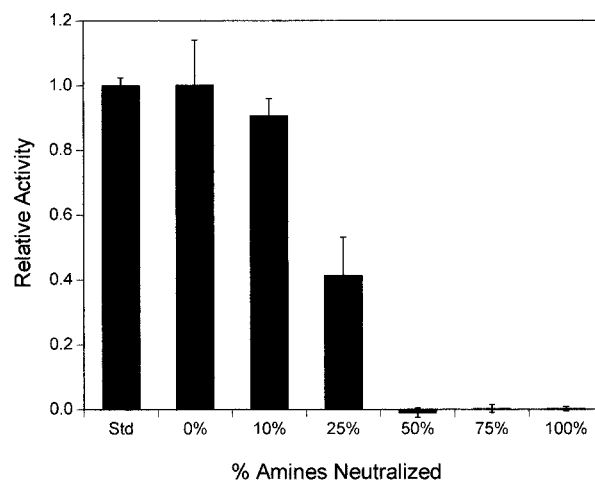


**Figure 7.** Transfection activity (relative to the untreated dendrimer) of acetic anhydride titrated dendrimers (fifth-generation ammonia core). Complexes were formed and added to CV-1 cells cultured in 96-well trays as described in the Experimental Procedures. The pCMV- $\beta$ Gal plasmid DNA was held constant at 0.6  $\mu$ g per well, and the ratio of all dendrimer terminal groups (modified or not) to DNA phosphates was varied from 0.5 to 24. The maximum activity level, regardless of terminal group to phosphate ratio used, is reported. Control sample is the unmodified dendrimer; the 0% sample underwent the same treatment as the other samples, but in the absence of acetic anhydride. Data reported are the mean and range of duplicate wells in one run which was representative of three similar experiments.

**Effect of Diameter of Heat-Activated Dendrimer on Transfection.** The SEC traces of several optimally heat-activated dendrimers (Figure 5) show that a significant amount of high molecular weight material remains, as indicated by the broad peaks at 100 mL but that significant fracturing has occurred, as shown by the gradual tailing of the high molecular weight peaks and strong salt peak signals at 170 mL. This observation suggests that transfection might actually be mediated by degradation byproducts of low molecular weight.

To determine whether low molecular weight fragments could be responsible for transfection, an optimally heat-activated sixth-generation TAEA-core dendrimer was fractionated by size using SEC. The high molecular weight fractions were dialyzed in water, lyophilized, and then redissolved in water before testing for transfection activity. The results are shown in Figure 6. In a separate experiment, the salt fraction, which was lyophilized and redissolved in water before the transfection assay, showed no measurable activity at any concentration. Only those fractions containing high molecular weight compounds showed transfection activity, thereby

**Table 2. Polymer Characteristics Associated with Dendrimers Heated to the Point of Highest Transfection Activity<sup>a</sup>**

| dendrimer | heating time (h) <sup>b</sup> | measd relative amine content <sup>c</sup> | computed % amide bonds cleaved <sup>d</sup> | initial molecular weight (kD) <sup>e</sup> | computed final molecular weight (kD) <sup>d</sup> | initial 1° amines/molecule <sup>e</sup> | computed final 1° amines/molecule <sup>d</sup> | computed net charge/molecule <sup>d</sup> | computed flexibility parameter (vol/wt) <sup>d</sup> | computed equivalent % defects <sup>f</sup> |
|-----------|-------------------------------|-------------------------------------------|---------------------------------------------|--------------------------------------------|---------------------------------------------------|-----------------------------------------|------------------------------------------------|-------------------------------------------|------------------------------------------------------|--------------------------------------------|
| 5-TAEA    | 8                             | 0.93                                      | 13                                          | 21                                         | 18.3                                              | 96                                      | 77                                             | 69                                        | 6.9                                                  | 5                                          |
| 6-TAEA    | 10                            | 0.83                                      | 35                                          | 43                                         | 28.5                                              | 192                                     | 107                                            | 80                                        | 7.6                                                  | 10                                         |
| 6-EDA     | 12                            | 0.76                                      | 50                                          | 58                                         | 28.9                                              | 256                                     | 97                                             | 57                                        | 7.5                                                  | 13                                         |
| 8-EDA     | 20                            | 0.62                                      | 70                                          | 233                                        | 70.1                                              | 1024                                    | 190                                            | 41                                        | 7.4                                                  | 17                                         |

<sup>a</sup> Dendrimers of various initial size were heat treated as described in Figure 2, dialyzed against water, then assayed for primary amine content as described in the Experimental Procedures. The amine content for each dendrimer, relative to that of its intact counterpart, was used to determine the extent of degradation based on the fractured dendrimer model, and thus, the model parameters as shown in Figure 11. <sup>b</sup> Heating time at which maximum activity was observed (refer to Figure 2.) <sup>c</sup> Amine content by weight relative to the corresponding intact dendrimer as determined by ninhydrin assay. <sup>d</sup> Parameters as determined from the model plotted in Figure 11. <sup>e</sup> Theoretical values as indicated in Scheme 1. <sup>f</sup> Computed defects per generation from Figure 11e.

**Table 3. Calculated Parameters for Dendrimers Deliberately Synthesized with Defects**

| % defects | measd relative amine content <sup>b</sup> | theor. relative amine content <sup>c</sup> | computed % defects incorporated <sup>d</sup> | theor. 1° amines/molecule <sup>c</sup> | theor. % amides remaining <sup>c</sup> | theor. molecular weight (kD) <sup>c</sup> | hydrodynamic diameter (Å) <sup>e</sup> | theor. flexibility parameter (vol/wt) <sup>c</sup> |
|-----------|-------------------------------------------|--------------------------------------------|----------------------------------------------|----------------------------------------|----------------------------------------|-------------------------------------------|----------------------------------------|----------------------------------------------------|
| 0         | 1.00                                      | 1.00                                       | 0                                            | 192                                    | 100                                    | 43.2                                      | 64                                     | 5.0                                                |
| 8         | 0.90                                      | 0.85                                       | 6                                            | 116                                    | 69                                     | 30.7                                      | 59                                     | 7.0                                                |
| 17        | 0.76                                      | 0.70                                       | 13                                           | 63                                     | 53                                     | 20.4                                      | 56                                     | 10.6                                               |
| 33        | 0.13                                      | 0.42                                       | 36                                           | 17                                     | 22                                     | 9.2                                       | 45                                     | 23.5                                               |

<sup>a</sup> Proportion of defective monomer (*N,N*-dimethylacrylamide) added during the synthesis as described in the Experimental Procedures. <sup>b</sup> Amine content by weight relative to the intact dendrimer as determined by ninhydrin assay. <sup>c</sup> Theoretical values as determined by the proportion of defects introduced at each generation (e.g., the number of primary amines per molecule for the 8% 6-TAEA defective dendrimer is  $192 \times (0.92)^6$ . <sup>d</sup> Calculated parameter value from Figure 11b,e. <sup>e</sup> Determined by size exclusion chromatography (see the Experimental Procedures).

ruling out the possibility that a low molecular weight fragment is mediating transfection. Thus, the agent that mediates transfection is the high molecular weight component of the degraded dendrimer product; this has been termed a "fractured" dendrimer.

**The Influence of Primary Amine Density upon Transfection.** The primary amine content of the fifth-generation ammonia core dendrimer was varied by direct reaction with acetic anhydride. Ninhydrin analysis showed nearly stoichiometric addition by acetic anhydride to terminal amines (data not shown). Transfection by these amine-titrated dendrimers showed a significant decrease in activity for a 25% reduction in available terminal amines (Figure 7). Complete abolishment of activity was seen at 50% reduction, even in the presence of sufficient excess positive charge to neutralize the DNA. Thus, if all primary amines are assumed to be protonated at physiological pH, a high surface charge density is necessary for dendrimer transfection.

However, the terminal amine contents in highly active, fractured dendrimers that had been dialyzed and lyophilized to remove low molecular weight components following heat treatment are significantly lower than their intact counterparts which are not active in transfection (Tables 1–3). Therefore, although a high number of terminal amines are required for transfection by dendrimers, other physical properties of the polymer also significantly contribute to their activity.

**Transfection by Dendrimers Synthesized with Defective Branches.** As shown in Figure 5, heat activation of dendrimers results in a heterogeneous population of fractured polymers varying in size, shape, structure, and charge which is difficult to characterize by classical methods. Efforts to determine the molecular weight and specific volume of the fractured dendrimer, for example, even with size fractionated samples, were unsuccessful using analytical ultracentrifugation because the polydispersity was unresolvable. In an effort to produce a monodisperse polymer that is active in trans-

fection, a series of chemically defective dendrimers were synthesized. We hypothesized that, if solvolysis were the main degradative process and all amide bonds (internal as well as terminal) were equally susceptible, then this synthesis approach should yield a polymer with similar degrees of branching as the fractured dendrimer.

Transfection activity is improved significantly with the 17% defective sixth-generation dendrimer which exhibited measurable activity with ONPG compared to its intact counterpart (Figure 8a). Although the relative activity was still considerably lower than what is attained by heat-activated, fractured dendrimers, this result clearly indicates that defects in dendrimer branching can induce higher transfection activity. Heating the chemically defective dendrimers improved activity further (Figure 8b). Moreover, the heating time which maximum activity is observed decreases with increasing percentage of defects, except for the 33% defective dendrimer, suggesting that the optimal value lies somewhere between 17% and 33%. This implies that the synthetic strategy employed to create defective dendrimers is a viable approach to providing material of greater activity, although it has not yet been optimized in this series of experiments.

**Random Bond Cleavage Model of the Fractured Dendrimer.** Degradation of the dendrimer resulting in improved transfection activity occurs with heat treatment only in solvolytic solvents. We also observed that the partly degraded, highly active dendrimers used in our original characterization (8) had been stored for several years as free base in water. These observations strongly suggest that degradation of the dendrimer occurs by solvolysis of amide bonds. The process of degradation was simulated assuming that all amide bonds were equally susceptible to solvolysis. Using a random number generator, a chosen amide bond was broken and the corresponding branch associated with that bond was removed. More amide bonds were broken until only a certain fraction of the total amide bonds remained

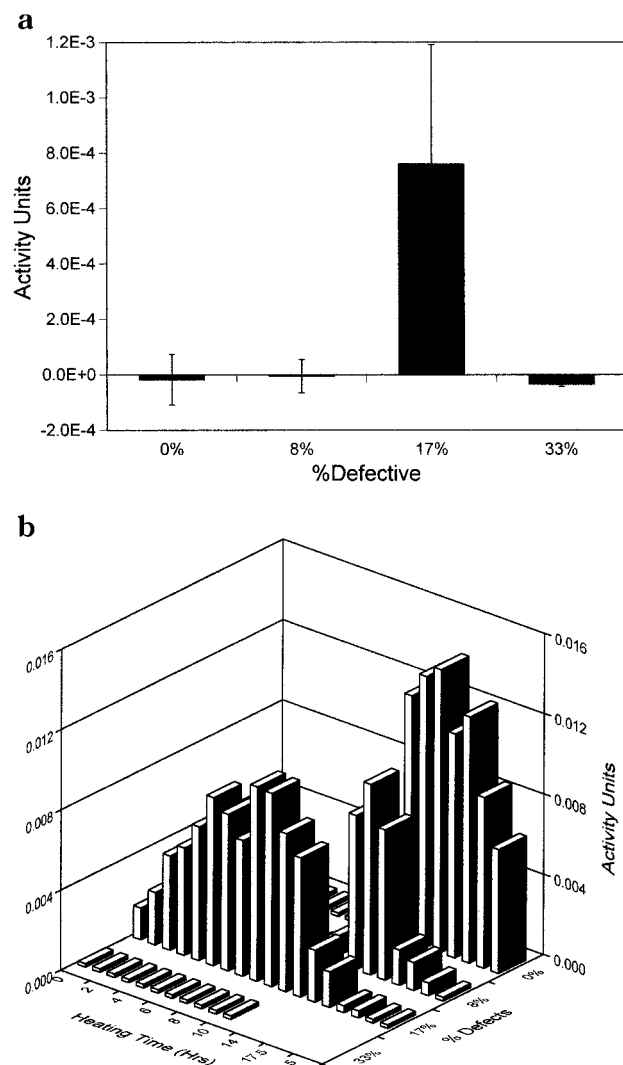


**Figure 8.** Transfection activity of dendrimers chemically synthesized with defective branches. (a.) Values reported are the mean and standard deviation of duplicate wells in three runs. (b) Transfection activity of defective-branch dendrimers heated in water for the times indicated. This series of dendrimers exhibited a strong concentration dependence upon transfection. Only the weight ratios at which maximum activities are observed are shown: 0% = 4:1; 8% = 12:1; 17% = 24:1. Although the 8% defective dendrimer also showed fairly good activity at 4:1, the 17% defective dendrimer showed little or no activity below 24:1. The mean of duplicate wells in one run representative of three trials is reported. The pooled standard deviation for this experiment is 0.000 17 activity units.

associated with the core. Two-dimensional representations of this model as applied to the 6-TAEA core dendrimer are shown in Figure 9. Certain features are clear in these fractured structures: (1) a substantial number of terminal amines remain, and thus much positive charge is retained, (2) the structure remains highly branched, and (3) the remaining branches are much less constrained by adjacent branches.

In this model, the molecular weight of the degraded polymer decreases almost linearly with the fraction of amide bonds cleaved (Figure 10a). The primary amine content decreases with amide bond cleavage, especially at high degrees of degradation, since the likelihood of cleaving an interior amide, which results in the loss of an entire polymer branch containing many terminal amines, increases (Figure 10b). Moreover, as degradation progresses, the net charge on the dendrimers decreases rapidly, due not only to the loss of primary amines but to the gain in carboxylic acid groups upon

hydrolysis at the amide bonds (Figure 10c). We have defined a flexibility parameter which is the volume of a sphere with a radius that increases linearly with generation, normalized by the molecular weight of the degraded polymer. We assume that, on average, the degraded dendrimer expands to occupy the volume occupied by the intact dendrimer. As shown in Figure 10d, the flexibility parameter increases as polymer degradation progresses.

Following dialysis, the primary amine content by weight of the fractured dendrimers can be easily and accurately measured, and one can then use the relationship illustrated in Figure 10b to determine the proportion of amide bonds that have been cleaved. From this and knowledge of the generation and core geometry of the initial polymer, the other parameters can be computed for each fractured dendrimer from the relations in Figure 10. The results for optimally heat-activated dendrimers of different generations and core geometry are summarized in Table 2. With increasing initial molecular weight of the dendrimer, the degree of degradation required to achieve maximum activity increases, as measured both by the relative terminal amine contents and extent of solvolysis indicated by the percentage of amide bonds cleaved. Although these samples exhibited nearly equivalent levels of high transfection activity (Figure 2), they exhibit widely different calculated final molecular weights and, surprisingly, final primary amine content and net charge. Their flexibility parameter values, however, are all within 7% of the mean value of 7.4 (Figure 10d).

In the completely random bond solvolysis model, the degree of branching that results is equivalent to that of a synthesis scheme in which a specific proportion of defects is introduced at every generation (Figure 10e). Thus, another test of this model is a comparison to the case of the dendrimers that had been deliberately synthesized with defects (see above). In Table 3, the measured terminal amine content of dendrimers synthesized with a defined percentage of branching defects is compared to the theoretical parameter values based on the proportion of defective NDMA monomer added at each synthesis step. A comparison between actual and expected values for relative amine contents indicates that the 8% and 17% defective dendrimers are slightly less, and the 33% somewhat more, defective than intended. When the computed flexibility parameters for the synthetically defective dendrimers are compared to those associated with the heat-activated dendrimers demonstrating high transfection activity, one would predict that the 8% defective dendrimer is in the range required for transfection activity whereas the 17% is too defective. However, this was not the case; the 17% exhibited some measurable activity, whereas the 8% did not. Moreover, heat treatment of both the 8% and 17% increased transfection activity, suggesting that even a greater degree of defects is required. Although introducing branching defects appears to improve transfection (Figure 8), the NDMA approach may not be the best synthetic model system for heat-activated dendrimers.

**Dilute Solution Viscosity of Polymer as a Function of pH.** Dilute solution viscosity measurements were used to quantify flexibility by exploiting the polyelectrolyte nature of these polymers (15). According to the first approximation of Einstein's law of viscosity, the specific viscosity ( $\eta_{sp}$ ) of a given solution is directly proportional to the volume occupied by the particles (modeled as spheres) dispersed in the solution (20). At near neutral pH, the mutual electrostatic repulsion between the protonated terminal amines cause the branches of the

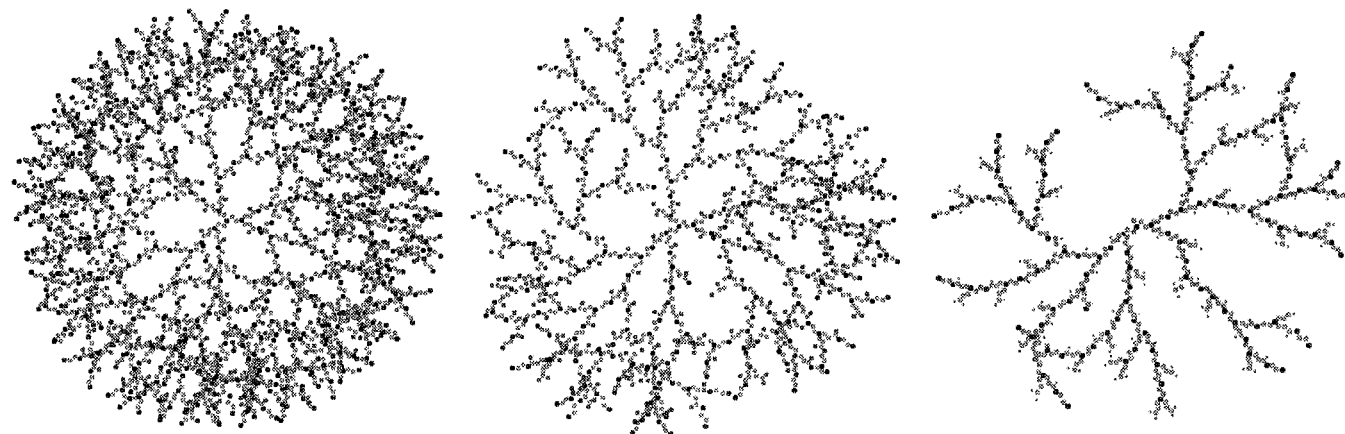


**Figure 9.** Simulation model of fractured dendrimers. These two-dimensional representations of various stages of dendrimer degradation are based on the results of a simulation in which amide bonds were hydrolyzed at random and the associated branches removed until only a specific proportion of amide bonds remained. These structures depict the average of 500 iterations of the simulation: (a, left) 100% amide bonds remaining; (b, center) 50% amide bonds remaining; (c, right) 25% of amide bonds remaining.

dendrimer to adopt a fully extended conformation. Above pH 9.5, the terminal amines are deprotonated; thus, the dendrimer adopts a more collapsed conformation. The relative difference in viscosities at a given concentration is proportional to the volume occupied in solution by the polymer and, therefore, the intrinsic flexibility of the polymer.

Reduced viscosity ( $\eta_{\text{red}}$ ), which is specific viscosity normalized by concentration, is plotted in Figure 11. Optimally fractured dendrimer (6-TAEA heated 42 h in 1-butanol) shows an increase in viscosity with decreasing pH that corresponds to a 2.4-fold increase in volume. The intact precursor, however, shows no overall change in volume with pH. The viscosity of the intact dendrimer at both pH's is the same. The effect of pH on the viscosity of polyethyleneimine, likewise shown to facilitate high transfection levels (9), was also studied and shows a 2.9-fold change increase in volume with decreasing pH. This correlation suggests that polymer flexibility plays an important role in the mechanism of transfection by cationic polymers.

## DISCUSSION

We previously reported that PAMAM cascade polymers could be used as a highly efficient transfection system for cells in culture but noted that certain lots of commercial dendrimer were degraded (8). Intact dendrimers subsequently obtained, much to our surprise, exhibited much lower transfection activities than expected. We suspected that the dendrimers used in the earlier characterization were activated for transfection either by extended storage in water at high pH or by the wiped film distillation purification step which we had replaced with dialysis in our synthesis. This activation process could be duplicated by heat treatment of the intact dendrimer, which improved transfection activity (Figure 2). Heat-activated dendrimers show considerable degradation as manifested by extensive polydispersity in their SEC traces (Figure 5). However, size-fractionated heat-activated dendrimer exhibits transfection activity only in the high molecular weight fractions, thereby ruling out low molecular weight fragments in mediating transfection. The imidazoline functionality formed from the end groups during synthesis or heat treatment in anhydrous solvents was also ruled out as activating transfection (unpublished observations). With these results, we conclude that a "fractured" polymer is the compound that mediates high levels of transfection activity observed in some dendrimers.

A comparison of the DNA complexes formed by the intact and fractured dendrimers does not immediately account for the difference in transfection activities observed. Both bind DNA to form compact structures of nearly the same size and morphology (Table 1), although the intact dendrimer/DNA complexes appear more prone to aggregation (Figure 3c). The larger size of the intact dendrimer/DNA aggregates alone, however, does not explain their low transfection activity because no clear correlation between DNA complex size and transfection activity could be detected for various dendrimers heated over time (Figure 4).

We have devised a model based on the assumption that the key degradation process is the random solvolysis of peptide bonds between polyamidoamine units. In proportion to their molecular weight, intact dendrimers surround a very large solvent-filled volume. Our sedimentation equilibrium studies, for example, show that an intact 6-TAEA dendrimer has a specific volume of 0.84 cm<sup>3</sup>/g, which is considerably larger than that of a typical protein (about 0.73 cm<sup>3</sup>/g). Moreover, modeling studies of the sixth-generation ammonia core dendrimer show that there is 124% more internal than external surface area available for solvent interaction (12). Thus, our assumption that all amide bonds, internal as well as those near the surface, are equally susceptible to solvolysis is consistent with the high degree of hydration of the intact dendrimers. Solvolysis of a terminal end amide would result in the loss of a primary amine as well as nominal mass. Solvolysis of an interior bond, however, would result in the loss of the entire corresponding branch. Although this would lead to an overall loss of surface charge which will adversely affect transfection activity (Figures 7 and 10c), this loss is accompanied by an increase in flexibility of the fractured dendrimer (Figure 10d).

Whatever the starting dendrimer, optimal transfection activity appears to be correlated to the flexibility parameter (Table 2, Figure 10d). For example, the intact 6-TAEA dendrimer differs structurally from the 6-EDA in that its branching has 3-fold rather than 4-fold symmetry and, so, possesses three-fourths of the mass and primary amines; however, their volumes are about the same as both are of the sixth generation. According to the model, at the point where maximum transfection activity is observed, the fractured counterparts of the 6-TAEA and 6-EDA dendrimers have the same apparent molecular weight and, therefore, flexibility. Thus, this model suggests that a key parameter influencing trans-

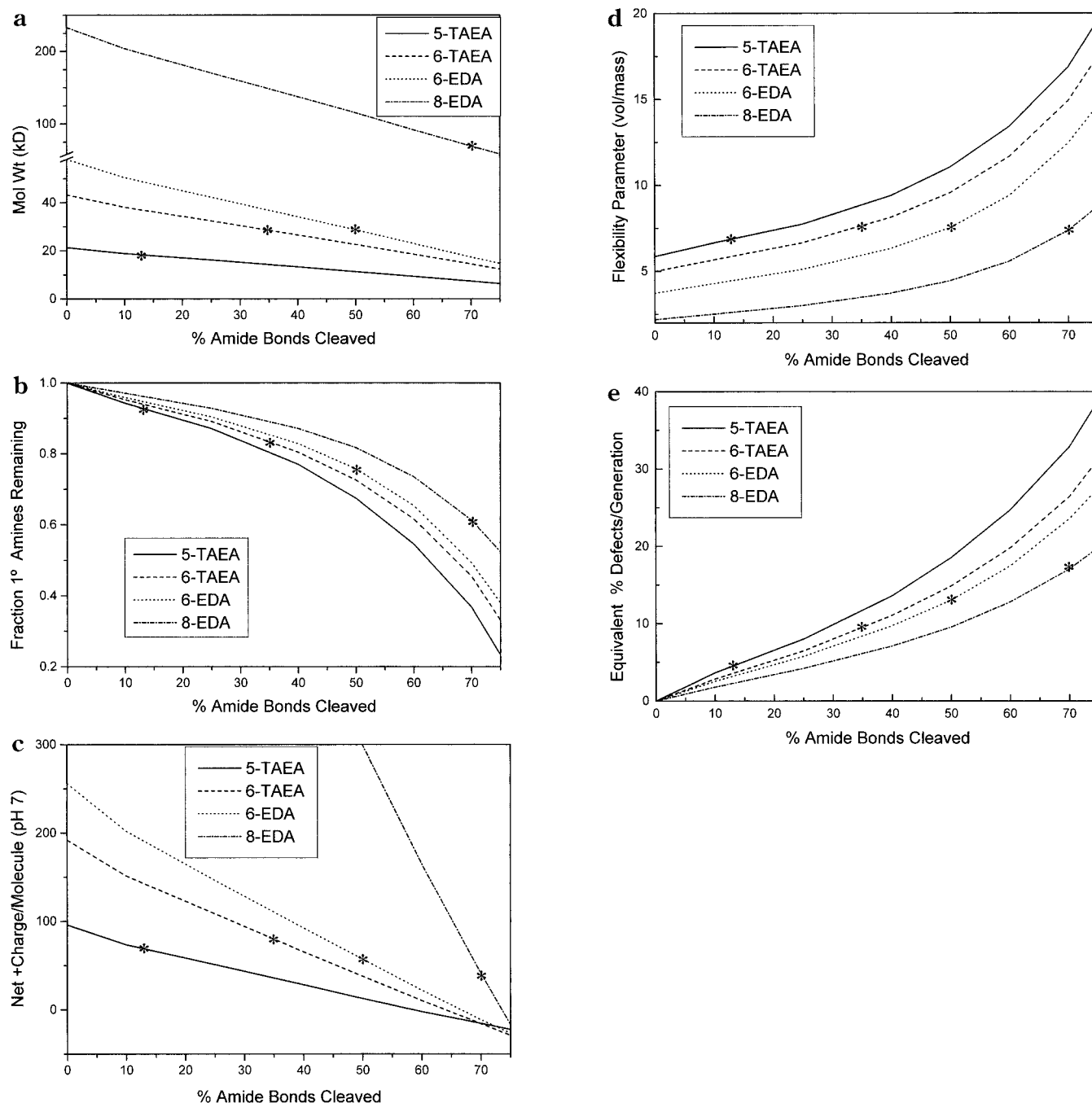


**Figure 10.** Simulation model parameters: (a) Molecular weight versus the proportion of amide bonds cleaved. (b) Proportion of primary amines remaining versus that of the amide bonds cleaved. This graph relates experimental data with model parameters. (c) Net positive charge at neutral pH vs amide bonds cleaved. Net positive charge is the difference between the number of primary amines remaining and the number of carboxylic acid groups created with amide bond hydrolysis. Because acid groups will influence titration behavior of nearby tertiary amines and may even interact to form zwitterion pairs, this is a minimum estimate of net positive charge. (d) Flexibility parameter as a function of amide bonds cleaved. (e) The percentage of defects at each generation that will yield the equivalent proportion of amide bonds cleaved. Asterisks (\*) indicate the values determined for the optimally fractured dendrimers described in Figure 2.

fection activity is polymer flexibility and that other polymer characteristics (amine content, net charge, and molecular weight), although important, may adopt a wide range of values without adversely affecting transfection. Viscosity measurements of polymers in their protonated and deprotonated states indicate a greater degree of flexibility in polymers that are active in transfection than one that is not (Figure 11) and, thus, support the hypothesis that transfection activity correlates with polymer flexibility.

The model, however, also predicts that a dendrimer with 8–10% defective branches should have high trans-

fection activity, which contradicts the observation that dendrimers synthesized with 8 and 17% NDMA had insufficient defects (Figure 8). This suggests two possibilities. First, degradation may not be a completely random process, so that this synthetic approach may not be appropriate for generating equivalently fractured dendrimers. Alternatively, the general synthetic approach may be valid; however, hydrolysis of amide bonds in fractured dendrimers generates carboxylic acid residues which may form zwitterion pairs with amines, whereas generation of defects by introduction of NDMA does not. Chemical differences between fractured den-

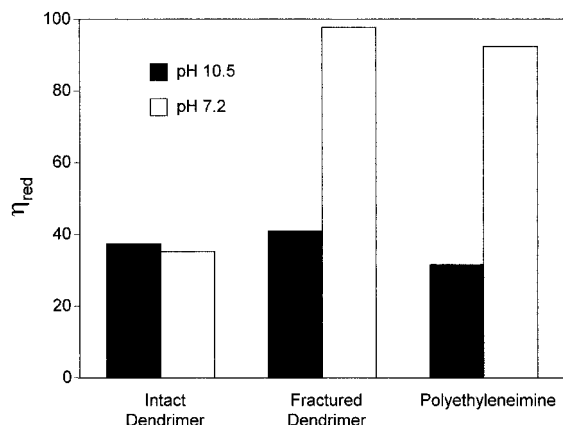


**Figure 11.** Dilute solution viscosity measurements. Reduced viscosity ( $\eta_{red}$ ) was determined as outlined in the Experimental Procedures. By starting from free base (pH 10.5), stock solutions of polymer at concentrations of 50 mg/mL in water were adjusted to acidic pH (pH 7.2) using 3 N HCl. Viscosity measurements of successive dilutions of the polymers in water showed a monotonic increase in  $\eta_{red}$  with decreasing concentration. Data shown are the  $\eta_{red}$  at polymer concentration of 0.5 mg/mL of the intact dendrimer (6-TAEA), fractured dendrimer (6-TAEA heated 43 h in 1-butanol), and polyethyleneimine (Aldrich, 25 kD). The pooled standard deviation for each value is less than 5%.

drimers and the synthetically defective dendrimers may account for different requirements for optimal transfection activity. In fact, viscosity measurements of the intact defective dendrimers in their protonated and deprotonated states shows little change in relative volume (unpublished observations), thus indicating a limited degree of flexibility in these polymers.

We, and others, have previously proposed that transfection obtained with branched cationic polymers is due to the high buffer capacity of the titratable amines present on these polymers which leads to release of the DNA from the endosome (8, 9). Such lysosomotropic agents have been observed to induce osmotic swelling and lysis in response to the pH drop in that compartment (21). In addition, we propose that polymer swelling and collapse facilitates transfection by fractured dendrimers. At neutral pH, electrostatic repulsion between protonated primary amines at the branch termini causes the fractured dendrimer to adopt a fully extended conformation; as shown in Figure 11, the apparent volume even exceeds that of the more sterically constrained intact dendrimer. When the terminal groups are charge neutralized by complexation with DNA, the fractured polymer collapses into a compact form. When taken up in an endosomal compartment of the cell, the interior, tertiary amines become protonated as the pH drops. As charge on the polymer increases, less polymer is required to maintain charge neutralization of the DNA; thus excess polymer is released from the complex. No longer charge-neutralized by DNA, the released polymer swells. Endosomal swelling, due both to polymer expansion and osmotic-induced swelling from protonation of the tertiary amines, causes endosomal rupture and release of the complex or DNA into the cytoplasm. This hypothesis explains the observation that there is a maximum observed in the transfection activity as dendrimer degradation progresses (Figure 2). Intact dendrimers and less-than-optimally fractured dendrimers contain tertiary amines and can thus likewise induce osmotic swelling; however, they are also sterically constrained and, so, do not themselves swell or collapse and are thus less effective in transfection. Dendrimers that have been degraded beyond the

optimum lack both the mass to occupy an effective volume upon protonation and charge density to effectively complex the DNA.

Behr and colleagues have shown that certain polyethyleneimine cationic polymers are also very effective transfection agents and proposed that these polymers facilitated a lysosomotropic mechanism (9). Polyethyleneimines are quite similar to the fractured dendrimers in that they are branched polymers with primary amines at termini and higher order amines at interior branch points. Their buffer capacity and degree of flexibility as manifested by viscosity behavior (Figure 11) are likewise very similar to the fractured dendrimer. Our findings support the lysosomotropic mechanism proposed by Behr and colleagues and make more explicit the potential role of polymer swelling, per se, in the lysis mechanism. Baker and colleagues (22) have recently shown that intact dendrimers, in the presence of DEAE-dextran, can efficiently deliver DNA to cells in culture. The DEAE-dextran is claimed to facilitate both the formation of small particles from the larger dendrimer/DNA aggregates and improve transfection efficiency by several orders of magnitude over the intact dendrimer alone, as assayed by luciferase expression (22). DEAE-dextran is a highly charged, heterodisperse, branched polymer, although without the buffer capacity of the dendrimers. We hypothesize that, in this system, the DEAE-dextran is charge-neutralized and collapsed by the DNA. In the endosome, the dendrimers buffer the pH drop and become protonated, thus allowing the complex to release the DEAE-dextran, which can then swell. Thus, this three-component system (intact dendrimer, DEAE-dextran, and DNA) could act by the same mechanism as the two-component systems of DNA and fractured dendrimer or polyethyleneimine.

In summary, the key physical properties that determine the activity of the cationic polymer systems are (1) high positive charge at physiological pH which facilitates electrostatic interaction with DNA to form complexes, (2) the presence of groups which are titratable in the endosome (pH 5–6), and (3) a highly flexible structure which can collapse and swell. Flexibly branched, cationic polymers provide an effective, reproducible, and relatively nontoxic method for transferring genes into animal cells *in vitro*. They have proven effective in several cell lines that have been typically difficult to transfect (8, 9). Studies of their physical properties described here help elucidate the possible mechanism for DNA delivery to cells and may facilitate the design of other polymers for this purpose.

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**Supporting Information Available:** Details of the synthesis of dendrimers is available in the supplements entitled "I. Preparation of PAMAM Dendrimers around a Core of Tris-(2-aminoethyl)amine" and "II. Procedure for the Synthesis of Partially Defective PAMAM Dendrimers" (21 pages). Ordering information is given on any current masthead page.

## LITERATURE CITED

- (1) Wilson, J. M. (1996) Adenoviruses as gene-delivery vehicles. *New Engl. J. Med.* 334, 1185–1187.
- (2) Behr, J.-P. (1993) Synthetic gene-transfer vectors. *Acc. Chem. Res.* 26, 274–278.
- (3) Wagner, E., and Cotten, M. (1993) Non-viral approaches to gene therapy. *Curr. Opin. Biotechnol.* 4, 705–710.
- (4) Curiel, D. T. (1994) High-efficiency gene transfer mediated by adenovirus-polylysine-DNA complexes. *Ann. N. Y. Acad. Sci.* 716, 36–56.
- (5) Wagner, E., Cotten, M., Foisner, R., and Birnstiel, M. L. (1991) Transferrin-polycation DNA complexes: the effect of polycation on the structure of the complex and DNA delivery to cells. *Proc. Natl. Acad. Sci. U.S.A.* 88, 4255–4259.
- (6) Stankovics, J., Crane, A. M., Andrews, E., Wu, C. H., Wu, G. Y., and Ledley, F. D. (1994) Overexpression of human methylmalonyl CoA mutase in mice after in vivo gene transfer with asialoglycoprotein/polylysine/DNA complexes. *Human Gene Therapy* 5, 1095–104.
- (7) Kabanov, A. V., and Kabanov, V. A. (1995) DNA Complexes with Polycations for the Delivery of Genetic Material into Cells. *Bioconjugate Chem.* 6, 7–20.
- (8) Haensler, J., and Szoka, F. C., Jr. (1993) Functionalization of polyamidoamine cascade polymers and their use for efficient transfection of cells in culture. *Bioconjugate Chem.* 4, 372–379.
- (9) Boussif, O., Lezoualc'h, F., Zanta, M. A., Mergny, M. D., Scherman, D., Demeneix, B., and Behr, J.-P. (1995) A versatile vector for gene and oligonucleotide transfer into cells in culture and in vivo: Polyethyleneimine. *Proc. Natl. Acad. Sci. U.S.A.* 92, 7297–7301.
- (10) Felgner, P. L., Tsai, Y. J., Sukhu, L., Wheeler, C. J., Manthorpe, M., Marshall, J., and Cheng, S. H. (1995) Improved cationic lipid formulations for in vivo gene therapy. *Ann. N. Y. Acad. Sci.* 772, 126–39.
- (11) Tomalia, D. A., Baker, H., DeWald, J., Kallos, G., Martin, S., Roeck, J., Ryder, J., and Smith, P. (1985) A new class of polymers: Starburst-dendritic macromolecules. *Polymer J.* 17, 117–132.
- (12) Tomalia, D. A., Naylor, A. M., and Goddard, W. A. (1990) Starburst dendrimers: Molecular-level control of size, shape, surface chemistry, topology, and flexibility from atoms to macroscopic matter *Angew. Chem., Int. Ed. Engl.* 29, 138–175.
- (13) DeWet, J. R., Wood, K. V., De Luca, M., Helinsky, D. R., and Subramani, S. (1987) Firefly luciferase gene: Structure and expression in mammalian cells. *Mol. Cell Biol.* 7, 725–737.
- (14) McGregor, G. R., and Caskey, C. T. (1989) Construction of plasmids that express *E. coli*  $\beta$ -galactosidase in mammalian cell lines. *Biotechniques* 7, 1116–1122.
- (15) Lovell, P. A. (1995) Dilute solution viscometry. *Comprehensive Polymer Science: The Synthesis, Characterization, Reactions, and Applications of Polymers. Volume 1: Polymer Characterization.* (C. Booth and C. Price, Eds.) pp 173–197, Pergamon Press, New York.
- (16) Moore, S., and Stein, W. H. (1948) Photometric ninhydrin method for use in the chromatography of amino acids. *J. Biol. Chem.* 176, 367–388.
- (17) Bloomfield, V. A. (1991) Condensation of DNA by multivalent cations Considerations on mechanism. *Biopolymers* 31, 1471–1481.
- (18) Laemmli, U. K. (1975) Characterization of DNA condensates induced by poly(ethyleneoxide) and polylysine. *Proc. Natl. Acad. Sci. U.S.A.* 72, 4288–4292.
- (19) Allison, S. A., Easterly, R. A., and Teller, D. C. (1980) The translational friction of toroids. *Biopolymers* 19, 1475–1489.
- (20) Hiemenz, P. C. (1986) *Principles of Colloid and Surface Chemistry*, 2nd ed.; pp 187–198, Marcel Dekker, Inc., New York.
- (21) DeDuve, C., DeBary, T., Poole, B., Trouet, A., Tulken, P., and Van Hoof, F. (1974) Lysosomotropic agents. *Biochem. Pharmacol.* 23, 2495–2531.
- (22) Kukowska-Latallo, J. F., Bielinska, A. U., Johnson, J., Spindler, R., Tomalia, D. A., and Baker, J. R., Jr. (1996) Efficient transfer of genetic material into mammalian cells using Starburst polyamidoamine dendrimers. *Proc. Natl. Acad. Sci. U.S.A.* 93, 4897–4902.

BC9600630



## **Part I:**

### **Preparation of PAMAM Dendrimers around a Core of Tris(2-aminoethyl)amine**

#### **Introduction**

Tomalia and co-workers (2,3,4,5,6) first described what they called "PAMAM starburst™ dendrimers." They told of starting with a central core of ammonia and building outwards. They alternately caused methyl acrylate to react by way of the Michael addition to form poly-methyl esters and then exchanged the methoxyl groups of these esters with a large excess of ethylenediamine, forming poly-N-(2-aminoethyl)-amides. The resultant N-(2-aminoethyl)amides were subsequently carried through the same pair of reactions in sequence until molecules of the desired molecular weight had been obtained.

Inasmuch as tris(2-aminoethyl)amine is commercially available, we felt that employing this material for the core of a PAMAM dendrimer would produce a material with properties very much like those observed for dendrimers built around an ammonia core and at the same time reduce the labor required to attain a given molecular weight product. It is this synthesis which we propose to describe in detail.

We found that the descriptions in Tomalia's papers and patents failed to make us fully aware of two pitfalls in the preparation of monodisperse materials of molecular weights in the 10,000 to 100,000 range. Because the same two sequential reactions are employed repeatedly before the desired molecular weight is achieved, it is extremely important that all residual starting materials be removed after every step of the synthesis, including the earliest ones. Otherwise, residual ethylenediamine will react with the methyl acrylate, and so on, causing lower molecular weight materials with a central core of ethylenediamine to form simultaneously with the desired structures.

For the most of the higher generation polyamides, extended dialysis against deionized water proved adequate for purification. For the very lowest molecular weight polyamides, however, the lack of dialysis membranes having a sufficiently low molecular weight cut-off precluded purification by dialysis. Vacuum evaporation, by itself, removed much of the residual free ethylenediamine. But it was necessary to dry the lower molecular weight polyamides in a vacuum desiccator over concentrated sulfuric acid supported on glass wool to reduce the ethylenediamine level to a value which would afford essentially mono-disperse products at the higher generation numbers built from this material. Vacuum evaporation alone did not suffice to remove all unreacted methyl acrylate from the intermediate polyesters. Additional removal of excess methyl acrylate was achieved by terminal treatment in a vacuum desiccator containing a bed of activated 13X molecular sieves.

As the molecular weight of the dendrimeric polyesters increases, the hydrolytic sensitivity (3, 4) increases. Because of this hydrolytic sensitivity, the intermediate polyesters were not dialyzed at all, dialysis having been reserved for the polyamides. Moreover, when the intermediate polyamides in aqueous solution were lyophilized in a commercially available apparatus with a condenser temperature of  $-52^{\circ}\text{C}$ , the products were not completely dry. Subsequent to our realizing this, all of our lyophilized polyamides were given a terminal drying in a vacuum desiccator over phosphorous pentoxide. After purification in this fashion, all our polyamides above the 2<sup>nd</sup> generation were nearly colorless friable solids.

## Materials and Methods

Tris(2-aminoethyl)amine was Aldrich Chemical's 99% pure material, used without further purification.

Ethylenediamine was J. T. Baker reagent grade. It was dried over activated 3A molecular sieves prior to use.

Methyl acrylate was Aldrich Chemical 99% pure material. It was further purified by passage through a 4 cm. column of J. T. Baker's neutral aluminum oxide powder immediately prior to being added to a reaction mixture.

Dialysis tubing was any of several Spectra/Por membranes manufactured by Spectrum Industries, Inc., and supplied to the laboratory trade by Van Waters and Rogers, Inc.

Glass wool was Pyrex glass wool, obtained from Aldrich Chemical Co.

Activated 3A molecular sieves were 8-12 mesh product supplied by J. T. Baker.

Activated 13X molecular sieves were 1/16 inch pellets supplied by Aldrich Chemical.

Phosphorous pentoxide was Fisher Scientific's certified A.C.S. grade.

Solvents were Fisher HPLC grade.

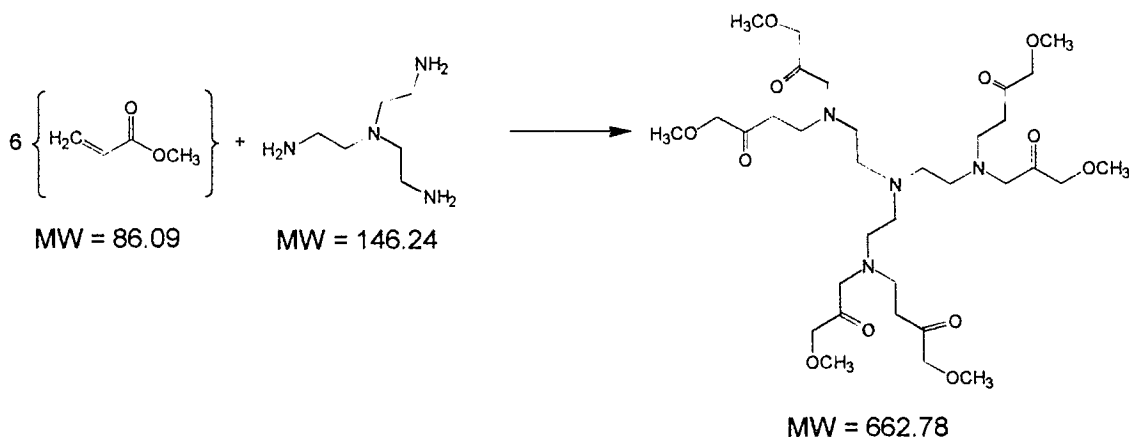
Sulfuric acid was A.C.S. reagent supplied by Fisher Scientific.

The NMR spectra reported were determined on a General Electric QE-300 Fourier-transform instrument.

## Experimental

### A. Preparation of 1<sup>st</sup> Generation Polyester.

Reaction:



Procedure:

Dissolve 7.31 g (50.0 mmol) of tris(2-aminoethyl)amine in 150 ml methanol, cooling in an ice bath to control the heat of solvation. Add thereunto 29.7 ml (330 mmol) of methyl acrylate. Displace the air in the reaction vessel with nitrogen or other inert gas. Stopper and permit to stand at room temperature (23° C) for 53 hours.

Remove the solvent and excess reactants on the rotary evaporator and dry under high vacuum until constant weight is achieved. In this way are obtained 32.55 g of nearly colorless viscous syrup.

Apparent yield: 98%

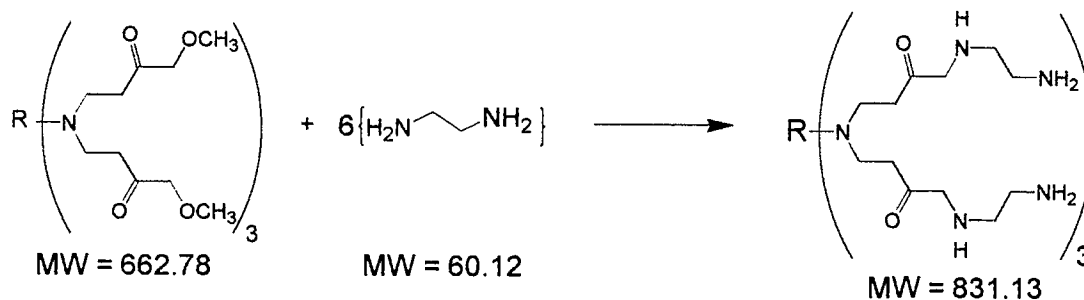
The mass spectrum shows a strong parent ion at  $m/e = 661.2$ .

The calculated  $[M-1]$  for a molecular formula of  $\text{C}_{30}\text{H}_{54}\text{N}_4\text{O}_{12}$  (1) is 661.37.

An NMR spectrum in  $\text{CDCl}_3$  solution showed the expected peaks: a 12 proton triplet at  $\delta = 2.45$  ppm, a 12 proton singlet at  $\delta = 2.5$  ppm, a 12 proton triplet at  $\delta = 2.8$  ppm, and about a 14 proton singlet at  $\delta = 3.7$  ppm (methoxyl). In view of the excellent mass spectrum, the authors tend to minimize the significance of the apparent 20% shortage of methoxyl signal.

B. Preparation of 1<sup>st</sup> Generation Polyamide

Reaction:



Procedure:

Dissolve 586 ml (8.75 mol) of ethylenediamine in 293 ml methanol. Cool the warm solution, first in an ice bath, and then in a dry ice bath, until the temperature falls to  $-30^\circ\text{C}$ .

In another reaction vessel, dissolve 32.19 g (48.6 mmol) of the 1<sup>st</sup> generation polyester in 50 ml methanol. Cool in a dry ice bath and add the cold solution gradually, with stirring, at such a rate that the reaction temperature remains below  $-25^\circ\text{C}$ . Displace the air above the reaction mixture with inert gas and let warm to room temperature spontaneously. Let stand at room temperature ( $23^\circ\text{C}$ ) for 48 hours.

Remove the solvent and excess ethylenediamine under vacuum, with a bath temperature of  $40^\circ\text{C}$ . (The reaction mixture will probably not reach constant weight within a single working day.)

Place the flask and the syrup-like product in a vacuum desiccator with an adjacent Petri dish filled with glass wool moistened with concentrated sulfuric acid. Continue to dry until a moistened strip of wide-range indicator paper suspended above the drying syrup fails to show an alkaline reaction. This may require several days. In this way are obtained 46.77 g of pale amber colored syrup.

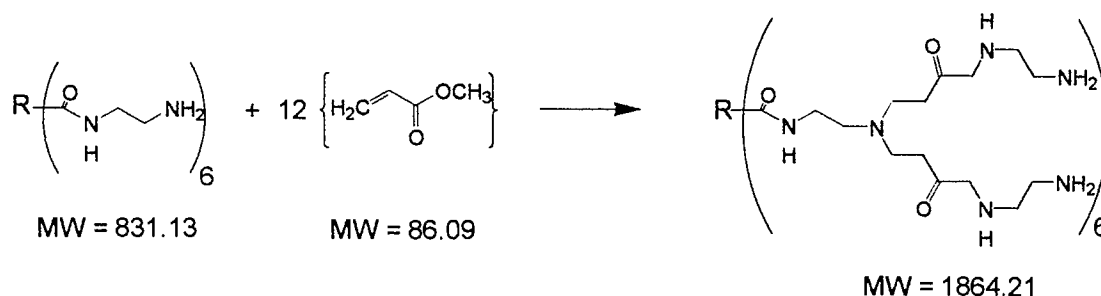
Yield: Greater than quantitative (absorbed solvent?)

Mass spectroscopy showed a strong parent ion at  $m/e = 831.5$ . This agrees with  $[\text{M} + 1]^+$  for a mass number of 830.5. That calculated a molecular formula  $\text{C}_{36}\text{H}_{78}\text{N}_{16}\text{O}_6$  is 830.63 (1).

The NMR spectrum of a sample dissolved in deuterium oxide showed the anticipated peaks at deltas of 2.45, 2.6, 2.7 and 3.25 ppm. The absence of a methoxyl singlet at delta of 3.7 ppm demonstrated that the exchange of methoxyls by ethylenediamine had gone to completion.

### C. Preparation of 2<sup>nd</sup> Generation Polyester.

Reaction:



Procedure.

Dissolve the entire 46.77 g of first generation polyamide resulting from 48.6 mmol of 1<sup>st</sup> generation polyester in 250 ml methanol. Cool to 4°C in a bath of crushed ice. Add dropwise, with stirring, at 4°C, 120 ml (1.33 mol) of methyl acrylate. Displace the air above the reaction mixture with an inert gas. Permit the temperature to rise spontaneously to room temperature. Let stand, stoppered, at room temperature for 20 hours. Warm the reaction mixture in a 50°C water bath for an additional 8 hours.

Evaporate the solvent and excess methyl acrylate on the rotating evaporator under vacuum to obtain 93.55 g of pale amber, very viscous syrup.

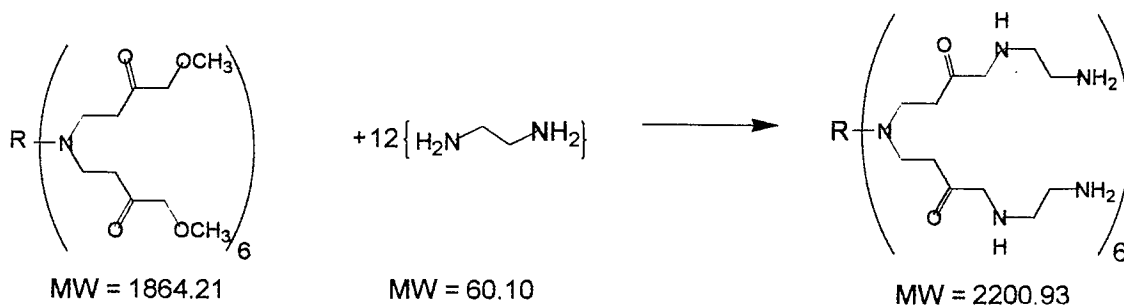
Apparent yield, 103%.

Mass Spectroscopy of this product failed to reveal any meaningful information. This was the case for all the higher generation dendrimers.

Integration of a proton NMR spectrum of a sample dissolved in deuterio-chloroform indicated that the polyester contained about 93% of the theoretical amount of methoxyl groups (Delta = 3.7 ppm(s)). Accordingly, the synthesis is continued with this intermediate. Other, expected, NMR peaks were observed at delta values of 2.36, 2.42, 2.54, 2.77 and 3.27 ppm.

#### D. Preparation of 2<sup>nd</sup> Generation Polyamide

Reaction:



Procedure:

Dissolve 37.28 g (approximately 20.0 mmol) of second generation polyester in 100 ml methanol. Cool in a bath of crushed ice. In another reaction vessel, dissolve 804 ml (12.0 mol) of ethylenediamine in 460 ml methanol. Cool to -25°C. Mix the two solutions slowly, as for the 1<sup>st</sup> generation polyamide. When the addition of reactants is complete, permit the temperature to rise to room temperature (23°C.) Displace the air in the flask with inert gas, stopper it, and let the reaction stand at room temperature for 5 days.

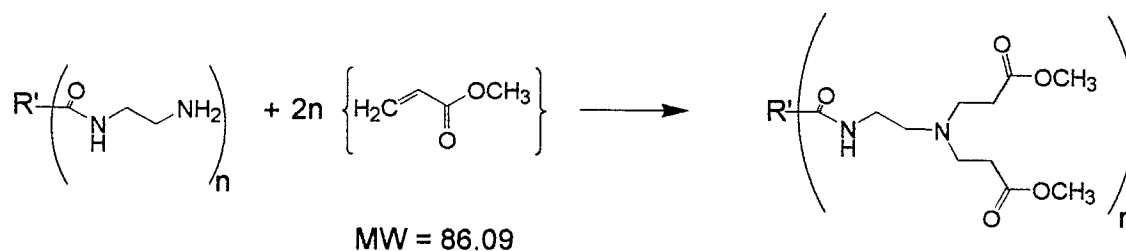
Remove the solvent and unreacted ethylenediamine on the rotating vacuum evaporator, with a bath temperature of 40°C. Dry the remaining viscous, pale amber syrup to constant weight in a vacuum desiccator adjacent to a Petri dish containing concentrated sulfuric acid supported on glass wool. In this way are obtained 46.49 g of highly viscous pale amber glass.

Apparent yield, 106%.

A proton NMR spectrum of the product dissolved in deuterium oxide demonstrated that the methoxyl groups of the 2<sup>nd</sup> generation polyester had completely reacted. The resonance peaks observed were at delta values of 2.4 (t), 2.64 (s), 2.37 (t), 2.83 (t), and 3.3 (t) ppm.

### E. Preparation of 3<sup>rd</sup> and Higher Generation Polyesters

Reaction:



Procedure

Dissolve 45.93 g (20.88 mmol) of 2<sup>nd</sup> generation polyamide in 302 ml methanol. Cool in a bath of crushed ice to 4° C and add slowly, with stirring, 88 ml of methyl acrylate, keeping the temperature at 4° C during the addition. Cover the reaction mixture with inert gas and let it stand at room temperature (23° C) for 20 hours. Then place the reaction vessel in a hot water bath and heat to 60° C for another 24 hours.

Remove the methanol and excess methyl acrylate on a vacuum rotary evaporator. Dry in high vacuum in a desiccator containing a tray of activated 13X molecular sieves to obtain 105.65 g of pale amber, viscous syrup.

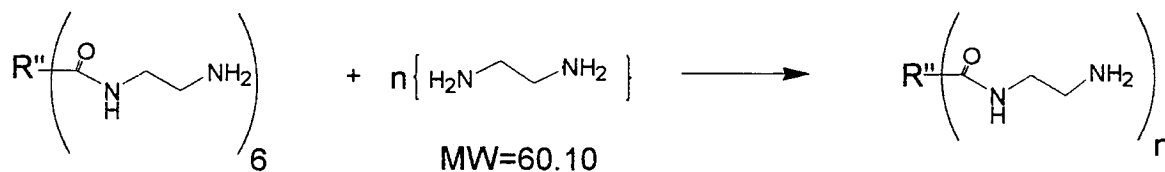
Apparent yield: 119%.

An NMR spectrum of the product dissolved in deuterio-chloroform displayed the anticipated peaks.

The parameters employed in adapting this same procedure to the preparation of the larger numbered generations are shown in Table I.

### F. Preparation of 3<sup>rd</sup> and Higher Generation Polyamides

Reaction:



Preparation of PAMAM Dendrimers Around a Core of Tris(2-aminoethyl)amine  
Redemann, Tang, Szoka

Procedure:

Dissolve 56.17 g (13.2 mol) of 3<sup>rd</sup> generation polyester in 148 ml methanol and cool in an ice bath. In another vessel, dissolve 1190 ml (17.8 mol) of ethylenediamine in 592 ml methanol and cool in an ice bath, followed by a dry ice bath, until the temperature reaches - 20° C. Then add slowly, with stirring and cooling, the methanolic solution of polyester, holding the temperature below - 20° C. When the addition is complete, replace the air in the reaction vessel with an inert gas. Stopper and permit the vessel to warm to room temperature spontaneously. Let stand at room temperature (23° C) for 48 hours.

Remove the solvent and excess ethylenediamine under vacuum, using a bath temperature of 60° C. After prolonged drying in a vacuum desiccator adjacent to a Petri dish supporting glass wool wetted with concentrated sulfuric acid, there will be obtained 75.93 g of a viscous syrup still not free of ethylenediamine.

Dilute this syrup by agitating it with 475 g of crushed ice. When the ice has melted, transfer the resulting solution to MWCO 6,000-8,000 tubing and dialyze against deionised water for 18 hours, changing the water frequently.

Lyophilize to obtain 27.83 g of colorless, friable solid. Dry in vacuum over phosphorous pentoxide to obtain 27.39 g of friable solid.

Yield: 66%. A considerable loss occurred, probably as a result of having used dialysis tubing with a MWCO about twice as large as the molecular weight of the anticipated product.

A proton NMR spectrum of this product dissolved in deuterium oxide displayed no trace of the methoxyl singlet at  $\Delta = 3.7$  ppm, indicating that the reaction had gone to completion. Observed, and expected peaks were at  $\Delta$ s of 2.4, 2.6, 2.8 and 3.3 ppm. These were all rather broad signals, giving little evidence of any order of multiplicity.

Higher generation polyamides may be prepared by the described procedure, using the parameters listed in Table II.



## **Part II:**

### **The Synthesis of Partially Defective PAMAM Dendrimers**

#### **Introduction**

In the original synthesis of PAMAM dendrimers described by Tomalia *et. al* (2-5), an excess of methyl acrylate is caused to enter into a Michael addition to a central primary amine-bearing core to form a considerably branched polyester. This polyester is subsequently treated with a large excess of ethylenediamine, displacing the methoxyl groups of the polyester to form poly-N-(2-aminoethyl) amides. The terminal amino groups of polyamides are subsequently caused to react with more methyl acrylate, and the whole process is repeated as many times as may be necessary to achieve a product of the desired molecular weight. Each pair of such reactions is termed a "generation".

If, along the way, either the Michael addition or the ester-displacement reaction are interrupted by introduction of a suitable competing reactant, intentionally defective dendrimers may be formed. If the core polyamine should be exposed to a suitable amount of N, N-dimethylacrylamide, instead of methyl acrylate, those terminal amino groups which react with the N, N-dimethylacrylamide will be prevented from entering into the branch formation through the methyl acrylate, ethylenediamine, methyl acrylate, ethylenediamine, etc., sequence. Such an interruption would provide what we will hereafter call an "ester-defective" dendrimer.

If one of the polyesters is caused to react with a suitable amount of a strong mono-amine, such as ethanolamine, those ester groups which have reacted with the mono-amine will be blocked from later entering into further branch formation through the ethylenediamine, methyl acrylate, ethylenediamine, methyl acrylate, etc., sequence. Such an interruption would provide what we hereafter will call an "amine-defective" dendrimer.

Although these defects could theoretically be introduced at any generation level during a dendrimer synthesis, we chose to introduce the ester defect at the level of the reaction immediately following the preparation of the first generation dendrimeric polyamide. This first generation polyamide resulted from the condensation of methyl acrylate with tris(2-aminoethyl)amine.

According to theory, each mole of this first generation dendrimer should be capable of reacting with 12 moles of methyl acrylate, as in Part I, section C. If each mole be first heated with 1 mole of N, N-dimethylacrylamide, then the reaction product will be unable to react with 1/12 of the potential theoretical amount of methyl acrylate in the subsequent reaction step. The product will be, roughly, 8% (1/12) ester-defective. A similar blocking step is later introduced immediately before each of the subsequent methyl acrylate reactions comprising the dendrimer synthesis.

The amine-defective step is first introduced at the level of the second generation polyester. Each mole of second generation polyester should theoretically be capable of reacting with 12 moles of ethylenediamine, as in Part I, section D.

If one mole of ethanolamine be heated with one mole of the polyester for a suitable time, the ethanolamine will displace 1/12 of the total number of methoxyl groups, rendering the dendrimeric polyester capable of reacting later with only 11/12 of the theoretical amount of ethylenediamine. The resulting dendrimer will, accordingly, be 8% amine-defective. A similar preliminary treatment with ethanolamine is used subsequently immediately before each of the reactions between polyester and ethylenediamine. Detailed procedures will be presented for each of these initial defect formation reactions.

## Material and Methods

Methanol was reagent grade solvent, used without further treatment.

Ethanolamine was Aldrich best grade, used without further treatment.

Ethylenediamine was Aldrich 99% material dried over activated 3A molecular sieves.

Both methyl acrylate and N, N-dimethylacrylamide were Aldrich material. Each was freed from inhibitory phenols by passage through a short column of neutral alumina immediately prior to use in the reactions.

NMR spectra were run on a G.E. QE-300 Fourier transform instrument.

## Experimental

### A. Preparation of "8% Ester-Defective" Second Generation Polyester

Dissolve 4.16 g (5.0 mmol) of the first generation dendrimer in 20 ml methanol. Cool the resulting solution in an ice bath and add 515 microliters (495 mg or 5.0 mmol) of N, N-dimethylacrylamide. Displace the air in the reaction vessel with nitrogen. Cap the vessel and heat in a 55°C water bath for 18 hour.

Cool the reaction mixture to 4°C in an ice bath. Add slowly, with agitation, 9.0 ml (100 mmol) of methyl acrylate. Let warm to room temperature. Displace the air in the flask with nitrogen, stopper the flask and let stand at room temperature for 18 hours.

Transfer the flask to a 55°C water bath and heat for another 24 hours.

Vacuum evaporate the resulting product to obtain 10.60 g of viscous amber syrup. On prolonged drying in a high vacuum desiccator containing activated 13 Å molecular sieves, the yield of "8% ester-defective" 2<sup>nd</sup> generation polyester will fall to about 10.39 g.

Apparent yield: 123%

A proton NMR spectrum of the product dissolved in deuterio-chloroform reveals two new singlets at  $\delta = 2.95$  and  $\delta = 3.05$  ppm., strong enough to account for almost 1/12 of the possible terminal methoxyl groups having been replaced with N,N-dimethylamino groups.

Figure 3 shows a comparison between the proton NMR spectrum for the 2<sup>nd</sup> generation "8% ester-defective" polyester and that of a 2<sup>nd</sup> generation non-defective polyester.

Parameters utilized for carrying the "8% ester-defective" dendrimers into higher generations than the second are listed in Tables III and IV.

#### B. Preparation of 2<sup>nd</sup> Generation "8% Amine Defective" Polyamide.

Dissolve 3.73 g (2.0 mmol) of 2<sup>nd</sup> generation polyester in 10 ml methanol. Add to this slowly, with stirring, 121  $\mu$ l (2.0 mmol) of ethanolamine.

Evaporate most of the solvent under reduced pressure. Displace the air in the reaction vessel with nitrogen and stopper. Place the vessel in a 55°C water bath for 18 hours. Cool to room temperature and dissolve in 10 ml methanol.

In another flask, dissolve 81 ml (1.20 mol) of ethylenediamine in 46 ml methanol. Cool to -25° C in a dry ice bath. To the resulting solution, add dropwise, with stirring, the methanolic solution of the ethanolamine reaction mixture, keeping the temperature below -25° C during the addition.

Displace the air in the reaction vessel with nitrogen, stopper, and permit to warm to room temperature for 21 hours.

Remove the solvent and excess ethylenediamine under reduced pressure to obtain 6.68 g of viscous, pale amber syrup.

Dissolve the syrup by agitating it with 120 g crushed ice. Transfer the solution to MWCO = 3500 dialysis tube. Dialyze against deionised water for 20 hours, changing the outer bath frequently until it was no longer strongly alkaline to wide-range indicator paper.

Centrifuge the contents of the dialysis tubing to remove suspended particles of molecular sieves. Lyophilize the clarified liquid to obtain 2.88 g of pale tan friable solid.

Yield: 65%

A proton NMR spectrum of the dialyzed product dissolved in deuterium oxide displays a new triplet resonance peak at  $\delta = 3.65$  ppm. This is attributable to the pair of protons immediately adjacent to the terminal hydroxyl of the ethanolamine. Integration peak areas suggest that about 8% of the C-CH<sub>2</sub>-CO-O functions have become C-CH<sub>2</sub>CO-NH-CH<sub>2</sub>-CH<sub>2</sub>-OH functions, rather than C-CH<sub>2</sub>-CONH-CH<sub>2</sub>-CH<sub>2</sub>-NH<sub>2</sub> functions.

Figure 4 shows a comparison between the proton NMR spectrum for the 2<sup>nd</sup> generation "8% amine-defective" polyamide and that of a 2<sup>nd</sup> generation non-defective polyamide.

The higher generation "8% amine-defective" polyamides are produced in a similar manner from the corresponding generation polyesters, using the parameters listed in Tables V and VI.

The higher generation "8% amine-defective" polyesters are prepared from the next lower generation polyamides, as already described in Part I for the preparation of non-defective polyesters.

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**References:**

1. Koenig, L.A., Mattauch, J.H.E., and Wapstra, A.H. "1961 Nuclidic Mass Table." *Nuclear Physics*, **31**, 18-42 (1962)
2. Naylor, A.M., Goddard, W.A., Kiefer, G.E. and Tomalia, D.A. "Starburst Dendrimers 5. Molecular Shape Control." *JACS*, **111**, 2339 (1989)
3. Tomalia, D.A., Baker, H., Dewald, J., Hall, M., Kallos, G. Martin, S., Roeck, J., Ryder, J., and Smith, P. "A New Class of Polymers: Starburst Dendritic Macromolecules." *Polymer Journal*, **17**, 117-132 (1985)
4. Tomalia, D.A., Baker, H., Dewald, J., Hall, M., Kallos, G. Martin, S., Roeck, J., Ryder, J., and Smith, P. "'Dendritic Macromolecules' Synthesis of Starburst Dendrimers." *Macromolecules*, **19**, 2466-246 (1985).
5. Tomalia, D.A., Hall, B.M., and Hedstrand, D.M. "'Starburst Dendrimers 4. Covalently fixed Unimolecular Assemblages Reminiscent of Spheroidal Micelles." *Macromolecules*, **20**, 1164-1167 (1987).
6. Tomalia, D.A., Naylor, A.M., and Goddard, W.A. U.S. Patent 4,410,688 (1983)

**Table I: Parameters for Preparation of Higher Generation Poly-esters**

| Gen of Poly-ester | Poly-amide used | Poly-amide (g) | Poly-amide (mmol) | MeOH (ml) | Methyl Acrylate (ml) | Methyl Acrylate (mmol) | Time at Room T (h) | Time at 60° C (h) | Yield (g) | % Yield |
|-------------------|-----------------|----------------|-------------------|-----------|----------------------|------------------------|--------------------|-------------------|-----------|---------|
| 4 <sup>th</sup>   | 3 <sup>rd</sup> | 23.85          | 4.83              | 118       | 43                   | 477                    | 24                 | 48                | 44.03     | 100.5   |
| 5 <sup>th</sup>   | 4 <sup>th</sup> | 25.0           | 2.40              | 250       | 45                   | 500                    | 16                 | 24                | 42.39     | 95      |
| 6 <sup>th</sup>   | 5 <sup>th</sup> | 14.26          | 0.667             | 143       | 23                   | 255                    | 20                 | 20                | 24.5      | 97      |

**Table II: Parameters for Preparation of Higher Generation Poly-amides**

| Gen of Poly-Amide | Poly-ester used | Poly-ester (g) | Poly-ester (mmol) | MeOH (ml)   | EDA (ml) | EDA (mol) | Time at Room T (h) | Crushed Ice (g) | MWCO Dialysis Tubing | Yield (g) | % Yield |
|-------------------|-----------------|----------------|-------------------|-------------|----------|-----------|--------------------|-----------------|----------------------|-----------|---------|
| 4 <sup>th</sup>   | 4 <sup>th</sup> | 41.94          | 4.62              | 434<br>62   | 751      | 11.2      | 18                 | 400             | 12,000<br>14,000     | 36.71     | 76      |
| 5 <sup>th</sup>   | 5 <sup>th</sup> | 41.0           | 2.19              | 1034<br>120 | 2,128    | 31.8      | 18                 | 500             | 25,000               | 42.56     | 91      |
| 6 <sup>th</sup>   | 6 <sup>th</sup> | 24.0           | 0.633             | 700<br>210  | 1,220    | 18.2      | 42                 | 450             | 50,000               | 22.895    | 84      |

**Table I: Parameters for Preparation of Higher Generation Polyesters**

| Gen of Poly-ester | Poly-amide used | Poly-amide (g) | Poly-amide (mmol) | MeOH (ml) | Methyl Acrylate (ml) | Methyl Acrylate (mmol) | Time at Room T (h) | Time at 60° C (h) | Yield (g) | % Yield |
|-------------------|-----------------|----------------|-------------------|-----------|----------------------|------------------------|--------------------|-------------------|-----------|---------|
| 4 <sup>th</sup>   | 3 <sup>rd</sup> | 23.85          | 4.83              | 118       | 43                   | 477                    | 24                 | 48                | 44.03     | 100.5   |
| 5 <sup>th</sup>   | 4 <sup>th</sup> | 25.0           | 2.40              | 250       | 45                   | 500                    | 16                 | 24                | 42.39     | 95      |
| 6 <sup>th</sup>   | 5 <sup>th</sup> | 14.26          | 0.667             | 143       | 23                   | 255                    | 20                 | 20                | 24.5      | 97      |



**Table II: Parameters for Preparation of Higher Generation Polyamides**

| Gen of Poly-Amide | Poly-ester used | Poly-ester (g) | Poly-ester (mmol) | MeOH (ml)   | EDA (ml) | EDA (mol) | Time at Room T (h) | Crushed Ice (g) | MWCO Dialysis Tubing | Yield (g) | % Yield |
|-------------------|-----------------|----------------|-------------------|-------------|----------|-----------|--------------------|-----------------|----------------------|-----------|---------|
| 4 <sup>th</sup>   | 4 <sup>th</sup> | 41.94          | 4.62              | 434<br>62   | 751      | 11.2      | 18                 | 400             | 12,000<br>14,000     | 36.71     | 76      |
| 5 <sup>th</sup>   | 5 <sup>th</sup> | 41.0           | 2.19              | 1034<br>120 | 2,128    | 31.8      | 18                 | 500             | 25,000               | 42.56     | 91      |
| 6 <sup>th</sup>   | 6 <sup>th</sup> | 24.0           | 0.633             | 700<br>210  | 1,220    | 18.2      | 42                 | 450             | 50,000               | 22.895    | 84      |

**Table III: Reaction Parameters for Preparation of Higher Generation "8% Ester-defective" Polyesters**

| Generation # | Gen Poly Amide Used | Poly-Amide (g)       | N, N-di-methyl acrylamide (ml) | MeOH (ml) | Time at 55°C (h) | Methyl Acrylate (ml) | Time at Room T (h) | Time at 55°C (h) | Yield of Product (g) | Apparent Yield % |
|--------------|---------------------|----------------------|--------------------------------|-----------|------------------|----------------------|--------------------|------------------|----------------------|------------------|
| 2nd          | 1st                 | 41.6<br>(5.0 mmol)   | 0.515<br>(5.0 mmol)            | 20        | 18               | 9.0<br>(100 mmol)    | 18                 | 24               | 10.39                | 111              |
| 3rd          | 2nd                 | 8.60<br>(3.9 mmol)   | 0.804<br>(7.8 mmol)            | 43        | 20               | 9.3<br>(103 mmol)    | 24                 | 72               | 15.29                | 92               |
| 4th          | 3rd                 | 9.91<br>(2.0 mmol)   | 0.824<br>(8.0 mmol)            | 40        | 20               | 17.3<br>(192 mmol)   | 72                 | 22               | 17.50                | 96               |
| 5th          | 4th                 | 10.34<br>(0.99 mmol) | 0.816<br>(7.92 mmol)           | 100       | 24               | 18.0<br>(200 mmol)   | 24                 | 24               | 17.12                | 93               |
| 6th          | 5th                 | 9.12<br>(0.427 mmol) | 0.704<br>(6.83 mmol)           | 45        | 96               | 15.0<br>(166 mmol)   | 24                 | 24               | 15.52                | 96               |

**Table IV: Reaction Parameters for Preparation of Higher Generation  
"8% Ester-defective" Polyamides**

| Generation # | Gen Poly-Ester | Poly-Ester (g)        | MeOH (ml) | EDA (ml) | EDA (mol) | Time at Room Temp (h) | Crushed Ice (g) | MWCO of Dialysis Tubing | Yield of Product (g) | Apparent Yield % |
|--------------|----------------|-----------------------|-----------|----------|-----------|-----------------------|-----------------|-------------------------|----------------------|------------------|
| 2nd          | 2nd            | 10.39<br>(5.57 mmol)  | 175       | 201      | 3.0       | 21                    | 150             | 3,500                   | 9.38                 | 76               |
| 3rd          | 3rd            | 14.55<br>(3.41 mmol)  | 198       | 307      | 4.58      | 48                    | 200             | 6,000-8,000             | 11.07                | 66               |
| 4th          | 4th            | 17.01<br>(1.87 mmol)  | 201       | 304      | 4.53      | 42                    | 240             | 12,000-14,000           | 13.98                | 72               |
| 5th          | 5th            | 12.55<br>(0.672 mmol) | 340       | 650      | 9.7       | 96                    | 200             | 25,000                  | 13.21                | 92               |
| 6th          | 6th            | 12.73<br>(0.336 mmol) | 482       | 647      | 9.66      | 96                    | 200             | 50,000                  | 13.000               | 89               |

**Table V: Reaction Parameters for Preparation of Higher Generation "8% Amine-defective" Polyesters**

| Generation # | Gen Poly-Amide | Poly-Amide (g)       | MeOH (ml) | Methyl Acrylate (ml) | Time at Room Temp (h) | Time at 55° C (hrs) | Yield of Poly-Ester (g) | Apparent Yield % |
|--------------|----------------|----------------------|-----------|----------------------|-----------------------|---------------------|-------------------------|------------------|
| 3rd          | 2nd            | 2.79<br>(1.27 mmol)  | 18        | 5.3<br>(58.9 mmol)   | 20                    | 24                  | 4.97                    | 91               |
| 4th          | 3rd            | 4.37<br>(0.88 mmol)  | 22        | 7.8<br>(86.7 mmol)   | 18                    | 24                  | 7.74                    | 97               |
| 5th          | 4th            | 7.52<br>(0.722 mmol) | 100       | 13.5<br>(150 mmol)   | 18                    | 24                  | 12.49                   | 93               |
| 6th          | 5th            | 8.22<br>(0.38 mmol)  | 40        | 13.5<br>(150 mmol)   | 20                    | 24                  | 14.31                   | 99               |

**Table VI: Reaction Parameters for Preparation of Higher Generation "8% Amine-defective" Polyamides**

| Generation # | Gen Poly-Ester | Poly-Ester (g)        | MeOH (ml) | Ethanol-Amine (ml)   | MeOH with EDA (ml) | EDA (ml)            | Time with EDA (h) | MVCO of Dialysis Tubing | Yield of Product (g) | Apparent Yield % |
|--------------|----------------|-----------------------|-----------|----------------------|--------------------|---------------------|-------------------|-------------------------|----------------------|------------------|
| 2nd          | 2nd            | 3.73<br>(2.0 mmol)    | 0.0       | 0.121<br>(2.0 mmol)  | 56                 | 81<br>(1.20 moles)  | 18                | 3,500                   | 2.88                 | 65               |
| 3rd          | 3rd            | 4.93<br>(1.2 mmol)    | 10.0      | 0.139<br>(2.3 mmol)  | 65                 | 134<br>(2.0 moles)  | 22                | 6,000-8,000             | 4.57                 | 77               |
| 4th          | 4th            | 7.74<br>(0.853 mmol)  | 20.0      | 0.206<br>(3.41 mmol) | 80                 | 139<br>(2.07 moles) | 18                | 12,000-14,000           | 7.79                 | 88               |
| 5th          | 5th            | 11.96<br>(0.640 mmol) | 25        | 0.622<br>(10.3 mmol) | 302                | 622<br>(9.28 Moles) | 20                | 25,000                  | 11.98                | 88               |
| 6th          | 6th            | 13.98<br>(0.369 mmol) | 30        | 0.354<br>(5.9 mmol)  | 410                | 710<br>(10.6 moles) | 96                | 50,000                  | 13.97                | 88               |

# Incorporation of Two Anthraquinonylmethyl Groups into the 2'-O-Positions of Oligonucleotides: Increased Affinity and Sequence Specificity of Anthraquinone-Modified Oligonucleotides in Hybrid Formation with DNA and RNA

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Oligonucleotide 15-mers containing one or two anthraquinonylmethyl groups at specified sugar residues have been prepared on an automated DNA/RNA synthesizer by using 5'-O-dimethoxytrityl 2'-O-(2-anthraquinonylmethyl)uridine 3'-O-(2-cyanoethyl)-*N,N*-diisopropylphosphoramidite. The purification of the modified oligonucleotides was done with denaturing polyacrylamide gel electrophoresis. The base compositions and the presence of anthraquinone group(s) in the oligonucleotides were verified with enzymatic digestion (snake venom phosphodiesterase and alkaline phosphatase) analysis and UV-vis spectral measurements. The UV melting behaviors indicate that all the oligonucleotides with anthraquinone group(s) can bind to both their complementary DNA and RNA in a manner similar to that of the unmodified oligonucleotide. All the oligonucleotides possessing anthraquinone group(s) have higher affinity for both DNA and RNA segments when compared with the unmodified oligonucleotide. The oligomer containing two anthraquinone substituents at sites separated by four nucleotides instead of six exhibits the highest affinity for both the complementary DNA and RNA. The stabilizing effect can be translated into a free energy cost of 7.1 kcal/mol for the DNA hybrid and 3.6 kcal/mol for RNA. It has been shown through mismatch/*T<sub>m</sub>* studies that modification of the oligonucleotide by anthraquinone groups does not alter the sequence specificity in binding to a RNA segment.

## INTRODUCTION

Increasing the affinity of oligonucleotides for DNA/RNA segments while maintaining the sequence specificity for hybridization has been the subject of intense research, since it is one of the important requirements for antisense oligonucleotide probes (1). One possible approach has been demonstrated by oligonucleotides carrying a pendant intercalating group which is non-specific for interaction with nucleic acids and provides an additional energy for stabilizing duplexes (2). Other approaches involve modification at the 2'-position by alkyl, allyl, and fluoro groups in which an increase in the stability of DNA-RNA duplexes was observed, depending upon the size and number of the substituents (3). Several other modifications of oligonucleotides (4) have been shown to be useful for increasing affinity of oligonucleotide derivatives for complementary nucleic acid sequences.

We have recently developed a new method for incorporation of intercalating polyaromatic fragments via a relatively short linker into the 2'-O-position of oligonucleotides (5). The attractive features of our method are that (i) more than two intercalating fragments may be easily attached and (ii) the incorporated polyaromatic fragments can be placed anywhere in the sequence. We and others have already shown that the duplexes of self-complementary oligonucleotides containing one anthraquinone group bound to a sugar residue have increased thermal stability owing to intercalation (5a, 6). However, no detailed effects of a 2'-anthraquinone substitution on the stability of duplexes formed with DNA and more importantly with RNA have yet been evaluated.

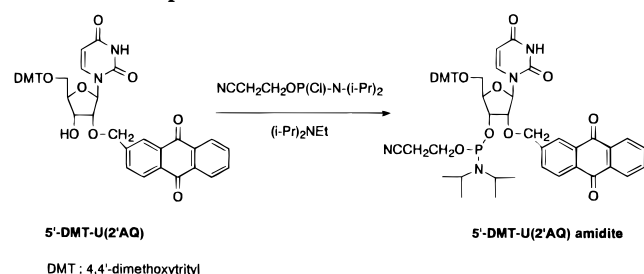
In this paper, we describe the synthesis of oligonucleotides containing two anthraquinonylmethyl groups at 2'-O-positions and focus on their binding to RNA as well as to DNA. Important findings of this research are that the oligonucleotides doubly modified with anthraquinone groups at two different sugar residues have greatly increased affinity for complementary RNA (DNA) segments without loss of sequence specificity. A similar effect in hybrid stability was not found in our present system when a 2'-O-methyl substitution at the same sites was employed.

## EXPERIMENTAL PROCEDURES

**General Methods.** <sup>31</sup>P NMR spectra were measured on a JEOL-JNM-GX-400 spectrometer using 85% H<sub>3</sub>PO<sub>4</sub> as an external standard. High-performance liquid chromatography (HPLC) was performed on a Waters 600E model apparatus equipped with a Hitachi LC 4200 UV-VIS detector at 254 nm, using a reversed phase YMC C<sub>18</sub> column (6 × 150 mm). Column chromatography and thin-layer chromatography (TLC) were carried out on Wako silica gel C-200 and Merck 60 PF<sub>254</sub>, respectively. Ultraviolet (UV) spectra were recorded with a Hitachi UV-3000 spectrophotometer equipped with a thermoelectrically controlled cell holder. Circular dichroism (CD) spectra were obtained on a JASCO CD J-720 spectrophotometer.

**Materials and Solvents.** 5'-O-Dimethoxytrityl 2'-O-(2-anthraquinonylmethyl)uridine [5'-DMT-U(2'AQ)] was synthesized in a manner previously described (5a). 5'-O-Dimethoxytrityl 2'-O-methyluridine was synthesized by published methods (3a). 2-Cyanoethyl diisopropylchlorophosphoramidite was obtained from Aldrich Chemical Co. Protected deoxy- and ribonucleoside 3'-O-(2-cyanoethyl)-*N,N*-diisopropylphosphoramidites and nucleoside-loaded controlled pore glass (CPG) supports were

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**Scheme 1. Synthesis of an Anthraquinone-Modified Uridine Phosphoramidite**


purchased from Cruachem. DNA and RNA oligomers were prepared by a standard phosphoramidite chemistry (7) on a Pharmacia LKB Gene Assembler Plus DNA/RNA synthesizer. For synthesis of the modified oligonucleotides, the X-bottle was used to supply the modified nucleoside amidite solutions. Snake venom phosphodiesterase (Sigma) and alkaline phosphatase (Funakoshi) were commercially obtained. Dichloromethane, diisopropylethylamine, and acetonitrile were dried by refluxing the  $\text{CaH}_2$  for at least 5 h under a nitrogen atmosphere, then distilled, and stored over  $\text{CaH}_2$  or molecular sieves.

**Synthesis of 5'-O-Dimethoxytrityl 2'-O-(2-Anthraquinonylmethyl)uridine 3'-O-(2-Cyanoethyl)-N,N-diisopropylphosphoramidite [5'-DMT-U(2' AQ) Amidite].** To a solution of 5'-DMT-U(2' AQ) (234 mg, 0.31 mmol) in dry dichloromethane (2.4 mL) containing dry diisopropylethylamine (0.24 mL) was added 2-cyanoethyl diisopropylchlorophosphoramidite (0.12 mL, 0.6 mmol). The solution was stirred at room temperature for 60 min. Methanol (1 mL) was added to the solution followed by diisopropylethylamine (0.2 mL) in ethyl acetate (5 mL). The solution was washed with 10%  $\text{NaHCO}_3$  in water (2 mL  $\times$  2). The organic phase was separated and dried over  $\text{Na}_2\text{SO}_4$  and then directly applied to a silica gel column (2.5  $\times$  30 cm). Elution was carried out with dichloromethane/ethyl acetate/triethylamine (45:45:10, v/v). The appropriate fractions were collected, and solvent was removed under reduced pressure to give a pale yellow powder (266 mg, 89%). 5'-DMT-U(2' AQ) amidite: TLC (45:45:10,  $\text{CH}_2\text{Cl}_2/\text{EtOAc}/\text{Et}_3\text{N}$ , v/v)  $R_f$  0.68;  $^{31}\text{P}$  NMR ( $\text{CH}_3\text{CN}$ ) 149.4, 149.9 ppm (diastereoisomers).

**Synthesis of Anthraquinone-Modified Oligonucleotides.** The synthesis of anthraquinone-modified oligonucleotides was accomplished by phosphoramidite chemistry (7), beginning with 5'-DMT-T (0.2  $\mu\text{mol}$ ) bound to a CPG support. For the coupling of normal deoxyribonucleoside phosphoramidites, the standard protocol (50  $\mu\text{L}$  of 0.1 M amidite and 50  $\mu\text{L}$  of 0.1 M tetrazole in acetonitrile, 2 min) was used. For the coupling of 5'-DMT-U(2' AQ) amidite, 120  $\mu\text{L}$  of the 0.13 M amidite and 120  $\mu\text{L}$  of 0.1 M tetrazole in acetonitrile (1.5 min) were used. With these conditions, the average coupling efficiency based on the DMT cation assay was 98% for each step in the oligonucleotide synthesis. The CPG-bound oligonucleotides were treated with concentrated ammonium hydroxide at 55  $^\circ\text{C}$  for 14 h.

Purification of the modified oligonucleotides was performed with denaturing 20% polyacrylamide gel (12  $\times$  12 cm, 1.5 mm thickness) electrophoresis (DPAGE) run at 350 V for 4 h. The oligonucleotides were visualized by UV shadowing and eluted from crushed gel slices by incubation at 37  $^\circ\text{C}$  in 0.5 M  $\text{NH}_4\text{OAc}$  and 1 mM EDTA for 15 h. The eluent was passed through a Sep-Pak  $\text{C}_{18}$  cartridge prewashed with  $\text{CH}_3\text{CN}$  and water (10 mL each). The Sep-Pak was then sequentially eluted with 10 mL of aqueous  $\text{NH}_4\text{OAc}$ , 10 mL of water, and 3 mL of

40%  $\text{CH}_3\text{CN}$  in water. The purified oligomers (5–10  $A_{260}$  units) were obtained by lyophilization.

**Synthesis of 2'-O-Methyl Oligonucleotides.** 5'-O-Dimethoxytrityl 2'-O-methyluridine 3'-O-(2-cyanoethyl)-N,N-diisopropylphosphoramidite [5'-DMT-U(2Me) amidite] was prepared by the same method as described for 5'-O-DMT-U(2' AQ) amidite. The synthesis of oligonucleotides having 2'-O-methyl groups at the sugar residues has been performed by using 5'-DMT-U(2' Me) amidite (100  $\mu\text{L}$  of a 0.1 M solution in acetonitrile and 100  $\mu\text{L}$  of 0.1 M tetrazole in acetonitrile, 5 min) and normal deoxyribonucleotide phosphoramidites (standard protocol described above) on a 0.2  $\mu\text{mol}$  scale synthesis. The average coupling efficiency exceeded 98% on the basis of a DMT cation assay. Deprotection and purification were carried out employing the same procedures described for the anthraquinone oligonucleotides.

**Enzymatic Digestion Analysis.** The modified oligonucleotides (0.2  $A_{260}$  unit) were subjected to digestion with snake venom phosphodiesterase (10 units/mL) and alkaline phosphatase (100 units/mL) in 50  $\mu\text{L}$  of 50 mM Tris-HCl buffer (pH 7.2) containing 10 mM  $\text{MgCl}_2$  at 37  $^\circ\text{C}$  for 1 h. The reaction mixtures were analyzed by reversed phase HPLC; elution was carried out with (i) 5%  $\text{CH}_3\text{CN}$  (5 min), (ii) a linear gradient of  $\text{CH}_3\text{CN}$  (0.67%/min, 5–35 min), and (iii) a linear gradient of  $\text{CH}_3\text{CN}$  (1%/min, 35–80 min) in triethylammonium acetate (pH 7.0) each at a flow rate of 1 mL/min. The calculations of nucleoside composition were done on the basis of peak integration of the HPLC profile by using analytical data obtained with a prepared mixture containing equimolar amounts of dC, dG, T, dA, and U(2' AQ) as a standard.

**Preparation of Oligonucleotide Solutions for Physical Measurements.** All solutions were prepared using a buffer containing 10 mM sodium phosphate and 100 mM NaCl, adjusted to pH 7.0. For preparation of RNA-containing solutions, the buffer was sterilized before use. Oligonucleotide concentrations were determined by absorbance at 260 nm and the calculated single-strand extinction coefficients based on a nearest neighbor model (8).

Except for thermodynamic studies, all duplex melting curves by UV spectra were measured at a common total single-strand concentration ( $4.3 \times 10^{-5}$  M) containing a 1:1 molar ratio of oligonucleotides with an increase in temperature from 0 to 80  $^\circ\text{C}$  at a rate of 0.5  $^\circ\text{C}/\text{min}$ . The solutions were heated to 80  $^\circ\text{C}$ , kept there for 5 min, and then gradually cooled before melting experiments. The thermodynamic data for duplex formation were obtained from UV melting curves by plotting the inverse melting temperature versus  $\ln C/4$ , where  $C$  is the total single-strand concentration (9). CD spectra were measured for the same solutions used for the UV melting studies at 23  $^\circ\text{C}$ .

## RESULTS

**Synthesis of Anthraquinone-Modified Oligonucleotides.** The synthesis of 2'-O-(anthraquinonylmethyl)uridine [U(2' AQ)] was established with a modified procedure reported by Moffat et al. (10). This procedure has also been effective for the synthesis of an anthraquinone-modified adenosine derivative (6). U(2' AQ) was converted by the usual method to a protected phosphoramidite which could be used for introduction of U(2' AQ) into the sequences of oligomer(AQ) 1–5. The oligonucleotides containing one or two anthraquinone groups were synthesized by the fully automated solid phase phosphoramidite methods. Efficient coupling (98%) of DMT-U(2' AQ) amidite was achieved when a large

**Table 1. Sequences, Abbreviations, Enzymatic Digestion Analysis Data, and UV Data for Anthraquinone-Modified Oligonucleotides<sup>a</sup>**

| abbreviation   | sequence                       | nucleoside |      |      |      |       | ratio of A <sub>340</sub> /A <sub>260</sub> |
|----------------|--------------------------------|------------|------|------|------|-------|---------------------------------------------|
|                |                                | dC         | dG   | T    | dA   | U(AQ) |                                             |
| oligomer(AQ) 1 | 5'-ACAU(2'AQ)GCAGTGTGAT        | 2.00       | 4.05 | 3.78 | 4.16 | 1.05  | 0.028                                       |
| oligomer(AQ) 2 | 5'-ACATGCAGU(2'AQ)GTTGAT       | 2.00       | 4.11 | 4.50 | 3.97 | 0.93  | 0.029                                       |
| oligomer(AQ) 3 | 5'-ACATGCAGTGU(2'AQ)TGAT       | 2.00       | 4.31 | 4.65 | 4.01 | 1.00  | 0.025                                       |
| oligomer(AQ) 4 | 5'-ACAU(2'AQ)GCAGU(2'AQ)GTTGAT | 2.00       | 3.61 | 3.21 | 3.95 | 1.82  | 0.043                                       |
| oligomer(AQ) 5 | 5'-ACAU(2'AQ)GCAGTGU(2'AQ)TGAT | 2.00       | 4.13 | 2.68 | 3.58 | 1.35  | 0.043                                       |

<sup>a</sup> Enzymatic digestion was carried out by snake venom phosphodiesterase and alkaline phosphatase in 50 mM Tris-HCl (pH 7.2) at 37 °C for 2 h. UV-vis spectra were obtained in a buffer containing 0.1 M NaCl and 0.01 M sodium phosphate, adjusted to pH 7.0. U(2'AQ) indicates 2'-O-(2-anthraquinonylmethyl)uridine.

**Table 2. Spectroscopically Measured *T<sub>m</sub>* Values (260 nm) for Duplexes of Anthraquinone- and Methyl-Modified Oligonucleotides with Complementary DNA or RNA<sup>a</sup>**

| abbreviation               | sequence                        | <i>T<sub>m</sub></i> (°C) |                 |
|----------------------------|---------------------------------|---------------------------|-----------------|
|                            |                                 | duplex with DNA           | duplex with RNA |
| unmodified oligonucleotide | 5'-dACATGCAGTGTGAT              | 54.3                      | 49.5            |
| oligomer(AQ) 1             | 5'-dACAU(2'AQ)GCAGTGTGAT        | 59.8                      | 53.2            |
| oligomer(AQ) 2             | 5'-dACATGCAGU(2'AQ)GTTGAT       | 56.8                      | 49.8            |
| oligomer(AQ) 3             | 5'-dACATGCAGTGU(2'AQ)TGAT       | 56.5                      | 50.5            |
| oligomer(AQ) 4             | 5'-dACAU(2'AQ)GCAGU(2'AQ)GTTGAT | 65.8                      | 56.1            |
| oligomer(AQ) 5             | 5'-dACAU(2'AQ)GCAGTGU(2'AQ)TGAT | 60.0                      | 52.2            |
| oligomer(Me) 1             | 5'-dACAU(2'Me)GCAGTGTGAT        | 53.2                      | 48.8            |
| oligomer(Me) 2             | 5'-dACATGCAGU(2'Me)GTTGAT       | 53.3                      | 48.6            |
| oligomer(Me) 3             | 5'-dACATGCAGTGU(2'Me)TGAT       | 53.9                      | 49.1            |
| oligomer(Me) 4             | 5'-dACAU(2'Me)GCAGU(2'Me)GTTGAT | 51.8                      | 47.4            |
| oligomer(Me) 5             | 5'-dACAU(2'Me)GCAGTGU(2'Me)TGAT | 52.3                      | 49.5            |

<sup>a</sup> *T<sub>m</sub>* was measured at a total strand concentration of  $4.3 \times 10^{-5}$  M in a buffer containing 0.1 M NaCl and 0.01 M sodium phosphate, adjusted to pH 7.0. U(2'AQ) and U(2'Me) indicate 2'-O-(2-anthraquinonylmethyl)uridine and 2'-O-methyluridine, respectively. DNA, 5'-dATCAACTGCATGT; RNA, 5'-rAUC AACACUGCAUGU.

excess of the amidite was used. The protected oligonucleotides bound to the supports were treated with concentrated ammonium hydroxide in the usual manner to afford deprotected oligomers. Final purification of oligomer(AQ) 1–5 was done with DPAGE. The nucleoside compositions of oligonucleotides and the presence of the anthraquinone group(s) in the oligomers were verified by enzymatic digestion analysis and UV-vis spectral measurements. Table 1 indicated the results of the enzyme assay and the ratio of A<sub>340</sub>/A<sub>260</sub> for each oligonucleotide. The expected molar ratio of the nucleosides was obtained for each synthesized oligomer. The ratios of A<sub>340</sub>/A<sub>260</sub> for the oligonucleotides containing two anthraquinone substituents were similar but larger to a similar extent (ca. 1.5 times) than those for the monosubstituted oligonucleotides.

**Duplexes with DNA and RNA.** All the UV melting profiles for the anthraquinone-modified oligonucleotide duplexes formed with DNA and RNA exhibited sigmoidal curves or monophasic transitions which were similar to those for the corresponding unmodified duplexes. The melting profiles for duplexes of oligomer(AQ) 1 and 4 are shown as examples in Figure 1. The melting curves for the methyl-modified oligonucleotide duplexes were also similar in shape and therefore are not shown. The melting temperature (*T<sub>m</sub>*) values derived from the UV melting profiles are listed in Table 2. The typical plots for dependency of inverse *T<sub>m</sub>* values on the logarithm of the oligonucleotide concentrations are shown in Figure 2. The thermodynamic data derived from the analysis (9) of the plots are listed in Table 3.

Figure 3 indicates the CD spectra at ambient temperature for the anthraquinone oligonucleotide duplexes formed with DNA and RNA. The CD profiles for the DNA duplexes of 1–3 and 5 exhibit almost the same shapes as that of the corresponding unmodified DNA duplex, showing that the global conformation of these duplexes is similar to a B-form helix. The doubly

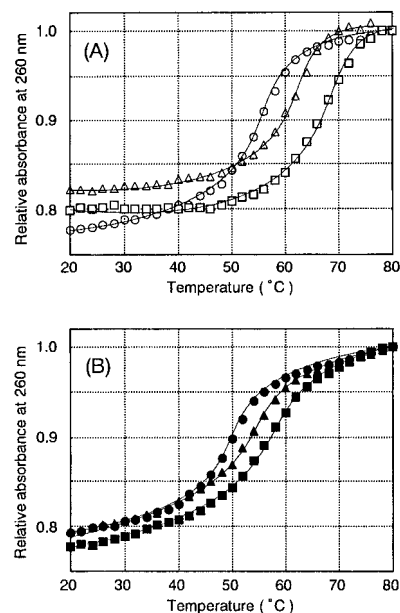


**Figure 1.** UV melting curves measured at 260 nm for duplexes at a common total strand concentration ( $4.3 \times 10^{-5}$  M). The buffer used contained 0.1 M NaCl and 0.01 M sodium phosphate, adjusted to pH 7.0. (A) Duplexes of oligomer(AQ) 1 ( $\Delta$ ) and 4 ( $\square$ ) and unmodified oligonucleotide ( $\circ$ ) with DNA. (B) Duplexes of oligomer(AQ) 1 ( $\Delta$ ) and 4 ( $\square$ ) and unmodified oligonucleotide ( $\circ$ ) with RNA. The sequences of oligonucleotides are shown in Table 2.

modified duplex derived from 4 shows slightly enhanced cotton effects at both negative and positive bands. This same tendency is seen in the case of the RNA duplexes. The duplex from 4 exhibits a slight shift to longer wavelengths in both positive and negative peaks as well as enhancement in peak intensity.

**Sequence-Specific Binding to RNA.** Sequence specificity in binding to RNA was examined for the



**Table 3. Thermodynamic Parameters for Duplexes of Anthraquinone-Modified Oligonucleotides (2 and 4) with Complementary DNA or RNA<sup>a</sup>**

| duplex                         | $\Delta G^\circ$ | $\Delta\Delta G^\circ$ | $\Delta H^\circ$ | $\Delta\Delta H^\circ$ | $\Delta S^\circ$ | $\Delta\Delta S^\circ$ |
|--------------------------------|------------------|------------------------|------------------|------------------------|------------------|------------------------|
| oligomer(AQ) <b>4</b> -DNA     | -23.2            | -7.1                   | -142.9           | -32.8                  | -398.9           | -85.5                  |
| oligomer(AQ) <b>2</b> -DNA     | -18.8            | -2.7                   | -131.4           | -21.3                  | -375.4           | -62.0                  |
| unmodified oligonucleotide-DNA | -16.1            |                        | -110.1           |                        | -313.4           |                        |
| oligomer(AQ) <b>4</b> -RNA     | -19.3            | -3.6                   | -141.8           | -14.1                  | -408.3           | -35.1                  |
| oligomer(AQ) <b>2</b> -RNA     | -16.5            | -0.8                   | -137.1           | -9.4                   | -402.1           | -28.9                  |
| unmodified oligonucleotide-RNA | -15.7            |                        | -127.7           |                        | -373.2           |                        |

<sup>a</sup> The free energy and enthalpy data are in kilocalories per mole. The entropy data are in calories per mole per Kelvin.  $\Delta G^\circ$  and  $\Delta\Delta G^\circ$  are at 27 °C.

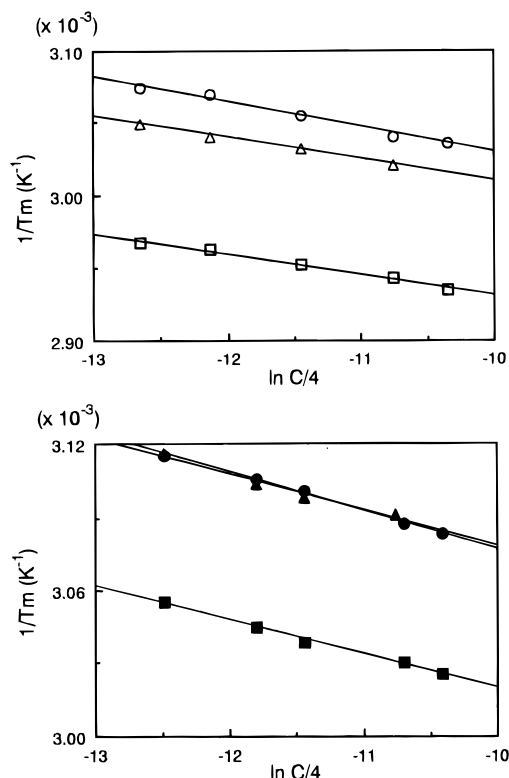


**Figure 2.** Dependency of inverse  $T_m$  values on the logarithm of the oligomer concentrations. The  $T_m$  measurements were carried out for duplexes of oligomer(AQ) **2** ( $\Delta$ ) and **4** ( $\square$ ) and unmodified oligonucleotide ( $\circ$ ) with DNA (upper) and oligomer(AQ) **2** ( $\blacktriangle$ ) and **4** ( $\blacksquare$ ) and unmodified oligonucleotide ( $\bullet$ ) with RNA (lower) in a buffer containing 0.1 M NaCl and 0.01 M sodium phosphate, adjusted to pH 7.0. The sequences of oligonucleotides are shown in Table 2.

oligonucleotide **4** containing two anthraquinone groups at the specified 2'-O-positions. Figure 4 indicates the  $T_m$  values of duplexes between the modified oligomer **4** and RNA in which X = A, C, G, or U; thus, three of them contained a noncomplementary base. A large decrease in thermal stability was observed for the duplex of **4** with a mismatch RNA, when compared with the duplex of **4** with a fullmatch RNA. The observed  $\Delta T_m$  by a mismatch base in the target RNA was in fairly good agreement with that obtained from the experiment on the unmodified oligonucleotide.

## DISCUSSION

The synthesis of oligonucleotides containing anthraquinonylmethyl groups at 2'-O-positions was accomplished with a DNA/RNA synthesizer by using a protected 2'-O-(2-anthraquinonylmethyl)uridine phosphoramidite. The purification of the modified oligonucleotides was successfully carried out with denaturing polyacrylamide gel electrophoresis. The anthraquinone oligo-

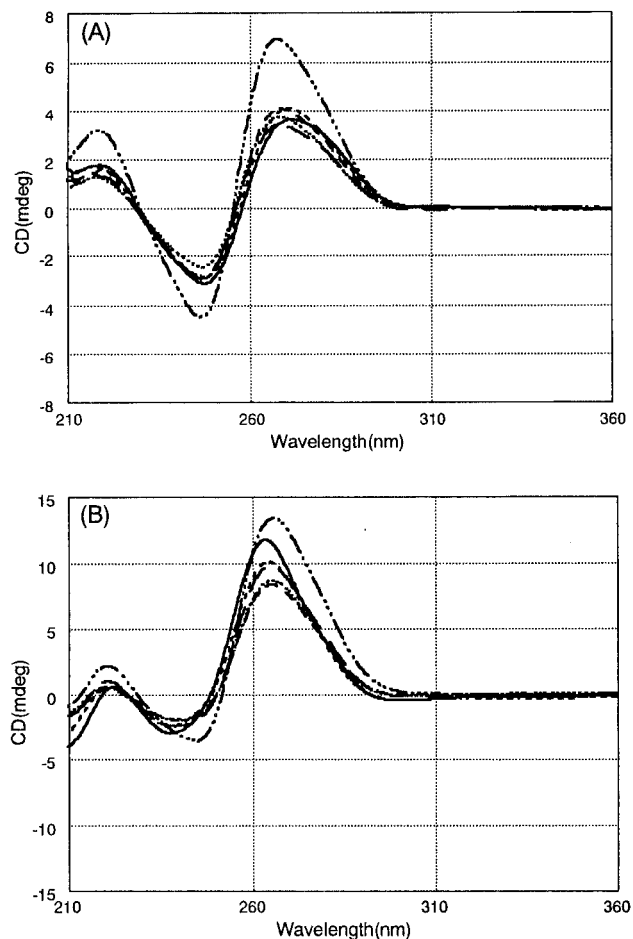


**Figure 3.** CD spectra for duplexes at a total strand concentration of  $4.3 \times 10^{-5}$  M. The measurements were carried out at 23 °C in a buffer containing 0.1 M NaCl and 0.01 M sodium phosphate, adjusted to pH 7.0. (A) Duplexes of oligomer(AQ) **1** (—), **2** (---), **3** (---), **4** (---), and **5** (···) and unmodified oligonucleotide (—) with DNA. (B) Duplexes of oligomer(AQ) **1** (—), **2** (---), **3** (---), **4** (---), and **5** (···) and unmodified oligonucleotide (—) with RNA. The sequences of oligonucleotides are shown in Table 2.

nucleotides were found to be completely digested by snake venom phosphodiesterase under the conditions that we used for hydrolysis of unmodified oligodeoxyribonucleotides. HPLC analysis of these digestion products verified the base compositions of the anthraquinone oligonucleotides.

For all the anthraquinone-modified duplexes with DNA and RNA, the UV melting profiles exhibited sigmoidal curves or monophasic transitions whose shapes were similar to those for unmodified duplexes. The plots of inverted  $T_m$  values versus the logarithm of concentrations of the modified duplexes showed good straight lines. These observations may indicate that melting of the anthraquinone-modified duplexes proceeds in a two-state (all or none) manner.

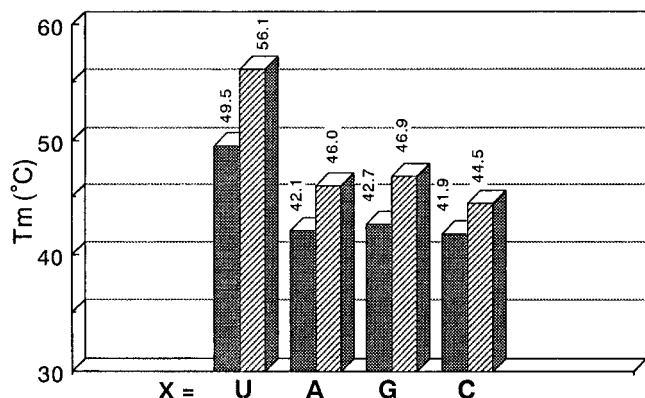


**Figure 4.**  $T_m$  values for hybrids of oligomer(AQ) **4**, 5'-dACAUG(2'AQ)GCAGU(2'AQ)GTTGAT (hatched bars), and unmodified oligonucleotide, 5'-dACATGCAGTGTGAT (stippled bars), with 5'-rAUCAACACXGCAUGU in which X = A, C, G, or U; thus, three of them contained a noncomplementary base. The  $T_m$  measurements were carried out at a total strand concentration of  $4.3 \times 10^{-5}$  M in a buffer containing 0.1 M NaCl and 0.1 M sodium phosphate, adjusted to pH 7.0.

All the anthraquinone-modified DNA and RNA duplexes have higher  $T_m$  values than those for the corresponding unmodified duplexes, although the effect of the anthraquinone substitution on the duplex stability appeared to be slightly dependent on the site(s) of modification. The effect of the substitution on the DNA duplex stability was larger than that on the RNA duplexes. The oligomer(AQ) **4** containing two anthraquinone substituents at sites separated by four nucleotides instead of six exhibits the highest affinity for both complementary DNA and RNA. The stabilizing effect can be translated into a free energy cost of 7.1 kcal/mol for the DNA hybrid and 3.6 kcal/mol for RNA. The  $\Delta\Delta G^\circ$  value for the stabilizing RNA duplex is about  $1/2$  of that for the PNA (protein-nucleic acids)-DNA stabilization (11). Close examination of our thermodynamic parameters reveals that the gain in enthalpy is a major contributor rather than the entropic factor to stabilizing the duplex of oligomer(AQ) **4** with both DNA and RNA. Although the oligoribonucleotides fully substituted with methyl groups at the 2'-O-positions have been shown to form duplexes with RNA having considerable stability (3), in the present study, we found little or no effect by partial substitution of methyl group(s) on duplex stability. This observation indicates that increased stability of DNA and RNA duplexes is indeed derived from the anthraquinone, a planar intercalative polyaromatic molecule, attached via the methylene linker to the sugar residue. A recent NMR study by Broom et al. has clearly established that intercalation of an anthraquinone moiety occurs in an oligodeoxynucleotide duplex (6).

Inspection of the CD spectra of the anthraquinone-modified duplexes reveals that the global conformation is not affected by the position of a single modification. Double modification, especially in the case of **4**, causes slight global distortion in both DNA and RNA duplexes while exhibiting high duplex stability. For all the modified duplexes, little or no induced CD at around 340 nm was observed. It has been shown (6) that a global structure of an anthraquinone-modified DNA duplex is maintained as B form while forcing the modified ribonucleoside into a C2'-endo conformation. Our CD data appear to be consistent with this observation. Since a 2'-endo conformation in oligonucleotides is more compatible for binding to DNA than to RNA, it might be reasonable that the present anthraquinone-modified oligonucleotides exhibit better affinity for DNA than RNA. In contrast, the oligonucleotide derivatives possessing

C3'-endo sugars tend to bind preferentially to RNA segments (3, 12).

When a base-pair mismatch was introduced in the target oligoribonucleotide at the position facing the middle nucleotide in the two-anthraquinone-modified oligonucleotide **4**, a large decrease in  $T_m$  was observed for the duplexes containing the mismatch. This tendency is comparable to the results on the corresponding unmodified DNA-RNA duplexes. On the basis of these observations, we conclude that modification of oligonucleotides by anthraquinone groups does not alter sequence specificity in recognizing a RNA segment.

#### ACKNOWLEDGMENT

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#### LITERATURE CITED

- (1) (a) Uhlmann, E., and Peyman, A. (1990) Antisense oligonucleotides: A new therapeutic principle. *Chem. Rev.* 90, 543-584. (b) Beaucage, S. L., and Iyer, R. P. (1993) The synthesis of modified oligonucleotides by the phosphoramidite approach and their applications. *Tetrahedron* 49, 6123-6194.
- (2) (a) Asseline, U., Delarue, M., Lancelot, G., Toulme, F., Thuong, N. T., Montenay-Garestier, T., and Helene, C. (1984) Nucleic acids-binding molecules with high affinity and base sequence specificity. *Proc. Natl. Acad. Sci. U.S.A.* 81, 3297-3301. (b) Yamana, K., and Letsinger, R. L. (1985) Synthesis and properties of oligonucleotides bearing a pendant pyrene group. *Nucleic Acids Symp. Ser.* (No. 16), 169-172. (c) Toulme, J. J., Krisch, M. M., Loreau, N., Thuong, N. T., and Helene, C. (1986) Specific inhibition of mRNA translation by complementary oligonucleotides covalently linked to intercalating agents. *Proc. Natl. Acad. Sci. U.S.A.* 83, 1227-1231. (d) Telser, J., Cruickshank, K. A., Morrison, L. E., Netzel, T. L., and Chan, C. (1989) DNA duplexes covalently labeled at two sites: synthesis and characterization by steady-state and time-resolved optical spectroscopies. *J. Am. Chem. Soc.* 111, 7226-7232. (e) Mann, J. S., Shibata, Y., and Meehan, T. (1992) Synthesis and properties of an oligonucleotide with a pyrene derivative at the 5'-phosphate. *Bioconjugate Chem.* 3, 554-558. (f) Ono, A., Dan, A., and Matsuda, A. (1993) Synthesis of oligonucleotides carrying linker groups at the 1'-position of sugar residue. *Bioconjugate Chem.* 4, 499-508. (g) Zhou, B. W., Sun, P. E., Garestier, J. S., and Helene, C. (1995) Stable triple helices formed by acridine-containing oligonucleotides with oligopurine tracts of DNA interrupted by one or two pyrimidines. *J. Am. Chem. Soc.* 117, 10425-10428.
- (3) (a) Inoue, H., Hayase, Y., Imura, A., Iwai, S., Miura, K., and Ohtsuka, E. (1987) Synthesis and hybridization studies on two complementary nona(2'-O-methyl)ribonucleotides. *Nucleic Acids Res.* 15, 6131-6148. (b) Iribarren, A. M., Sproat, B. S., Neuner, P., Sulston, I., Ryder, U., and Lamond, A. I. (1990) 2'-O-Alkyloligonucleotides as antisense probes. *Proc. Natl. Acad. Sci. U.S.A.* 87, 7747-7751. (c) Kawasaki, A. M., Casper, M. D., Freier, S. M., Lesnik, E. A., Zounes, M. C., Cummins, L. L., Gonzalez, C., and Cook, P. D. (1993) Uniformly modified 2'-deoxy-2'-fluoro phosphorothioate oligonucleotides as nuclease-resistance compounds with high affinity and specificity for RNA targets. *J. Med. Chem.* 36, 831-841. (d) Lesnik, E. A., Guinosso, C. J., Kawasaki, A. M., Sasmor, H., Zounes, M., Cummins, L. L., Ecker, D. J., Cook, P. D., and Freier, S. M. (1993) Oligodeoxynucleotides containing 2'-O-modified adenosine: Synthesis and effects on stability of DNA:RNA duplexes. *Biochemistry* 32, 7832-7838.
- (4) (a) Murakami, A., Blake, K. R., and Miller, P. S. (1985) Characterization of sequence-specific oligodeoxyribonucleoside methylphosphonates and their interaction with rabbit globulin mRNA. *Biochemistry* 24, 4041-4048. (b) Rumney,

- S., and Kool, E. T. (1992) DNA recognition by hybrid oligo-ether-oligodeoxyribonucleotide macrocycles. *Angew. Chem., Int. Ed. Engl.* 31, 1617–1619. (c) Salunkhe, M., Wu, T., and Letsinger, R. L. (1992) Control of folding and binding of oligonucleotides by use of a nonnucleotide linker. *J. Am. Chem. Soc.* 114, 8768–8772. (d) Nara, H., Ono, A., and Matsuda, A. (1995) Nucleosides and Nucleotides. 135. DNA duplex and triplex formation and resistance to nucleotidic degradation of oligodeoxynucleotides containing *syn*-nor-spermidine at the 5-position at 2'-deoxyuridine. *Bioconjugate Chem.* 6, 54–61. (e) Rumney, S., and Kool, E. T. (1995) Structural optimization of non-nucleotide loop replacements for DNA and triplex DNAs. *J. Am. Chem. Soc.* 117, 5635–5646.
- (5) (a) Yamana, K., Nishijima, Y., Ikeda, T., Gokota, T., Ozaki, H., Nakano, H., Sangen, O., and Shimidzu, T. (1990) Synthesis and interactive properties of an oligonucleotide with anthraquinone at the sugar fragment. *Bioconjugate Chem.* 1, 319–324. (b) Yamana, K., Ohashi, Y., Nunota, K., Kitamura, M., Nakano, H., Sangen, O., and Shimidzu, T. (1991) Synthesis of oligonucleotide derivatives with pyrene group at sugar fragment. *Tetrahedron Lett.* 32, 6347–6350. (c) Yamana, K., Gokota, T., Ozaki, H., Nakano, H., Sangen, O., and Shimidzu, T. (1992) Enhanced fluorescence in the binding of oligonucleotides with a pyrene group in the sugar fragment to complementary polynucleotide. *Nucleosides Nucleotides* 11, 383–390. (d) Yamana, K., Aota, R., and Nakano, H. (1995) Oligonucleotides having covalently linked anthracene at specific sugar residue: Differential binding to DNA and RNA and fluorescence properties. *Tetrahedron Lett.* 36, 8427–8430.
- (6) Deshmukh, H. M., Joglekar, S. P., and Broom, A. D. (1995) Self-complementary oligodeoxyribonucleotides containing 2'-O-(anthraquinone-2-methyl)adenosine. *Bioconjugate Chem.* 6, 578–586.
- (7) Atkinson, T., and Smith, M. (1984) Solid-phase Synthesis of Oligodeoxyribonucleotides by the Phosphite Triester Method. In *Oligonucleotide Synthesis: A Practical Approach* (M. J. Gait, Ed.) pp 35–81, IRL Press, Oxford, U. K.
- (8) Fasman, G. D., Ed. (1985) *Handbook of biochemistry and molecular biology: Nucleic Acids*, 2nd ed., Vol. 1, p 589, CRC Press, Cleveland, OH.
- (9) (a) Turner, D., and Sugimoto, N. (1988) RNA structure prediction. *Annu. Rev. Biophys. Chem.* 17, 167–192. (b) Breslauer, K. J. (1986) Methods for obtaining thermodynamic data on oligonucleotide transitions. In *Thermodynamic Data for Biochemistry and Biotechnology* (H. Hinz, Ed.) pp 377–399, Academic Press, New York.
- (10) Wagner, D., Verheyden, J. P. H., and Moffatt, J. G. (1974) Preparation and synthetic utility of some organotin derivatives of nucleosides. *J. Org. Chem.* 39, 24–30.
- (11) Eghoim, M., Buchardt, O., Christensen, L., Behrens, C., Freler, S., Driver, D. A., Berg, R. H., Kim, S. K., Nordon, B., and Nielsen, P. E. (1993) PNA hybridizes to complementary oligonucleotides obeying the Watson-Crick hydrogen-bonding rules. *Nature* 365, 566–568.
- (12) Damha, M. J., Meng, B., Wang, D., Yannopoulos, C. G., and Just, G. (1995) Structural basis for the RNA binding selectivity of oligonucleotide analogues containing alkylsulfide internucleoside linkages and 2'-substituted 3'-deoxyribonucleosides. *Nucleic Acids Res.* 23, 3967–3973.

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# CORRECTIONS

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## A NEW RADIOLIGAND FOR THE EPIDERMAL GROWTH FACTOR RECEPTOR: $^{111}\text{IN}$ LABELED HUMAN EPIDERMAL GROWTH FACTOR DERIVATIZED WITH A BIFUNCTIONAL METAL-CHELATING PEPTIDE

Page 686. The amino acid sequence of human EGF-(1–51) originally presented in Figure 3 was incorrect. The correct sequence of hEGF(1–51) and the three possible sites of attachment of the metal-chelating peptide MCP-4-EDTA to hEGF(1–51) are now presented in this revised version of Figure 3.

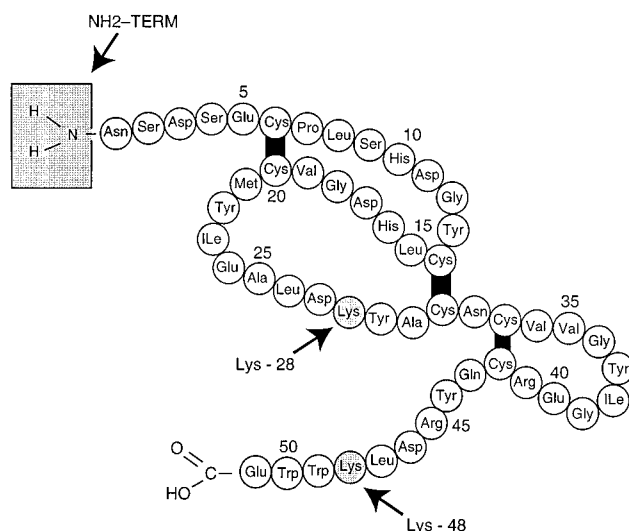


**Figure 3.** Primary sequence of human EGF(1–51). The amino terminus (gray box) and the  $\epsilon$ -amino groups of lysine 28–48 (arrows) represent the three possible sites of attachment of the metal-chelating peptide MCP-4-EDTA to hEGF(1–51). The coupling reaction was performed at pH 7.4 to favor the addition of MCP-4-EDTA at the N terminus site of hEGF(1–51). The coupling of a single mole of MCP-4-EDTA per mole of hEGF(1–51) was confirmed by amino acid analysis. Three-letter codes were used for all amino acids.

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