C. Neuraminidase Assay. Fluorescent α-sialosides (1 mM) in phosphate-buffered saline were incubated with influenza virus X-31[25] (0.1 mg mL^{-1}) at 37 °C. Portions were removed after 0.25, 0.5, 1, 2, 12, and 24 h and analyzed by thin-layer chromatography on silica gel using a mixture of 2-propanol, water, and glacial acetic acid (30:8:1 by volume) as eluant.[26] Fluorescent compounds were visualized in the UV and had the following R_f values: **1a**, 0.71; **1b**, 0.59; **9**, 0.55; the fluorescent cleavage product of **9**, 0.79. A control experiment contained, in addition to the α-sialosides and virus, the neuraminidase inhibitor 2-deoxy-2,3-didehydro-*N*-acetylneuraminic acid (1 mM).

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Supplementary Material Available: Derivation of eq 1 (3 pages). Ordering information is given on any current masthead page.

(26) Kleineidam, R. G.; Furuhata, K.; Ogura, H.; Schauer, R. *Biol. Chem. Hoppe-Seyler* **1990**, *371*, 715–719.

Trimesoyltris(3,5-dibromosalicylate): Specificity of Reactions of a Trifunctional Acylating Agent with Hemoglobin[1]

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Abstract: Trimesoyltris(3,5-dibromosalicylate)(TTDS) was prepared and evaluated as a trifunctional site-directed protein cross-linking reagent. It is synthesized by reaction of trimesoyl chloride with *tert*-butyl 3,5-dibromosalicylate, followed by deprotection with trifluoroacetic acid. TTDS reacts with the deoxy form of human hemoglobin A and with carbonmonoxyhemoglobin to produce amides from the ε-amino groups of Lys-82 of each of the β chains of hemoglobin. The third 3,5-dibromosalicylate ester group from TTDS reacts much more slowly, principally undergoing hydrolysis. Minor products include materials with α-chain modification and triply linked trimesoyl (β82Lys, β1Val, β′82Lys)hemoglobin. Comparison of these results with results from other reagents indicates that the nature of the leaving group and the structure of the acylating core control the observed specificity.

The chemical cross-linking of proteins can produce stabilized materials for use in a wide variety of applications.[2,3] Products of the method can be complementary to those obtained with site-directed mutagenesis.[4–7] A limitation of chemical cross-linking is its tendency to produce highly heterogeneous materials. Functional group specificity (chemoselectivity) is insufficient to give homogeneity since proteins contain many instances of the same functional group. However, reagents can be developed which have added specificity for small regions of a protein.[8,9] Such site-directed group-specific cross-linking reagents can improve the chances of reducing heterogeneity.

Hemoglobin is an important target protein for modification, being the basis for the production of a red-cell substitute to be used in transfusions.[4,7,10] The protein is a tetramer consisting of αβ dimers. Amino groups are good targets for directing cross-linking reagents, but in the case of hemoglobin, each αβ dimer has 24 primary amino groups which can react, giving diverse products unless the reagent has further specificity.[11–14] It has been observed that anionic acylating reagents show selectivity for certain amino groups within hemoglobin, particularly those located in the site which binds the polyanionic regulator 2,3-diphosphoglycerate.[8,9,13–22] The charge of such a reagent directs it to cationic sites on the protein, consistent with the known importance of electrostatic control of association in protein binding sites.[23]

In addition to electrostatic effects, selectivity and efficiency can be enhanced by taking advantage of steric effects and the presence of additional functional groups. Steric effects play an important role in determining the regioselectivity of organic reagents,[24] and it can be expected that such effects can increase the selectivity of a protein reagent. The combination of charge direction, steric bulk, and multifunctionality in a single reagent should lead to

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(2) Martinek, K.; Torchilin, V. P. *Methods Enzymol.* **1988**, *137*, 615.
(3) Ji, T. H. *Methods Enzymol.* **1983**, *91*, 580.
(4) Looker, D.; Abbott-Brown, D.; Cozart, P.; Durfeee, S.; Hoffman, S.; Mathews, A. J.; Miller-Roehrich, J.; Shoemaker, S.; Trimble, S.; Fermi, G.; Komiyama, N. H.; Nagai, K.; Stetler, G. L. *Nature* **1992**, *356*, 258.
(5) Perry, L. J.; Wetzel, R. *Science (Washington D.C.)* **1984**, *226*, 557.
(6) Perry, L. J.; Wetzel, R. *Biochemistry* **1986**, *25*, 733.
(7) Ogden, J. E. *Trends Biotechnol.* **1992**, *10*, 91.
(8) Kluger, R.; Grant, A. S.; Bearne, S. L.; Trachsel, M. R. *J. Org. Chem.* **1990**, *55*, 2864.
(9) Zaugg, R. H.; King, L. C.; Klotz, I. M. Biochem. *Biophys. Res. Commun.* **1975**, *64*, 1192.
(10) Vandegriff, K. D.; Winslow, R. M. *Chem. Ind. (London)* **1991**, 497.
(11) Dickerson, R. E.; Geis, I. *Hemoglobin: Structure, Function, Evolution, and Pathology*; The Benjamin/Cummings Publishing Co.: Menlo Park, CA, 1983; p 32.
(12) Fermi, G.; Perutz, M. F. *J. Mol. Biol.* **1984**, *175*, 159.
(13) Manning, J. M. *Adv. Enzymol.* **1991**, *64*, 55.
(14) Kavanaugh, M. P.; Shih, D. T. B.; Jones, R. T. *Biochemistry* **1988**, *27*, 1804.
(15) Stark, G. R. *Biochemistry* **1965**, *4*, 1030.
(16) Stark, G. R.; Stein, W. H.; Moore, S. *J. Biol. Chem.* **1960**, *235*, 3177.
(17) Benesch, R.; Benesch, R. E.; Yung, P. S.; Edalji, R. *Biochem. Biophys. Res. Commun.* **1975**, *63*, 1123.
(18) Benesch, R.; Triner, L.; Benesch, R. E.; S., K. P.; Verosky, M. *Proc. Natl. Acad. Sci. U.S.A.* **1984**, *81*, 2941.
(19) Bucci, E.; Razynska, A.; Urbaitis, B.; Fronticelli, C. *J. Biol. Chem.* **1989**, *264*, 6191.
(20) Chatterjee, R.; Welty, E. V.; Walder, R. Y.; Pruitt, S. L.; Rogers, P. H.; Arnone, A.; Walder, J. A. *J. Biol. Chem.* **1986**, *261*, 9929.

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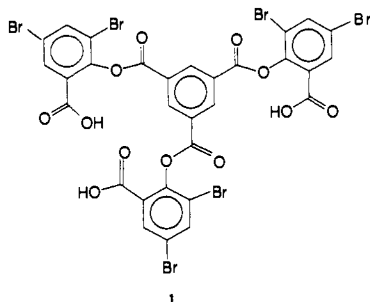
improved selectivity and yield, if the desired product results from reaction at the most accessible site.

We have prepared trimesoyltris(3,5-dibromosalicylate) (TTDS, 1), a reagent which combines all the features discussed above: it is anionic, bulky, and trifunctional. In this paper we report that TTDS reacts with both deoxy and carbonmonoxy human hemoglobin A to give products which indicate that the reaction is effectively controlled by both charge and steric bulk of the reagent. The additional functional group provides statistical advantages in the initial reaction and entropic advantages in forming the cross-link.



Experimental Section

Materials and Methods. Commercial reagents were utilized without further purification. Solvents were dried prior to use. Deuterated solvents were from MSD Isotopes Ltd. and the Aldrich Chemical Company. Organic reagents and solvents were purchased from BDH Canada Ltd. and Caledon Laboratories Ltd. Inorganic materials were purchased from Fisher Scientific. The purity of samples of newly synthesized materials was assessed by a combination of NMR spectroscopy, mass spectrometry, analytical thin layer chromatography, and elemental analysis. The latter was done by Galbraith Laboratories, Knoxville, TN.

Molecular Mechanics. HyperChem (version 1.9, from HyperCube division of Autodesk, Inc.), operating on a 486 computer (MS-DOS), was used to obtain energetic minima for iterative conformational perturbations based on several initially estimated structures of TTDS using mixed-gradient optimization. It was also used to visualize the structure of deoxyhemoglobin on the basis of information from the Brookhaven Protein Data Bank.

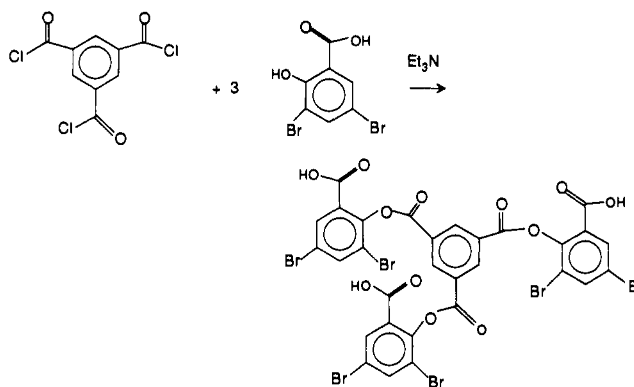
Spectra and Kinetics. Proton and carbon NMR spectra were recorded on a Varian Gemini (200 MHz) spectrometer. Infrared spectra were recorded on a Nicolet 5DX FTIR spectrometer. UV spectra and kinetics were done on a Perkin-Elmer Lambda 2 spectrometer interfaced to a MS-DOS 386 computer. Spectra were collected with the Perkin-Elmer PECSS program and kinetics analyzed using GraFit (Erithacus Software, Ltd.)

Synthesis of Trimesoyltris(3,5-dibromosalicylate) (TTDS). The reaction of 3,5-dibromosalicylic acid with trimesoyl trichloride in anhydrous THF in the presence of triethylamine gave a very low yield of the desired product, which was isolated by HPLC, so an alternative route was used.

Trimesoyltris(1-(*tert*-butoxycarbonyl)-3,5-dibromosalicylate). This carboxyl-protected salicylate was prepared according to the procedure of Klotz and co-workers.[25] Potassium *tert*-butoxide (0.30 g, 2.69 mmol) was added to a solution of *tert*-butyl 3,5-dibromosalicylate (0.95 g, 2.69 mmol) in anhydrous THF (30 mL). The mixture was stirred at room temperature for 15 min. A solution of trimesoyl trichloride (0.24 g, 0.90 mmol) in tetrahydrofuran (10 mL) was added dropwise over 15 min. The reaction mixture was stirred overnight at room temperature. Ether (60 mL) was added, and the mixture was washed with water (2 × 100 mL). The organic phase was dried over Na_2SO_4 and concentrated in vacuo to give the product as a white solid (0.98 g, yield 90%). ^{1}H NMR ($CDCl_3$): δ 9.32 (s, 3 H, ArH), 8.01 (AB, 6 H, ArH), 1.42 (s, 27 H, $C(CH_3)_3$). FAB-MS: 1213 (M + 1). IR (KBr): 2980 (m), 1771 (s), 1722 (s), 1185 (s) cm^{-1}.

Trimesoyltris(3,5-dibromosalicylate). Trimesoyltris(1-(*tert*-butoxycarbonyl)-3,5-dibromosalicylate) (0.88 g, 0.73 mmol) was dissolved in anhydrous trifluoroacetic acid (20 mL) and kept at room temperature for 1 h. Diethyl ether (20 mL) was added to induce crystallization. The solution was kept at 5 °C overnight. The product was collected by filtration (white crystals, 0.62 g, yield 82%). IR (KBr): 3416 (br, s), 2355 (s), 1743 (s), 1002 (s) cm^{-1}. ^{1}H NMR (acetone-d_6): δ 9.25 (s, 3 H, ArH), 8.25 (AB, 6 H, ArH), 4.40 (br s, 3 H, CO_2H). ^{13}C NMR (acetone-d_6): δ 164.01 (s), 163.01 (s), 148.59 (s), 140.64 (s), 137.20 (s), 135.16 (s), 132.18 (s), 128.17 (s), 120.69 (s), 120.50 (s). MS (negative FAB): 1043 (M − 1) Anal. Calcd for $C_{30}H_{12}O_{12}Br_6$: C, 34.52; H, 1.16; Br, 45.93. Found C, 34.26; H, 1.43; Br, 45.58.

Scheme I. Direct Synthesis of TTDS



Biochemical Materials. HPLC-grade acetonitrile and water were obtained from Mallinckrodt, and trifluoroacetic acid (TFA) was from Pierce Chemical Co. (Rockford, IL). Trypsin from Worthington Biochemical Co. (Freehold, NJ) and *Staphylococcus aureus* V8 endoproteinase Glu-C from Boehringer Mannheim Biochemical (Indianapolis, IN) were used for enzymic digestion of globin chains. Sephadex G-25, DEAE-Sephacel, and CM-Sephadex were from Pharmacia Fine Chemicals AB (Uppsala, Sweden). Other reagents for preparation of buffers and developers for the modifications of hemoglobin and for chromatography were all of analytical grade or better.

Hemoglobin was prepared using procedures described previously.[14,21] Solutions were contained in a 50-mL round-bottom flask which was immersed in a water bath and connected to a rotary evaporator with the condenser replaced by a septum through which passed outlet and inlet tubes (for gas flow). Stock solutions of carbonmonoxyhemoglobin were converted to oxyhemoglobin by photoirradiation under a stream of humidified oxygen for 60 min at 0 °C in a rotating flask.[26] Oxyhemoglobin was converted to deoxyhemoglobin by passing a stream of humidified nitrogen over the oxyhemoglobin solution in the rotating flask for 2 h at 35 °C.

Reaction of TTDS with Hemoglobin. A solution of TTDS in buffer was added to the 1 mM hemoglobin solution described above so that the final concentrations of TTDS and hemoglobin were 1.0 mM and 0.5 mM, respectively, in a 0.1 M pH 7.2 MOPS buffer. The reactions were run at 35 °C or 60 °C for 2-3 h in the rotating flask with hemoglobin in either the deoxy or the carbonmonoxy form. For reactions with deoxyhemoglobin, TTDS was introduced into the reaction vessel under nitrogen. Nitrogen flowed continuously during the reaction over the hemoglobin solutions in the rotating flask to maintain the hemoglobin in the deoxy state. For reactions of carbonmonoxyhemoglobin, the hemoglobin solution was saturated with carbon monoxide and the reaction run without adding more gas. The reaction continued for two more hours after the completion of the addition of the reagents.

After reaction, the hemoglobin in each sample was separated from unreacted reagent by passing the sample through a Pharmacia PD-10 Sephadex G-25M column at 4 °C, and then it was converted to carbonmonoxyhemoglobin by addition of carbon monoxide. Products were analyzed by a combination of polyacrylamide gel electrophoresis and ion exchange HPLC of intact tetramers, reverse-phase HPLC of separated globin chains, and tryptic/Glu-C digest of globin chains with peptide mapping as described previously.[14,21]

Results

Syntheses. The direct reaction of trimesoyl trichloride with 3 equiv of 3,5-dibromosalicylic acid in the presence of 6 equiv of base gives an incomplete reaction (Scheme I). Purification of the mixture using reverse phase HPLC provides the product in low yield.

(21) Kluger, R.; Wodzinska, J.; Jones, R. T.; Head, C.; Fujita, T. S.; Shih, D. T. *Biochemistry* **1992**, *31*, 7551.
(22) Massil, S. E.; Shi, G.-Y.; Klotz, I. M. *J. Pharm. Sci.* **1984**, *73*, 418.
(23) Warshel, A. *Proc. Natl. Acad. Sci. U.S.A.* **1978**, *75*, 5250.
(24) Newman, M. S. *Steric Effects in Organic Chemistry*; Wiley; New York, 1956.
(25) Delaney, E. J.; Massil, S. E.; Shi, G. Y.; Klotz, I. M. *Arch. Biochem. Biophys.* **1984**, *228*, 627.
(26) Shih, T.-B.; Jones, R. T.; Johnson, C. S. *Hemoglobin* **1982**, *6*, 153.

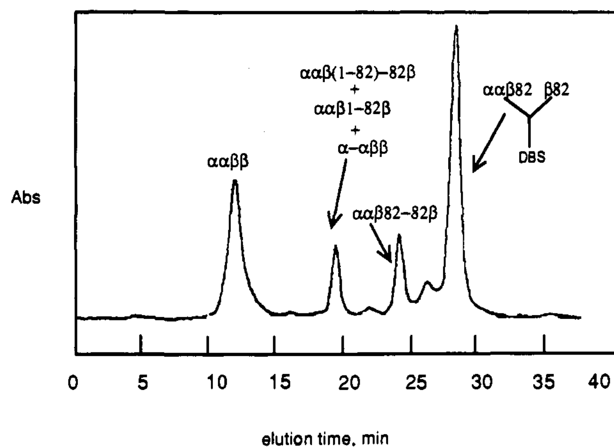
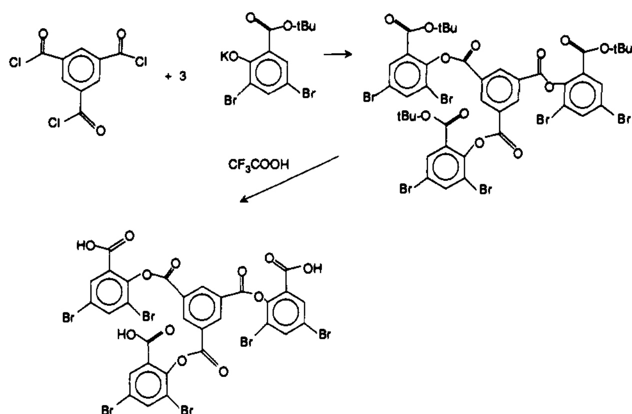


Figure 1. AX-300 ion exchange chromatography of reaction products from TTDS and deoxyhemoglobin, reacted at 35 °C for 2 h. Native hemoglobin is $\alpha\alpha\beta\beta$. The peaks are indicated by the modification of hemoglobin. $\alpha\alpha\beta$82–82β is cross-linked by the trimesic functionality and has the third salicylate hydrolyzed to the free acid. DBS indicates that the salicylate ester is still intact at the third carboxyl group (while the other two groups have formed amides with hemoglobin). Peak identification is by globin chain separation and peptide analysis. The presence of α–α cross-linked species was also detected, but the reaction site was not determined (α–$\alpha\beta\beta$).

Scheme II. Synthesis of TTDS from *tert*-Butyl Ester



The method developed by Klotz to prepare salicylates of unreactive carboxylic acids was used successfully.[25] This involves protection of the carboxyl group of 3,5-dibromosalicylic acid as the *tert*-butyl ester, coupling, and deprotection.[25,27] Reaction of trimesoyl trichloride with potassium *tert*-butyl 3,5-dibromosalicylate provides the carboxyl-protected coupling product in high yield (90%). The *tert*-butyl group was readily removed with anhydrous trifluoroacetic acid to give analytically pure TTDS in 82% yield (Scheme II).

The rate of hydrolysis of TTDS was measured under the conditions in which the material is used to cross-link hemoglobin (0.1 M MOPS, pH 7.2). The first ester hydrolysis follows first-order kinetics with a half-life of 6.0 h ($k_{obs} = 3.2 \times 10^{-5}$ s^{-1}). The hydrolysis of the second and third ester groups is much slower. To estimate the reactivity of TTDS toward amino groups of hemoglobin, the reaction with *n*-hexylamine was measured (in 0.1 M pH 7.2 buffer, $k_2 = 32$ M^{-1} s^{-1}). If, for example, hemoglobin is present at a concentration of 1 mM and TTDS is 2 mM, then the apparent initial first-order rate constant for acylation of an amino group will be 3.2×10^{-2} s^{-1}, corresponding to a half-life of about 20 s. The further reaction with other amino groups to form the crosslink should be rapid due to the reduced translational entropy of the bound reagent. The reaction of the reagent with accessible amino groups in hemoglobin should also be faster than

(27) Razynska, A.; Rak, J.; Fronticelli, C.; Bucci, E. *J. Chem. Soc., Perkin Trans. 2* **1991**, 1531.

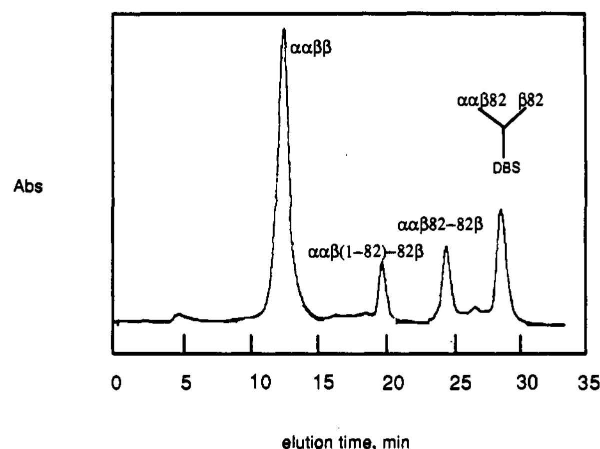


Figure 2. AX-300 profile for reaction of TTDS with carbonmonoxyhemoglobin at 35 °C, after 2 h.

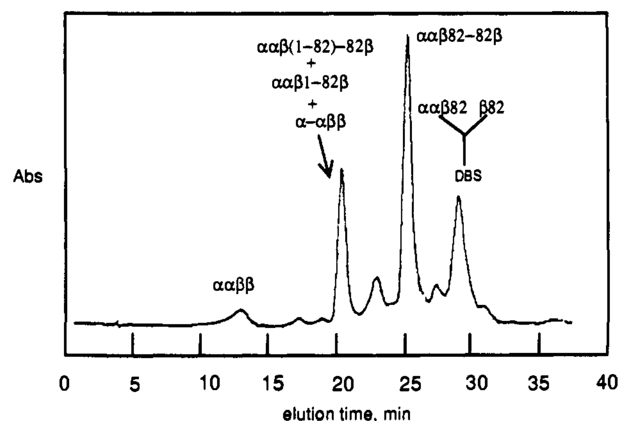


Figure 3. AX-300 profile for reaction of TTDS with deoxyhemoglobin at 60 °C for 2 h.

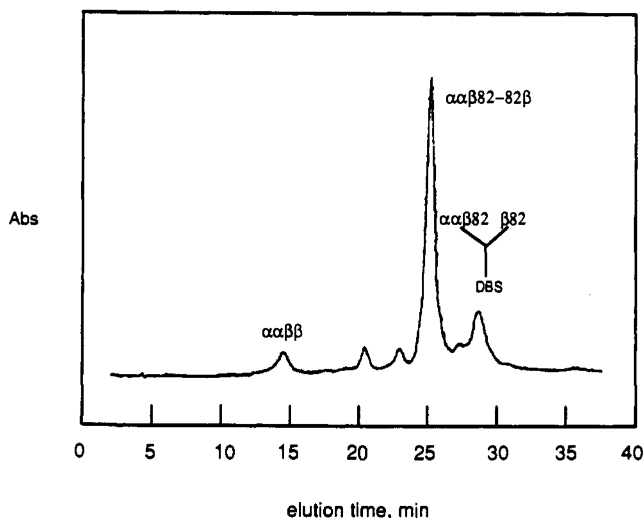


Figure 4. AX-300 profile for reaction of TTDS with carbonmonoxyhemoglobin at COHb 60 °C for 2 h.

hydrolysis, but, due to the size of the molecule, the rate should be considerably slower. Thus, based on experience with other reagents, the reactions with hemoglobin were allowed to proceed for at least several hours.

Product Analysis. Deoxyhemoglobin and carbonmonoxyhemoglobin were treated with TTDS at 35 °C and at 60 °C. The AX-300 ion exchange HPLC chromatograms of the reaction products (showing intact hemoglobin tetramers and modified tetramers) after 2 h of reaction (with reagents removed) are shown in Figures 1–4. In addition, the deoxyhemoglobin and carbonmonoxyhemoglobin reaction mixtures which had been run at 35

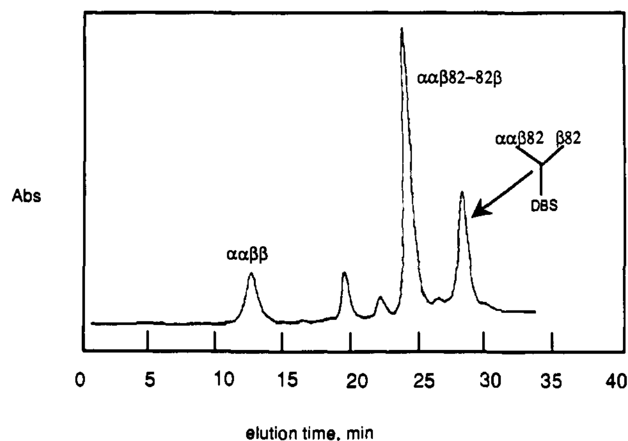


Figure 5. AX-300 profile for reaction of TTDS with deoxyhemoglobin at 35 °C for 2 h, analyzed after 15 days at 0 °C.

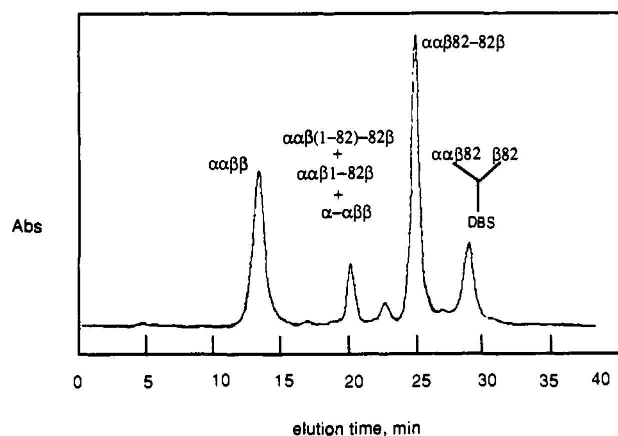


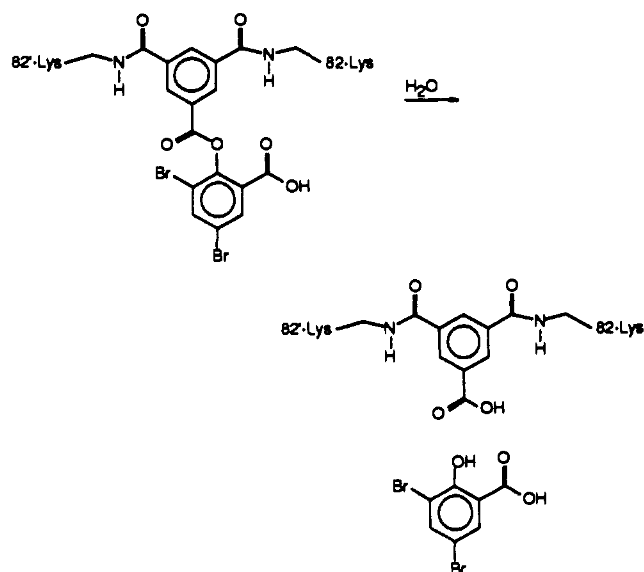
Figure 6. AX-300 profile for reaction of TTDS with carbonmonoxyhemoglobin at 35 °C for 2 h, analyzed after 15 days at 0 °C.

°C were stored on ice for 15 days and analyzed chromatographically after 1 week and after 15 days using the AX-300 column (Figures 5 and 6 are the chromatograms of the products after 15 days). The peaks were identified by globin chain separations on a C-4 reverse-phase column and peptide pattern analysis. In all cases, products were identified by comparison with those obtained from the reaction of hemoglobin with trimesoyl tris(methyl phosphate) which had included amino acid analysis.[21]

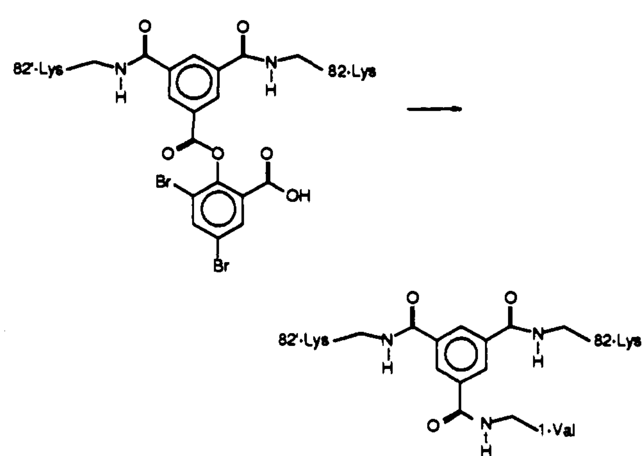
Analysis of the product distribution from the reaction of deoxyhemoglobin with TTDS shows that the primary cross-linked product has a trimesoyl group bridging Lysβ82 to the corresponding residue in the other β chain with the third group unreacted. The product pattern changes slowly with time, releasing dibromosalicylic acid from the third carboxyl group during the 15 days following reaction. This slow hydrolysis is evidenced by the elution of free dibromosalicyclic acid (as determined by UV absorbance of the eluant). The major product after loss of dibromosalicyclic acid is $\alpha\alpha\beta$82Lys-trimesoyl-β'82Lys hemoglobin, accounting for about 85% of the total protein present. There is a very small amount of triply-linked ($\alpha\alpha\beta$(82Lys,1Val)-trimesoyl-β'82Lys) hemoglobin[21] and also material modified in the α chain. The hydrolysis reaction is shown in Scheme III. The formation of the triply-linked species is summarized in Scheme IV.

TTDS reacts more readily with deoxyhemoglobin than with carbonmonoxyhemoglobin at 35 °C, but both form the same modified hemoglobins as products. The main difference is that deoxyhemoglobin produces much more of the β82–82β cross-linked hemoglobin with the third dibromosalicylate group still present. After 15 days at 0 °C, however, the product from deoxyhemoglobin also has the third trimesoyl acyl group as the free acid. Complete hydrolysis occurs during this time rather than reaction with other amino groups of the protein. The reaction of TTDS

Scheme III



Scheme IV



with carbonmonoxyhemoglobin and with deoxyhemoglobin at 60 °C is complete after the initial 2-h reaction period. The major product in each case is $\alpha\alpha\beta$Lys82-trimesoyl-82Lysβ with the third acyl group as the free acid (Scheme IV).

Discussion

The reaction of TTDS with hemoglobin is highly selective for the ϵ-amino groups of the Lys-82 residues of each of the β subunits. Integration of the chromatograms of the reaction products indicates a yield of about 85% of the products resulting from cross-linking of these residues. These are in the site which normally binds the polyanionic effector 2,3-diphosphoglycerate (DPG). The residue is very accessible to bulky reagents, since its ϵ-amino group is at the end of a primary alkyl chain in a large cavity of the protein tetramer.[12] In deoxyhemoglobin, the DPG binding site is expanded relative to the carbonmonoxy and oxy forms, separating the β chains and moving the Lys-82 residues. If the third dibromosalicylate ester group is less mobile in the deoxy cross-linked species, then it is also less accessible for hydrolysis than in the carbonmonoxy form where the residues are more flexible. Another possibility is that in the carbonmonoxyhemoglobin the third carboxyl could be held outside the DPG site by steric limitations.

The bifunctional cross-linker fumaroylbis(3,5-dibromosalicylate) (FBDS) reacts with oxyhemoglobin to give as a primary product (β82Lys-fumaroyl-Lys82β')hemoglobin in which the cross-link is between ϵ-amino groups.[28,29] The reaction of deoxyhemoglobin

(28) Walder, J. A.; Walder, R. Y.; Arnone, A. *J. Mol. Biol.* **1980**, *141*, 195.

with FBDS gives a mixture of major products.[28] Thus, the trifunctional nature of TTDS adds specificity beyond that of a related bifunctional material with the same leaving group.

The reactions of trimesoyl tris(methyl phosphate) (TTMP) with hemoglobin under the same reaction conditions have also been studied.[21] In those cases, the principal reaction products result from cross-linking the terminal amino group of the β subunits, Val-1, to the ϵ-amino group of Lys-82, with the principal product being the triply cross-linked species ($\alpha\alpha\beta$(82Lys,1-Val)-trimesyl-β'82Lys). TTMP reacts with carbonmonoxyhemoglobin to produce a small amount (15%) of the $\alpha\alpha\beta$82Lys-trimesoyl-Lys82β. Since the acyl core is the same in TTMP and TTDS, the nature of the leaving group must determine the cross-linking site.

Examination by structural modeling of TTDS clearly shows that the reaction site is well-shielded by the aryl groups. The 3,5-dibromosalicylate moiety is an unusually bulky leaving group, and this partially controls the regioselectivity of its derivatives as cross-linking reagents for hemoglobin. Analysis of the structure of deoxyhemoglobin[12] shows that the 82βLys amino groups are accessible, even to a bulky reagent. Thus, electrostatic forces direct TTDS to the DPG binding site, and the bulkiness of the reagent causes its opportunities for reaction to be limited once it is within the site.

(29) Shibayama, N.; Imai, K.; Hirata, H.; Hiraiwa, H.; Morimoto, H.; Saigo, S. *Biochemistry* 1991, 30, 8158.

Conclusions

The reaction of TTDS with hemoglobin demonstrates that high-yield production of cross-linked proteins can be achieved using a reagent which combines aspects of chemical selectivity. Applications of the reagent to stabilization of other proteins will depend on the specific structural features of those proteins.

Acknowledgment. We thank Leuming Feng for preliminary studies on the synthesis of TTDS.

Redox-Controlled Bergman Cycloaromatizations. Designed Enediynes with DNA-Cleaving Properties and Antitumor Activity

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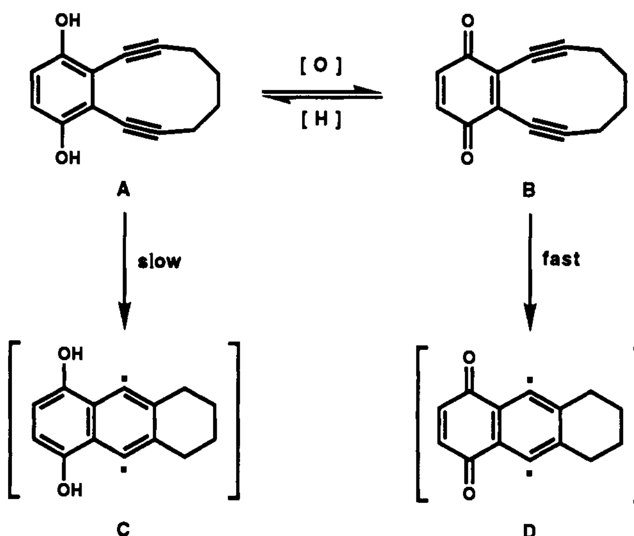
Abstract: Enediynes **7** and **9** were designed for their potential to act as radical-generating species upon oxidation to the corresponding quinone. Their synthesis entailed a chromium–nickel-mediated ring closure of iodo aldehyde **6**. Investigations with these molecules and their derivatives **8** and **10** demonstrated the anticipated acceleration of the Bergman cycloaromatization of the oxidized species as compared to the reduced compounds and potent DNA-cleaving and antitumor properties.

Introduction

Naturally occurring enediyne anticancer antibiotics[1,2] are triggered to exert their biological actions by bioreductive processes which initiate Bergman cycloaromatization[3] leading to DNA cleavage. Several designed enediynes[1,4] have been shown to undergo the Bergman reaction upon activation with acid, base, or UV irradiation. In this paper, we report the design, synthesis, and evaluation of a series of cyclic enediynes in which the Bergman cycloaromatization is controlled by the redox process hydroquinone ⇄ quinone (Scheme I). Given the ease by which the processes are driven in either direction both in vitro and in vivo, the synthesis and evaluation of such systems was deemed important and may have considerable potential.

On the basis of previous observations with arene-substituted enediynes,[5-7] we postulated that hydroquinone systems of type A (Scheme I) should be quite stable toward cycloaromatization to form benzenoid diradical C, whereas oxidation to the quinone (B) should result in lowering of the activation energy for this process and, therefore, faster cyclization to diradical D. This hypothesis was tested by synthesizing compounds **7–10** as shown in Scheme II.

Scheme I. Redox-Controlled Bergman Cycloaromatization



Results and Discussion

The readily available diiodide **1**[8] was sequentially coupled with $Me_3SiC{\equiv}CH$ and $^tBuMe_2SiO(CH_2)_4C{\equiv}CH$ under the catalytic influence of Pd(O)–Cu(I) to afford the diacetylenic compound **3** via **2**. Formation of the dipivaloyl ester of **3** followed by desilylation furnished compound **4** in 71% overall yield. Iodination

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(1) Nicolaou, K. C.; Dai, W.-M. *Angew. Chem., Int. Ed. Engl.* 1991, 30, 1387.
(2) Lee, M. D.; Ellestad, G. A.; Borders, D. B. *Acc. Chem. Res.* 1991, 24, 235.

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