time of 1.71 min under these conditions. Anal. Calcd for $C_{58}H_{68}N_8O_{14}S_2 \cdot 6HCl$: C, 50.33; H, 5.39; N, 8.10. Found: C, 50.62; H, 5.30; N, 8.06. In the absence of a Cl analysis these data are also consistent with $C_{58}H_{68}N_8O_{14}S_2 \cdot 3HCl \cdot CF_3CO_2H \cdot 2H_2O$: C, 50.58; H, 5.37; N, 7.86. A second lot of material prepared by this procedure and stored in the freezer for approximately 1 year was analyzed: calcd for $C_{58}H_{68}N_8O_{14}S_2 \cdot 2HCl \cdot CF_3CO_2H \cdot H_2O$: C, 52.59; H, 5.36; N, 8.17; Cl, 5.17. Found: C, 52.89; H, 5.38; N, 8.11; Cl, 5.45.

**Enzymatic Hydrolysis of Cephalosporins.** $\beta$-Lactamase activity of the MoAb–enzyme immunoconjugate was measured by monitoring the change in absorbance of the chromogenic substrate PADAC (Calbiochem) at 570 nm in a stirred cuvette at 37 °C in PBS. Absorbance was measured every 5 s for 120 s using a Hewlett-Packard 8451A spectrophotometer, and linear portions of the rate plots were used to obtain reaction velocities. $K_M$ and $k_{cat}$ were determined from the slope and intercept of Lineweaver–Burk plots of the velocity data. PADAC $\epsilon_{570}$ was taken to be $4.8 \times 10^4$ (product label).

Due to PADAC's low solubility and high extinction coefficient, the assay was run under conditions in which the rate is dependent on the substrate concentration. Consequently, the runs comparing the activity between different preparations were all performed with a starting $A_{570}$ of approximately 0.5.

Kinetic parameters for cephalothin (4), 11, and 18 were measured as for PADAC, except that the change in absorbance was monitored at around 260 nm. Compounds 11 and 18 have a residual absorbance after hydrolysis so $\Delta\epsilon$s were obtained by completely hydrolyzing a known concentration of the substrate and calculating $\Delta\epsilon$ from the change in absorbance: $\Delta\epsilon_{258}$ for 11 = $6.6 \times 10^3$ (cm M)$^{-1}$; and $\Delta\epsilon_{260}$ for 18 = $8.4 \times 10^3$ (cm M)$^{-1}$.

The spectral change upon $\beta$-lactamase catalyzed hydrolysis of prodrug 20 was too small to be useful for quantitative determination of the rates. Consequently, HPLC methods were developed to monitor this reaction. Vials containing 1.5-mL solutions of varying concentrations of substrate at 37 °C in PBS, pH 7.4, were treated with 0.11 nM of the MoAb–enzyme immunoconjugate. Samples were quenched after 90 s by adding 0.5 mL of the reaction solution to 0.5 mL of 34% $CH_3CN$ in 200 mM potassium phosphate, pH 3.0. Control experiments were performed to determine appropriate concentrations so that less than 10% of the substrate would be consumed during the 90-s reaction to assure a linear reaction rate. Duplicate samples of the quenched reaction mixtures were injected onto a 0.46- × 15-cm C18 reversed-phase HPLC column eluted (34% $CH_3CN$ in 200 mM potassium phosphate) at 1 mL/min. The prodrug and product concentrations were monitored by absorbance at 266 nm.

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# Use of a Thiol Tether for the Site-Specific Attachment of Reporter Groups to DNA

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The sequence-specific incorporation of a thiol tether into oligodeoxynucleotides provides for the rapid and facile attachment of a wide variety of reporter groups employing thiol-specific alkylating functionalities. The thiol residue is introduced into the DNA by oxidation of an internucleotide H-phosphonate at a unique site within the sequence in the presence of cystamine. After synthesis and purification of the cystamine-containing oligodeoxynucleotide, the sulfhydryl residue is unmasked by a short treatment with dithiothreitol. The tethered sulfhydryl residue is amenable to modification by a variety of thiol-specific reporter groups. Nuclease digests of the modified and unmodified sequences confirm that labeling occurs at the site of the tether. Duplex sequences containing a variety of fluorophores covalently bound through this thiol tether exhibit thermal stabilities that are very similar to that of the unlabeled sequence.

## Introduction

The site-specific attachment of reporter groups to DNA would facilitate the detailed study of the structure and dynamics of unusual nucleic acid forms as well as ligand–DNA or protein–DNA complexes. In many previous studies (for a recent review see Goodchild[1]), the introduction of reporter groups has relied upon either the chemical synthesis of a modified nucleoside residue carrying the reporter group attached to the base residue[2] or a variety of related procedures which exploit the reactivity of functional groups attached to the 5′ or 3′ terminus of the DNA fragment.[3]

Reporter groups tethered to the base residue can be introduced site-specifically (depending upon the sequence location of the base). These procedures typically employ a linker arm attached to the C5-position of thymine or the N4-position on cytosine, the latter being a functional group normally involved in Watson–Crick hydrogen bonding. This approach can lead to destabilization of the helix

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structure, even in the absence of the incorporated label. Presumably the linker itself can interfere with interstrand hydrogen bonding and/or base stacking.[2k,m] For example, Telser et al.[2k] report a net destabilization of 1 kcal/mol for octamer duplexes containing a primary amine linker bound to the C5-position of thymine or to the N4-position of cytosine. In a similar approach, McMillian and Verdine[2m] report a decrease of between 6 and 7 °C in Tm values for self-complementary decamer duplexes containing a primary amine linker attached to the N4-position of cytosine. The attachment of the reporter group to the linker can further destabilize duplex structures, or in some cases provide significant stabilization providing that the attached group interacts favorably with the duplex structure (see for example the work on intercalative prosthetic groups as reviewed recently by Helene and Toulme[4]).

Attachment of the reporter group to the terminus of the fragment has been a popular approach to the functionalization of DNA fragments. Placing the label at either the 3′ or 5′ terminus of a duplex structure does not typically interfere with hydrogen bonding or base stacking interactions, but labeling at either of the termini does eliminate the possibility of site-specific placement of the label within the sequence.

More recently, the DNA backbone has been exploited as a site for covalent binding of reporter groups.[5] This approach allows for the site-specific introduction of the label into a DNA sequence at a position that does not generally interfere with duplex stability. Some of these methods require that the moiety of interest be incorporated during the DNA synthesis procedures; it must then withstand the acid, base, and oxidizing conditions present during the assembly of the desired sequence. Relatively few procedures allow for the site-specific introduction of reporter groups to the DNA backbone after synthesis and purification of the desired sequence.[5e,f] We wish to report a simple procedure for the site-specific introduction of a thiol functionality tethered to the DNA backbone. DNA fragments functionalized in this manner are amenable to labeling by a variety of thiol specific fluorophores, spin labels, or other prosthetic groups.

## Experimental Section

**Materials.** HPLC-grade solvents were either obtained from Aldrich (Milwaukee, WI) or from Fisher Scientific (Fair Lawn, NJ). High-performance liquid chromatography (HPLC) was carried out using columns of ODS-Hypersil (4.6 × 250 mm) or MOS-Hypersil (9.4 × 250 mm). 5′-dimethoxytrityl nucleoside phosphoramidite monomers as well as all ancillary reagents for nucleic acid synthesis were obtained from Cruachem (Sterling, VA) or American Bionetics (Hayward, CA). Nuclease S1 from *Aspergillus oryzae* was obtained from United States Biochemical Corporation (Cleveland, OH). Nuclease P1 purified from *Penicillium citrinum* was obtained from GIBCO BRL (Gaithersburg, MD). Alkaline phosphatase from calf intestine as well as phosphodiesterase from *Crotalus durissus* were obtained from Boehringer Mannheim (Indianapolis, IN). Hydrogen phosphonate monomers were purchased from Glenn Research (Sterling, VA). Anhydrous pyridine, anhydrous carbon tetrachloride, 1-adamantanecarbonyl chloride, and cystamine dihydrochloride were all obtained from Aldrich (Milwaukee, WI). All fluorophores were obtained from Molecular Probes (Eugene, OR). The iodoacetamido-PROXYL spin label was from Aldrich (Milwaukee, WI). Quantities of nucleic acid were measured and reported in $A_{260}$ units, where 1 $A_{260}$ unit is absorbance obtained for a sample of DNA dissolved in 1.0 mL of distilled water and measured in a spectrophotometer at 260 nm in a 1.0-cm-pathlength cell (1 $A_{260}$ unit is approximately 50 μg of DNA).

**Methods. Synthesis of d[Ap(NHCH$_2$CH$_2$S-SCH$_2$CH$_2$NH$_2$)A].** To 2.5 g (0.011 mol) of cystamine dihydrochloride (Aldrich) dissolved in 3 mL of water was added approximately 1 mL of 10 M sodium hydroxide. With a few drops of hydrochloric acid, the pH of this mixture was adjusted to 10–12, and the aqueous solution was evaporated to dryness. The residue was washed with dichloromethane several times to extract the free amine, and the resulting solution was filtered to remove any insoluble material. The dichloromethane solution was dried with potassium carbonate and the solvent removed to yield a pale yellow oil that was further dried in a desiccator under high vacuum overnight. Yield: 1.6 g (96%).

A 10 μmol (284 mg, 35.2 μmol/g) portion of protected 2′-deoxyadenosine bound to the CPG support was packed in a glass DNA synthesis column and placed on the synthesizer. The terminal trityl group was removed with trichloroacetic acid, and the support was washed with anhydrous acetonitrile. A 100 μmol (82 mg) portion of the appropriately protected H-phosphonate derivative of 2′-deoxyadenosine (as the triethylamine salt) was dissolved in 800 μL of pyridine/acetonitrile (1:1 v/v). Immediately before use the H-phosphonate was activated by the addition of 500 μmol (99 mg) of 1-adamantanecarbonyl chloride, and the resulting mixture was swirled for 30 s. This solution was added to the CPG support with a glass syringe, and the reaction was allowed to proceed for 18 min at ambient temperature. The CPG support was then washed with dry acetonitrile for approximately 4–5 min. The column was removed from the synthesizer, and the internucleotide H-phosphonate was oxidized by adding a solution of 400 mg of cystamine in 1.0 mL of pyridine and 200 μL of carbon tetrachloride using a glass syringe. The outflow of the column was fitted with an additional glass syringe to seal the reaction vessel. The oxidation reaction proceeded for 1 h and was mixed regularly by slowly pushing the cystamine solution back and forth between the two syringes. After 1 h, the support was washed with several mL of dry pyridine to remove excess cystamine from the CPG beads, placed on the DNA synthesizer, and washed with pyridine/acetonitrile (1:1) for at least 5 min and then with acetonitrile for 5 min. The support was further treated with the "standard" $I_2/H_2O$/lutidine/THF solution for 2 min to oxidize any unreacted hydrogen phosphonate diester to the phospho diester. Finally, the terminal trityl group was removed with trichloroacetic acid. The CPG support was placed in concentrated aqueous ammonium hydroxide overnight at 50 °C. After cooling and removal of the ammonium hydroxide, the dimer was isolated on a 9.4- × 250-mm column of MOS-Hypersil (3.0 mL/min) using 50 mM triethylammonium acetate (pH 7.0) and a gradient of

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acetonitrile (0–70% in 60 min). The product (eluting as two closely running peaks at 18.7 and 19.1 min) was collected, evaporated to dryness a number of times from water to remove the buffer, and lyophylized to dryness. Yield: 132 $A_{260}$ units, ~4.8 μmol (48% from the initial CPG-bound dA). UV ($H_2O$): $\lambda_{max}$ = 256 nm, $\lambda_{min}$ = 231 nm. $^1$H-NMR ($D_2O$) (TMS ext). The proton NMR spectrum was complicated by the presence of two diastereomers. Key groups of resonances are noted as follows: δ 2.23–2.75 (m, 8 H, $CH_2$, $H_{2'}$), 2.90 (m, 2 H, $CH_2$), 3.05 (m, 2 H, $CH_2$), 3.95–4.95 (m, carbohydrate H, HOD), 5.98 (m, 1 H, $H_{1'}$), 6.09 (m, 1 H, $H_{1'}$), 7.88–8.09 (multiple singlets, $H_2$, $H_8$) ppm. $^{31}$P-NMR ($D_2O$) ($H_3PO_4$ ext): δ 10.1, 10.2 ppm.

**Synthesis of d[Ap($NHCH_2CH_2S$-$SCH_2CH_2NHCOCH_3$)A].** This derivative was prepared in an analogous manner using 1 μmol (43 mg, 24 μmol/g) of protected 2′-deoxyadenosine derivatized CPG support packed into a DNA synthesis column. After the terminal trityl group was removed, the support was washed with anhydrous acetonitrile and 25 μmol (20.6 mg) of the H-phosphonate derivative of 2′-deoxyadenosine (as the triethylamine salt) was dissolved in 200 μL of pyridine/acetonitrile (1:1 v/v). Immediately before use 125 μmol (24.8 mg) of 1-adamantanecarbonyl chloride was added, and the H-phosphonate solution and the reaction mixture were swirled for 30 s. This solution was added to the support as described above. After a 6-min reaction time the support was washed, removed from the synthesizer, and oxidized with 200 mg of cystamine in 200 μL of pyridine and 100 μL of carbon tetrachloride. After a 1-h reaction time the support was washed as described above. At this point the terminal amine of the linker was capped by treatment with acetic anhydride and (dimethylamino)pyridine. The support was washed with acetonitrile and treated with $I_2$/$H_2O$/lutidine/THF, and the terminal trityl group was removed by a short treatment with trichloroacetic acid. The dimer was deprotected and isolated as described above. Yield: 13.5 $A_{260}$ units (0.49 μmol, 49%).

This material was obtained as a mixture of the acetylated and deacetylated disulfide linker and was further analyzed as described in the text.

**Incorporation of a Protected Thiol Residue during Assembly of an Oligodeoxynucleotide.** An oligodeoxynucleotide was elongated at a 1 μmol scale using standard phosphoramidite coupling procedures up to a residue located at the 5′ side of the tether. A hydrogen phosphonate derivative (25 μmol) was then introduced at a single site by coupling for 5 min in the presence of 5 equiv (125 μmol) of 1-adamantanecarbonyl chloride. The phosphonate diester thus formed was oxidized in the presence of 200 mg of cystamine in 300 μL of pyridine/carbon tetrachloride (2:1) for 1 h and then treated with acetic anhydride (or trifluoroacetic anhydride) to cap the terminal amine of the tether and any unreacted 5′ hydroxyl residues. Oligodeoxynucleotide synthesis was then continued in the normal manner,[6] followed by standard deprotection and HPLC isolation procedures.[7] This procedure typically resulted in yields of purified product that ranged from 30 to 50 $A_{260}$ units depending upon the sequence.

HPLC analysis (buffer A: 0.02 M $KH_2PO_4$, pH 5.5, B: 0.02 M $KH_2PO_4$, pH 5.5 containing 70% methanol, gradient: 0–100% B in 60 min) revealed an oligomer eluting as two closely related peaks in approximately equal proportions due to the chirality imparted upon the oligomer by the presence of the phosphoramidate diastereoisomers (*R*p and *S*p) linking the disulfide-containing tether.

**Nucleoside Analysis of d[CGCA($NHCH_2CH_2SSCH_2CH_2NHCOCH_3$)AAAAAGCG].** To 1 $A_{260}$ unit of the thiol-containing oligodeoxynucleotide was added 2 units of nuclease P1, and the mixture was incubated 30 min in 25 mM sodium acetate, pH 5.3, at 37 °C. The solution was then rebuffered to pH 8.0 using 200 mM Tris·HCl, 20 mM $MgCl_2$, and 3 units of snake venom phosphodiesterase, and 2 units of bacterial alkaline phosphatase were added followed by an additional incubation at 37 °C for 2–3 h. Using the HPLC conditions noted above, analysis of the resulting hydrolysate indicated the presence of five distinct species confirmed by comparison with authentic standards: 6.3 min (dC), 10.6 min, (dG), 15.0 min, (dA), two peaks at 28.4 and 29.7 min corresponding to the *S*p and *R*p diastereoisomers of d[Ap($NHCH_2CH_2SSCH_2CH_2NH_2$)A], and peaks centered at 30.7 min corresponding to the *S*p and *R*p diastereomers of d[Ap($NHCH_2CH_2SSCH_2CH_2NHCOCH_3$)A].

**Unmasking of the Tethered Thiol Residue.** Postsynthetic removal of the thiol protecting group was performed by incubating the disulfide-containing oligomer in a solution containing 11 mM dithiothreitol and 25 mM Tris·HCl pH 8.0 for 15 min at ambient temperature. The cleavage of the disulfide could be monitored by reversed-phase HPLC (Buffer A: 0.02 M $KH_2PO_4$, pH 5.5; B: 0.02 M $KH_2PO_4$, pH 5.5 containing 70% methanol; gradient: 0–100% B in 60 min). Analysis of the reaction indicated the presence of two closely related peaks reflecting the diastereomeric character of the oligomer. The degree of diastereomeric resolution varies with the particular sequence and the location of the tether within the strand. In a typical example, the *R*p and *S*p diastereomers of the dodecamer d[CGCA($NHCH_2CH_2SSCH_2CH_2NHCOCH_3$)AAAAAGCG] eluted from the column as two closely running peaks with a retention times of 20 and 20.6 min. After 15 min of reaction, the deprotected fragments, *R*p and *S*p d[CGCA($NHCH_2CH_2SH$)AAAAAGCG], eluted approximately 1 min earlier but with less resolution of the diastereomers.

**Nucleoside Analysis of d[CGCA($NHCH_2CH_2SH$)AAAAAGCG].** Treatment of a thiol-containing oligodeoxynucleotide with nuclease P1, snake venom phosphodiesterase, and bacterial alkaline phosphatase was performed as described above. Using the HPLC conditions noted above, analysis of the resulting hydrolysate indicated the presence of five distinct species confirmed by comparison with authentic standards: 6.3 min (dC), 10.6 min, (dG), 15.0 min (dA), and two peaks at 29.5 and 30.6 min corresponding to the *S*p and *R*p diastereoisomers of d[Ap($NHCH_2CH_2SH$)A].

**Labeling of a Thiol-Containing Dodecamer with 4-Chloro-7-nitrobenz-2-oxa-1,3-diazole.** A reaction mixture containing 0.08 mM d[CGCA($NHCH_2CH_2SH$)AAAAAGCG], excess DTT, and 8 mM 4-chloro-7-nitrobenz-2-oxa-1,3-diazole (NBD-Cl) was incubated at ambient temperature in 30 mM Tris pH 8.0 in a solution containing 33% DMF for 15 min. HPLC analysis (Buffer A: 0.02 M $KH_2PO_4$, pH 5.5; B: 0.02 M $KH_2PO_4$, pH 5.5 containing 70% methanol; gradient: 0–100% B in 60 min) after 15 min (see above) indicated that the oligodeoxynucleotide was >90% labeled (see Figure 3a). The products were isolated by HPLC using a 4.6- × 250-mm column of ODS-Hypersil, desalted using Sephadex G-10 or Sep-Pak (C18) cartridges and lyophilized to dryness.

**Tm Measurements.** Tm values were obtained in 10 mM sodium phosphate (pH 7.0) 1.0 M NaCl at duplex concentrations in the low micromolar range (see details in Table I). Absorbance values were measured with a Perkin-Elmer Lambda 3B UV/vis spectrophotometer equipped with digital temperature control. The solution temperatures were measured directly with a thermister probe (OMEGA Engineering, Stanford, CT). Absorbance and temperature data were collected after analog to digital conversion (DT-2800, Data Translation, Marlboro, MA) using an IBM-XT computer and the ASYST (version 1.53) scientific software package (MacMillian Software, New York, NY). Tm values were determined from first- and second-order derivatives of the absorbance vs temperature plots.

**Fluorescence Measurements.** Fluorescence spectra were collected on a Shimadzu RF5000U fluorescence spectrophotometer containing a Shimadzu DR-15 microprocessor and graphics display terminal. All measurements were done with the following list of parameters: slit width: $E_x/E_m$ = 5 nm/5 nm, high sensitivity, fast speed.

The thiol-labeled oligomers were isolated directly from the HPLC in the phosphate/methanol buffers, and the fluorescence scans were performed in this solvent without further treatment.

## Results

The method chosen for the incorporation of a thiol residue site specifically into oligodeoxynucleotides relies upon the amine oxidation chemistry originally developed by Todd and co-workers[8] and extended more recently by

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**Table I. Retention Times and Tm Values for Oligodeoxynucleotides Containing a Reporter Group Covalently Bound to the Backbone through a Thiol Tether**

| | reporter group (R) | retention time (min)<br>5′-d(CGCApAAAAAGCG) with $NHCH_2CH_2S{-}R$ on the phosphate | Tm (°C)[a,c]/[total strands] (μM)<br>3′-d(GCGTTTTTTCGC)<br>5′-d(CGCApAAAAAGCG) with $NHCH_2CH_2S{-}R$ on the phosphate |
|---|---|---|---|
| 1 | -H | 17.9, 18.4 | 52.4/4.0 |
| 2 | $-SCH_2CH_2NHCOCH_3$[b] | 18.9, 19.4 | 53.8/10 |
| 3 | | 19.6, 19.7[d] | 52.8/7.8 |
| 4 | | 19.3, 20.3 | 49.5/7.8 |
| 5 | | 20.2, 20.8 | 50.2/9.0 |
| 6 | | Isomer A 26.0<br>Isomer B 28.7 | 55.3/9.5<br>52.2/9.5 |
| 7 | | 26.4, 27.7 | 51.3/8.1 |
| 8 | | 33.7, 34.4<br>35.1, 35.6 | 51.2/9.0 |

[a] Error in Tm values is estimated to be ±0.5 °C. [b] A portion of the acetylated terminal amine is lost during ammonium hydroxide hydrolysis (see text and Figure 2). [c] With the exception of the pyrene-labeled sequences, all Tm values characterize the thermal stability of diastereomerically labeled sequences. [d] Although attachment of the PROXYL spin label adds a second stereogenic center to the labeled dodecamers, only two diastereomers were resolved.

Froehler,[9] Letsinger,[10] and Agrawal.[5f] In this approach an internucleotide H-phosphonate linkage is oxidized in the presence of a primary amine carrying the reporter group or linker of interest:

$$N{-}O{-}P(=O)(H){-}O{-}N \xrightarrow{R\text{-}NH_2,\ py,\ CCl_4} N{-}O{-}P(=O)(NHR){-}O{-}N$$

In the present case, a thiol residue can be introduced by choosing the appropriately protected aliphatic mercapto amine derivative. We have found that the simplest such derivative is cystamine, although other related derivatives should function equally well. Disulfides have been used effectively in other reports to protect thiol moieties during subsequent assembly of the oligodeoxynucleotide. For example, disulfides have been used previously to link (as well as protect) a 3′-terminal thiol residue to the solid-phase DNA synthesis support.[3r,11] The free thiol residue can then be unmasked after deprotection and purification of the DNA fragment by treatment with dithiothreitol (DTT) or other disulfide reducing agents.

**Preparation and Analysis of DNA Sequences Containing a Thiol Tether.** The procedure used to introduce the tethered thiol residue is analogous to other published work.[5f] The oligodeoxynucleotide was initially elongated using standard solid-phase-based phosphoramidite techniques. At the site of functionalization, a single H-phosphonate coupling was performed that was then oxidized in the presence of cystamine. The phosphonate monomer was incorporated after activation with adamantanecarbonyl chloride (Figure 1). The coupling efficiency at the step was indistinguishable from that at other positions using nucleoside phosphoramidites activated with tetrazole. After oxidation, the terminal amino group was acetylated (capped) and the sequence further elongated using standard phosphoramidite procedures. The capping step could be performed with acetic anhydride or with trifluoroacetic anhydride.[5f] We have typically used acetic anhydride at this step because the solution is readily available on the DNA synthesizer, but the acetamide formed at the terminus of the linker is largely resistant to the subsequent deprotection conditions employing ammonium hydroxide hydrolysis. However, after DTT treatment to unmask the free thiol, this portion of the linker containing the acetylated amine is lost (see below).

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**Figure 1.** Introduction of the protected thiol tether through oxidation of an H-phosphonate internucleotide linkage.

After completion of the synthesis, deprotection and isolation of the fragment generated two phosphoramidate diastereoisomers (*S*p and *R*p) linking the disulfide through a two-carbon chain to the DNA backbone. A small portion of the diastereomeric functionalized DNA fragments was degraded with nuclease P1 followed by treatment with snake venom phosphodiesterase and bacterial alkaline phosphatase. HPLC analysis of the hydrolysate resulting from degradation of the dodecamer d[CGCA($NHCH_2CH_2S-SCH_2CH_2NHCOCH_3$)AAAAAGCG] containing the protected thiol tether yielded, in addition to the appropriate peaks for the common nucleosides, a group of peaks eluting near 30 min (Figure 2a). Two dimers were prepared as standards for identification of the products obtained after nuclease degradation. In the first case, dApA was prepared with an H-phosphonate linkage and subsequently oxidized in the presence of cystamine. After deprotection, this dimer eluted from the HPLC column as a pair of diastereomers [e.g., d[Ap($NHCH_2CH_2S-SCH_2CH_2NH_2$)A]] (Figure 2c). In a similar synthesis, we prepared the same dimer but acetylated the terminal amine of the cystamine linker. After deprotection under standard conditions, this material eluted from the HPLC column as the series of peaks in Figure 2b. On this basis, we assigned the later eluting peaks of Figure 1a and 1b to the two diastereomers of the dimer containing the acetylated phosphoramidate linker [e.g. d[Ap($NHCH_2CH_2S-SCH_2CH_2NHCOCH_3$)A]] while the peaks eluting a few minutes earlier correspond to the deacylated derivative. The chromatograms of Figure 2a and 2b suggest that the terminal acetamide of the simple dimer (Figure 2b) is largely converted to the free amine during ammonium hydroxide deprotection (compare Figure 2b with 2c), while that of the dodecamer remains largely as the acetylated derivative (see Figure 2a). The two minor peaks eluting near 20 min (see Figure 2a) remain unassigned at present.

The phosphoramidate linkage was refractory to S1 nuclease hydrolysis as well as snake venom phosphodiesterase or nuclease P1 hydrolysis in agreement with previous work.[5d] The thiol residue was deprotected in a final step using dithiothreitol (DTT) to generate the DNA fragment containing the thiol tether. HPLC analysis of the thiol-containing DNA typically indicated the presence of two closely eluting peaks reflecting the diastereomeric character of the product. The degree of resolution of the diastereomers was dependent on both the DNA sequence and the location of the tether within the sequence. In some cases the diastereomeric character of the sequence was readily evident from the presence of two peaks in the HPLC chromatogram, while other diastereomeric sequences eluted almost as a single peak (see, for example, Figure 3a). Nuclease hydrolysis was employed to analyze the material obtained after DTT treatment. For example, nuclease and phosphatase hydrolysis of a small portion of d[CGCA($NHCH_2CH_2S-SCH_2CH_2NHCOCH_3$)AAAAAGCG] after DTT treatment followed by HPLC analysis generated the chromatogram shown in Figure 2d. The pair of peaks that eluted from the column near 30 min had the same retention time as that of the independently prepared dimer, d[Ap($NHCH_2CH_2S-SCH_2CH_2NH_2$)A], after treatment with DTT (see Figure 2e). On this basis we assigned the two peaks appearing in Figure 2d near 30 min as the two diastereomers of the thiol-containing dimer d[Ap($NHCH_2CH_2SH$)A].

**Reactions of Oligodeoxynucleotides Containing a Thiol Tether.** The functionalized DNA fragments prepared in this manner did not undergo degradation with the release of mercaptoethanol at pH 8.0 as described previously for similar phosphodiester derivatives.[3k,r,11b,12] Degradation in these cases presumably results from attack of the thiolate anion on the $\alpha$-carbon with elimination of the phosphate monoester; hydrolysis of the thiiran generated in this manner results in the production of the observed mercaptoethanol. This reaction would be disfavored in the present case where the phosphoramidate must function as the leaving group. We were also unable

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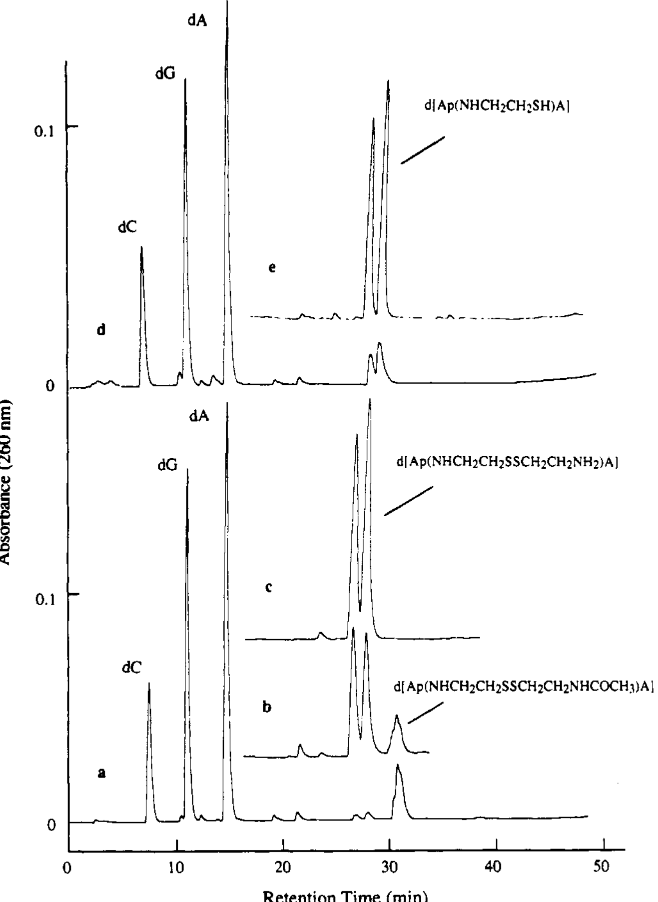


**Figure 2.** (a) HPLC analysis of the hydrolysate produced after the treatment of the dodecamer d[CGCA($NHCH_2CH_2SSCH_2CH_2NHCOCH_3$)AAAAAGCG] with nuclease P1, snake venom phosphodiesterase, and bacterial alkaline phosphatase (for specific conditions see Experimental Section). (b) HPLC analysis of the dimer d[A($NHCH_2CH_2SSCH_2CH_2NHCOCH_3$)A] after standard deprotection conditions. (c) HPLC analysis of the dimer d[A($NHCH_2CH_2SSCH_2CH_2NH_2$)A] after standard deprotection conditions. (d) HPLC analysis of the hydrolysate produced after the treatment of the dodecamer d[CGCA($NHCH_2CH_2SH$)-AAAAAGCG] with nuclease P1, snake venom phosphodiesterase, and bacterial alkaline phosphatase. (e) HPLC analysis of the dimer d[A($NHCH_2CH_2SH$)A] generated by treatment of d[A($NHCH_2CH_2SSCH_2CH_2NH_2$)A] or d[A($NHCH_2CH_2SSCH_2$-$CH_2NHCOCH_3$)A] with DTT.

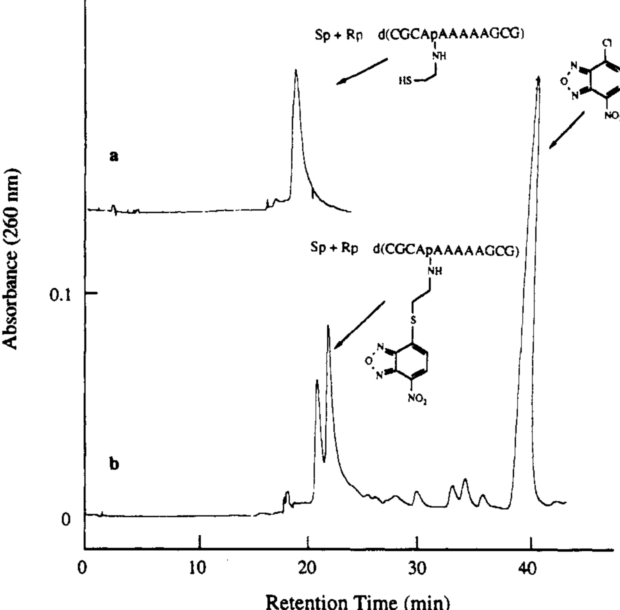


**Figure 3.** (a) HPLC analysis of the dodecamer d[CGCA-($NHCH_2CH_2SH$)AAAAAGCG] (for specific conditions see Experimental Section). (b) HPLC analysis of the mixture resulting from the reaction of the dodecamer of (a) with 4-chloro-7-nitrobenz-2-oxa-1,3-diazole.

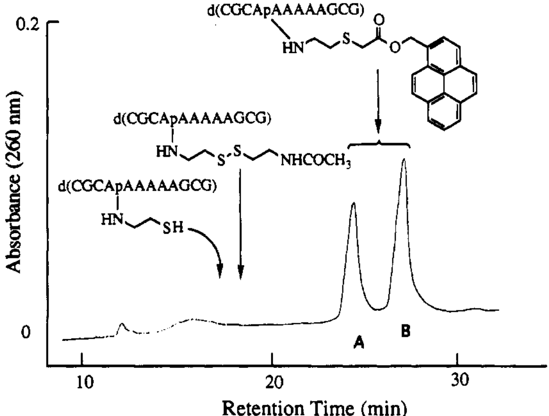


**Figure 4.** HPLC analysis of the reaction between the *R*p and *S*p diastereomers of the dodecamer d[CGCA($NHCH_2CH_2SH$)-AAAAAGCG] and 1-pyrenemethyl iodoacetate (the pyrene label was not eluted from the column during the gradient.

to detect any degradation products resulting from attack of the thiolate anion at phosphorus.

The thiol functionality linked by two carbons to the phosphoramidate of the DNA backbone is amenable to alkylation by a wide variety of thiol-specific reporter groups. Fluorophores, spin labels, or other reagents containing bromo(iodo)acetamido (or the corresponding acetate derivative), bromobenzyl, $\gamma$-bromo-$\alpha,\beta$-unsaturated carbonyls, maleimides, $\alpha,\beta$-unsaturated carbonyls, isothiocyanates, and chloro-substituted nitrobenzoxadiazole reactive groups could be employed to label the thiol functionality, typically with >90% yield in less than 15 min at pH 8.0 and ambient temperature. Under these conditions we could not detect any corresponding labeling of sequences lacking the thiol tether. Alkylation of the thiol residue could be performed without removal of the excess DTT, and attachment of the reporter groups to the sequence often enhanced the chromatographic resolution of the two diastereoisomers (compare Figure 3a with 3b). It is likely that some of the DTT is alkylated in these reactions, but these products did not appear to elute from the column or were minor in nature and did not affect isolation of the modified oligodeoxynucleotides (see, for example, the minor peaks eluting near 30 min in Figure 3b). In some cases the individual phosphorus diastereomers could be resolved and purified separately as illustrated for the analysis of the crude reaction mixture containing the pyrene derivative in Figure 4. The analysis of the pyrene-labeling reaction (Figure 4) is typical for reactions with hydrophobic reporter groups. The unreacted 1-pyrenemethyl iodoacetate as well as any reaction products resulting from the labeling of the excess DTT did not elute from the column during the course of the gradient. However, as indicated by the chromatograms of both Figures 3b and 4, unmasking the thiol with DTT followed by reaction with a variety of labels occurs site-specifically with very high yields.

Alkylation of the oligodeoxynucleotides by all of the fluorophores and spin labels examined to date resulted in an increase in HPLC retention time for the product DNA sequences carrying the reporter group (for selected examples see Table I). In general, two diastereomeric products,

corresponding to the *R*p and *S*p isomers of the phosphoramidate, were obtained during the reaction (see Figure 3b). However, with the use of the PROXYL and maleimide derivatives (see entries 2 and 8 in Table I), four diastereomers result since the PROXYL derivative is prepared as an enantiomeric mixture and alkylation of the maleimide functional group generates an additional stereogenic center. Only two of the four diastereomers of the PROXYL-labeled dodecamer could be resolved by HPLC while labeling with the maleimide derivative resulted in four chromatographically different species (see Table I). The thiol tether can be modified with a variety of thiol-specific alkylating agents that have generally been developed for site-specific modification for cysteine residues in proteins. As illustrated in Table I, we have examined the reaction of thiol-containing oligodeoxynucleotides with a variety of reporter groups including the iodacetamido-PROXYL spin label (entry 3), the fluorophores 4-chloro-7-nitrobenz-2-oxa-1,3-diazole (entry 4), monobromobimane (entry 5), 1-pyrenemethyl iodoacetate (entry 6), 6-acryloyl-2-(dimethylamino)naphthalene (entry 7), and 7-(diethylamino)-3-(4′-maleimidylphenyl)-4-methylcoumarin (entry 8).

The isolated modified oligodeoxynucleotides were examined spectroscopically. They all had UV absorption characteristics typical of DNA and exhibited fluorescence (or ESR) spectra characteristic of the label used (data not shown). Additionally, after labeling and isolation of the oligodeoxynucleotide, nuclease digestion as described above could be used to confirm the site of reaction in some cases. For example, after isolation of the 7-nitrobenz-2-oxa-1,3-diazole alkylated dodecamer (see Figure 3b and entry 4, Table I), a small portion of material was treated with nuclease P1, snake venom phosphodiesterase, and bacterial alkaline phosphatase. HPLC analysis of the resulting mixture indicated the presence of the three common 2′-deoxynucleosides (analogous to Figure 2d), but the peaks corresponding to the diastereomers of d[Ap($NHCH_2CH_2SH$)A] (at 29.0 and 30.0 min in Figure 2d) were absent and had been replaced by two new peaks at 41.7 and 42.3 min. Reaction of the simple dimer d[Ap($NHCH_2CH_2SH$)A with 4-chloro-7-nitrobenz-2-oxa-1,3-diazole also produced peaks with these retention times. This analysis could not be used with some of the large hydrophobic reporter groups since the labeled dimer could not be eluted from the column within the 60 min gradient. However, this analysis strongly suggests that the labels used were covalently bound to the oligodeoxynucleotides by alkylation of the thiol tether.

**Properties of the Labeled Oligodeoxynucleotides.** Most of the fluorescent and spin labels described here have not been reported to interact with duplex DNA. With few exceptions, annealing of the complementary native DNA strand to the labeled sequence produced a duplex with stability that was very similar to that of the unmodified thiol-containing duplex (Table I). This suggests that attachment of reporter groups to the DNA backbone through the described thiol functionality linked to an internucleotide phosphorus residue by two carbon atoms does not dramatically disrupt the helical structure or stability of the nucleic acid. Although 1–2 °C differences in Tm values between the free thiol linker and various labeled derivatives is larger than the estimated error (±0.5 °C), it is unclear if such slight changes represent a significant change in helix stability.

With one of the pyrene derivatives (see entry 6, Table I), an increase in Tm of approximately 3 °C was measured. The DNA dodecamer d[CGCA($NHCH_2CH_2SH$)AAAAAGCG]·d[CGCTTTTTTGCG] exhibited a Tm of 52.4 °C at a strand concentration of approximately 10 μM (1.0 M NaCl, pH 7.0), and this value was essentially the same as that obtained for the native dodecamer duplex. However, the stability of one of the pyrene isomers increased slightly, exhibiting a Tm value of 55.3 °C (isomer A) while the Tm for the second isomer (B) remained unchanged (see Table I). The 3 °C increase in Tm for isomer A is consistent with a stabilizing interaction, possibly intercalation, similar to that described by Yamana and Letsinger,[5b] Telser et al.,[2k] and Yamana et al.[13] for related pyrene-containing sequences.

We have additionally undertaken preliminary spectroscopic characterization of the two pyrene-labeled diastereomers. The absorbance maxima of the pyrene moiety ($\lambda_{max}$ = 333 and 349 nm) for the single stranded dodecamer (isomer A) was shifted by 9 nm upon complexation with the unlabeled complementary oligodeoxynucleotide ($\lambda_{max}$ = 324 and 340 nm for the pyrene labeled duplex). Isomer B exhibited a similar difference in absorbance maxima for the pyrene ligand bound to the single-stranded ($\lambda_{max}$ = 332 and 348 nm) and double-stranded complex ($\lambda_{max}$ = 324 and 340 nm). The pyrene fluorescence emission spectrum ($\lambda_{ex}$ = 338 nm) for the single-stranded fragment exhibited characteristic emission peaks at 376 and 395 nm. Upon annealing of the complementary sequence to isomer A, the emission maxima remained unchanged but the quantum yield for the duplex decreased approximately 50%. In a similar fashion, binding of the complementary sequence to isomer B did not significantly alter either of the emission maxima, but the quantum yield of pyrene fluorescence decreased 50%. This moderate decrease in quantum yield does not appear to be consistent with an intercalative interaction with the duplex DNA as has been suggested for other pyrene-containing duplexes.[2k,5b,13] The derivatives prepared by Telser et al.[2k] or Yamana and Letsinger[5b] exhibit more dramatic fluorescence quenching effects than we have observed in the present case. The fluorescence characteristics of DNA sequences containing a pyrene derivative bound to the 2′-hydroxyl of a uridine residue have not been reported in detail, but a preliminary paper[13] suggests that these authors observe a significant increase in fluorescence quantum yield upon duplex formation. In the present case, we observe only a slight quenching of the pyrene fluorescence upon duplex formation. Although, the 3 °C difference in Tm values does suggest that tethering a pyrene ligand to one of the diastereomeric sites on the internucleotide phosphorus residue facilitates a slight stabilizing interaction with the duplex nucleic acid, the fluorescence data obtained for this complex is not consistent with an intercalative mechanism.

## Conclusions

The incorporation of a thiol linker through an internucleotide phosphoramidate residue on the DNA backbone can be accomplished rapidly, simply, and sequence-specifically using the described procedures. This functionalization permits the incorporation of a wide variety of fluorophores, spin labels and other moieties into the DNA sequence.

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