to use. The stock solution was further diluted with DMSO and used immediately. The DMSO solution (10 μL) was added with a microsyringe to a solution (90 μL) containing the ^{32}P-labeled DNA in Tris·HCl buffer (25 mM, pH 7.4) to give a final concentration of 0.045 and 0.09 mM. After incubation at room temperature (1 h), the DNA was precipitated.

λ-Exo Digestion of Mitomycin C (1)-, 10-Decarbamoylmitomycin C (3)-, and 7-Aminoaziridinomitosene (4)-Modified DNA. The method used was the same as described previously.[6,15]

Purification of UVRA, UVRB, and UVRC Proteins. UVRA, UVRB, and UVRC proteins were isolated from *E. coli* K12 strain CH296 (*recA*, *endA*/F'lacI^{Q}) carrying plasmids pUNC45 (*uvrA*), pUNC211 (*uvrB*), and pDR3274 (*uvrC*).[26] The methods of purification were the same as described previously.[27]

UVRABC Nuclease Reactions. The UVRABC nuclease reactions were carried out in a reaction mixture (25 μL) containing 50 mM Tris·HCl (pH 7.5), 0.1 mM EDTA, 10 mM $MgCl_2$, 1 mM ATP, 100 mM KCl, 1 mM dithiothreitol, 15 mM UVRA, 15 mM UVRB, 15 mM UVRC, and substrate DNA. The mixtures were incubated at 37 °C (1 h), and the reactions were stopped by phenol–chloroform extractions followed by ethanol precipitation in the presence of aqueous NH_4OAc (2.5 M). The precipitated DNA was recovered by centrifugation and washed with 80% ethanol.

DNA Sequencing, DNA Sequencing Gel Electrophoresis, and Autoradiography. The 3' and 5' end-labeled DNA fragments were sequenced by the method of Maxam and Gilbert.[18] The ^{32}P-labeled fragments with or without various enzyme treatments were suspended in sequencing tracking dye (80% v/v deionized formamide, 10 mM NaOH, 1 mM EDTA, 0.1% xylene cyanol, and 0.1% bromophenol blue), heated at 90 °C (3 min), and quenched in an ice bath. The samples were applied to a 8% denaturing sequencing gel in parallel with the Maxam and Gilbert sequencing reactions. After electrophoresis the gels were exposed to Kodak X-Omat AR film with intensifying screen at −70 °C.

Densitometric Scanning. The intensities of UVRABC nuclease incision bands were determined with a Bio-Image Visage 100 System consisting of a high-resolution digitizing camera and whole band analysis software.

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Supplementary Material Available: Autoradiograms of UVRABC nuclease cutting and λ-exo digestion of mitomycin C (**1**) and 10-decarbamoylmitomycin C (**3**)-modified 3' end-labeled DNAs: Figure 7a, 146 base pair fragment from pBR322 plasmid (bottom strand); Figure 7b, 237 base pair fragment from pBR322 plasmid (top strand); Figure 7c, 142 base pair fragment from ØX 174 RF I DNA (top strand); Figure 7d, 178 base pair fragment from ØX 174 RF I DNA (bottom strand) (7 pages). Ordering information is given on any current masthead page.

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Site-Specific Labeling of DNA Sequences Containing Phosphorothioate Diesters

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Abstract: A phosphorothioate diester can be incorporated site-specifically into DNA sequences to provide a nucleophilic site that is amenable to alkylation by labels containing haloacetamide, aziridine sulfonamide, or γ-bromo-α,β-unsaturated carbonyl functionalities. Labeling reactions proceed most efficiently at 50 °C in the pH range 5.0–8.0 and require a number of hours for completion. HPLC techniques employing reversed-phase columns can be used to rapidly purify the labeled materials, and a variety of examples are shown including the purification of a site-specifically labeled 30-mer. Sequences containing a single diastereomeric phosphorothioate (R_p or S_p) can be prepared by synthesizing the appropriate dNp(s)N dimer block followed by resolution of the diastereomers and incorporation of either the S_p or R_p dimer into the sequence of interest. Oligodeoxynucleotides labeled in this manner are quite stable near neutral pH values, but the phosphorothioate triesters formed undergo hydrolysis under alkaline conditions. Double-stranded sequences containing the labeled phosphorothioate are less prone to base catalyzed hydrolysis than single-stranded sequences. Duplex structures containing a single backbone label are shown to have thermal stabilities that are generally very similar to those of the unlabeled sequences suggesting that little structural perturbation is present for sequences labeled by this technique. Labeling internucleotidic phosphorothioate diesters provides a rapid and simple procedure for the introduction of fluorophores, spin labels, or other moieties site-specifically without significant changes in standard phosphoramidite DNA synthesis techniques.

Introduction

A wide variety of techniques has been developed for the functionalization of oligodeoxynucleotides with reporter groups (for a recent review see Goodchild[1]). The 3'- or 5'-termini of short DNA fragments are amenable to chemical modification, and a number of procedures have described the incorporation of an appropriate amino or thiol tether that subsequently can be modified with the moiety of interest.[2] The use of end labeling procedures has been valuable in a number of subsequent studies, but this approach is less optimal in cases where the reporter group would be more effective if present internally within the sequence. The introduction of prosthetic groups to internal sites of a sequence

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can be accomplished by using the appropriately modified nucleoside residue.[3] In some cases, this employs specific functional groups present on the heterocyclic bases. For example, 4-thiouridine can be used for the covalent attachment of fluorophores containing α-haloacetamides to the sulfur residue at the 4-position.[4] A more general approach involves the chemical modification of base residues in order to introduce a tether terminating in a functional group that subsequently can be modified with the reporter group of choice. Tethers of this type have been attached most commonly to the C5-position of thymine[3b] or the N4-position of cytosine[3b,d] and typically terminate in amino or sulfhydryl residues. During the chemical synthesis of short DNA fragments, such modified pyrimidines can be introduced into the sequence to permit the site-specific attachment of reporter groups. One disadvantage with the use of modified bases to tether reporter groups is that the tether itself can result in some disruption of helical structure as indicated by decreases in thermal stability. For example, Telser et al.[3k] have reported that the introduction of an amino-terminated tether to either the C5-position of thymine or the N4-position of cytosine results in biphasic melting curves and an approximate change in free energy for helix formation of about 1 kcal/mol for a series of octanucleotides. MacMillian and Verdine[3m] have additionally reported a 6–7 °C decrease in T_m values for decamers containing a series of tethers attached to the exocyclic amino group of cytosine.

Labeling techniques directed toward the DNA backbone have been examined more recently.[5] A number of potential advantages are available with backbone labeling in comparison to end-labeling or base-labeling procedures. The use of internal phosphodiester residues instead of terminal phosphates means that the desired functionality can be placed at virtually any position within the sequence. Since the phosphodiester residues are not involved in interstrand DNA base pairing, the presence of a label on the DNA backbone may not alter drastically the stability (or structure) of duplex nucleic acids. The modification of the prochiral phosphodiester residue with a single moiety creates a chiral site and two phosphorus diastereomers (R_p and S_p). For many applications individual diastereomeric sequences may not be essential to obtain the desired information, but in other cases the control of such chirality may be critical. One of the phosphorus diastereomers will orient the reporter group toward the major groove-side of the duplex structure, while the other isomer will orient the attached group out, away from the helix. If the desired agent binds or reacts preferentially in one of the grooves, stereogenic labeling of the backbone should assist in enhancing such interactions (reactions).

One approach to the labeling of the DNA backbone has employed the oxidation of internucleotidic H-phosphonate derivatives in the presence of amines; a procedure developed initially by Todd and co-workers[6] and advanced more recently by Froehler,[7] Letsinger,[8] and Agrawal[5f] as well as ourselves.[5g] Oxidation of H-phosphonates in the presence of amines produces the corresponding phosphoramidates. When the amine carries the reporter group of interest[8] or functionality suitable for the introduction of such groups,[5f,g] site-specific labeling of the DNA sequence results. In a similar approach, an internucleotide phosphite triester can be oxidized in the presence of an amine and also generate a phosphoramidate with specific residues linked to the DNA backbone through the nitrogen residue.[5d]

The enzymatic incorporation of phosphorothioate diesters at multiple positions provides nucleophilic sites available for reaction with appropriate alkylating agents[9] or complexation with some transition metals.[10] We wish to report that single internal phosphorothioate diesters can be exploited for site-specific labeling of DNA sequences with fluorophores or spin labels. Oligodeoxynucleotides containing a single phosphorothioate diester can be prepared by simply using standard DNA synthesis procedures, and the sulfur residue generated in this fashion is susceptible to selective covalent labeling as we have described in an earlier communication.[5e]

Experimental Section

Materials and Equipment. Thin-layer chromatography (TLC) was performed on 5 × 10 cm silica gel 60 F_{254} glass-backed plates, 10 × 20 cm silica gel 60 F_{254} glass-backed plates with concentrating zone (for analytical use), or 20 × 20 cm silica gel 60 F_{254} glass-backed plates with concentrating zone (for preparative use, E. Merck, Darmstadt, Germany). For flash chromatography silica gel 60 (particle size less than 0.063 mm, E. Merck, Darmstadt, Germany) was used under positive pressure from nitrogen gas. High performance liquid chromatography (HPLC) was carried out on ODS-Hypersil (4.6 × 250 mm) or MOS-Hypersil (9.4 × 250 mm) (Shandon Southern, England), using a system of two Beckman 114M pumps, 163 variable wavelength detector, and 421A controller. Chromatograms were recorded and integrated with a Shimadzu C-R3A Chromatopac. ^{31}P NMR spectra were obtained with Varian XL-300 multinuclear spectrometer at 121.421 MHz. Absorption spectra were recorded by Perkin-Elmer Lambda 3B UV–vis spectrophotometer. Cell temperature was controlled by digital temperature controller. Oligodeoxynucleotides were synthesized by a phosphoramidite method on an Applied Biosystems 381A DNA synthesizer. Protected nucleoside 3′-*O*-(diisopropylaminomethoxyphosphine) and protected nucleoside 3′-*O*-(diisopropylamino-β-cyanoethoxyphosphine) were purchased from ABN or Cruachem (through Fisher Scientific, Pittsburgh, PA). N^6-Benzoyl-3′-(methoxyacetyl)-2′-deoxyadenosine was prepared by the previously described method[11] except that methoxyacetic anhydride was prepared in dichloromethane and was used for the preparation of N^6-benzoyl-3′-(methoxyacetyl)-2′-deoxyadenosine without purification. Nuclease P1 was purchased from GIBCO BRL (Gaithersburg, MD). Snake venom phosphodiesterase and alkaline phosphatase were purchased from Boehringer Manheim Biochemicals (Indianapolis, IN). The fluorophores monobromobimane, 5-{[2-[(iodoacetyl)amino]ethyl]amino}-naphthalene-1-sulfonic acid (1,5-I-AEDANS), *N*-{[2-(iodoacetoxy)-ethyl]-*N*-methyl]amino}-7-nitrobenz-2-oxa-1,3-diazole (IANBD), 5-iodoacetamidofluorescein (5-IAF), and 5-iodoacetamidoeosin (5-IAE) were obtained from Molecular Probes Inc. (Eugene, OR). The spin label 3-[2-(iodoacetamido]-2,2,5,5-tetramethyl-1-pyrrolidinyloxy, free radical (PROXYL) was obtained from Aldrich (Milwaukee, WI), and *N*-dansylaziridine was a product of Sigma (St. Louis, MO). Both of the dihydropyrroloindole derivatives of CC-1065 were generous gifts of Prof. Dale Boger, Department of Chemistry, Purdue University. All other reagents were purchased either from Aldrich (Milwaukee, WI) or Sigma (St. Louis, MO).

Methods. Preparation and Stability of the Diastereomeric Mixture of 5′-*O*-(N^6-Benzoyl-2′-deoxyadenosyl) 3′-*O*-[5′-*O*-(Dimethoxytrityl)thymidine] *O*-Methyl (or *O*-β-Cyanoethyl) Phosphorothioate [DMT-dTp(=S)(OCH_3)A^{Bz}, or DMT-dTp(=S)(OCH_2CH_2CN)A^{Bz}]. A solution of 5′-*O*-(dimethoxytrityl)thymidine 3′-*O*-(diisopropylaminomethoxyphosphine) (500 mg, 0.71 mmol) in dry acetonitrile was added dropwise over a period of 10 min to the suspension of N^6-benzoyl-3′-(methoxyacetyl)-2′-deoxyadenosine (147 mg, 0.46 mmol) and tetrazole (130 mg, 1.86 mmol) in dry acetonitrile under argon at room temperature. After stirring for 1 h, a solution of sulfur (150 mg, 4.65 mmol) in 1/1 dry carbon disulfide/2,6-lutidine (3 mL) was added to the reaction solution,

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and the mixture was stirred for additional 1 h. Excess sulfur was removed by filtration, and the filtrate was evaporated to dryness. The residue was dissolved with 50 mL of chloroform and washed with a 5% aqueous sodium bicarbonate solution and water. The organic layer was dried over sodium sulfate, and the solvent was removed by evaporation. The crude mixture of this fully protected dinucleoside was treated briefly with ammonium hydroxide to hydrolyze the methoxyacetyl group, and the product phosphorothioate was isolated by flash chromatography on silica gel column: yield, 240 mg (54%); ^{31}P NMR ($CDCl_3$) δ = 67.4, 67.1 (1% H_3PO_4 in D_2O ext).

To 33 mg of the diastereomeric mixture DMT-dTp(=S)$(OCH_3)A^{Bz}$ dissolved in 133 μL lutidine and 533 μL THF was added 17 mg of iodine and 17 μL D_2O. During the next 24 h, the ^{31}P NMR spectrum (peaks at 70.5 and 70.3 ppm) of this solution was monitored over the range −30 to 200 ppm (H_3PO_4 ext).

DMT-dTp(=S)$(OCH_2CH_2CN)A^{Bz}$ was prepared by an analogous procedure. It was dissolved in the oxidation solution, and the ^{31}P resonances observed (67.5 and 67.3 ppm) were monitored over the range −30 to 200 ppm (H_3PO_4 ext).

Preparation of R_p (3) and S_p (4) Diastereomers of 5′-*O*-(N^6-Benzoyl-2′-deoxyadenosyl) 3′-*O*-[5′-*O*-(Dimethoxytrityl)thymidine] *O*-Methyl Phosphorothioate. The diastereomers **1** and **2** were synthesized by procedures similar to those described previously.[12] A solution of 5′-*O*-(dimethoxytrityl)thymidine 3′-*O*-(diisopropylaminomethoxyphosphine) (882 mg, 1.25 mmol) in dry acetonitrile (5.0 mL) was added dropwise over a period of 10 min to the suspension of N^6-benzoyl-3′-(methoxyacetyl)-2′-deoxyadenosine (427 mg, 1.0 mmol) and tetrazole (280 mg, 4.0 mmol) in dry acetonitrile (5.0 mL) under argon at room temperature. After stirring for 1 h 15 min, a suspension of sulfur (320 mg, 10 mmol) in dry pyridine (15 mL) was added to the reaction solution, and the mixture was stirred for an additional 1 h. The reaction was worked up as described above. The two diastereomers were separated by silica gel chromatography using a gradient of methanol in chloroform (beginning with 1% methanol/chloroform and using 0.5% increments). Three fractions were collected to give a pure "fast" moving fraction, a "slow" moving fraction, and a fraction consisting of both the slow and the fast isomer. This latter fraction was further separated by preparative TLC (silica gel plate with a concentration zone) using chloroform–methanol–ether–water (200:20:60:1, v/v) as an eluent. After consecutive developments, the three zones were extracted with acetone to give a pure "fast" moving zone, "slow" moving zone, and middle mixture zone. Each of the pure isomers was combined with each pure isomeric fraction obtained by flash chromatography. Analyses were carried out by TLC on silica gel plates containing concentrating zones. After evaporation of each pure isomeric fraction, each fraction was treated with dioxane (8 mL) and 28% aqueous ammonia solution (2 mL) at room temperature for 1–2 h to remove the 3′ protecting group. The obtained 3′ deprotected dimers were purified by silica gel columns: yield, "fast" eluting isomer, 178 mg (18%), ^{31}P NMR ($CDCl_3$) δ = 67.5 ppm; "slow" eluting isomer, 221 mg (22%), ^{31}P NMR ($CDCl_3$) δ = 66.9 ppm.

Preparation of R_p (5) and S_p (6) 5′-*O*-(N^6-Benzoyl-3′-*O*-(diisopropylaminomethoxyphosphino)-2′-deoxyadenosyl) 3′-*O*-[5′-*O*-(Dimethoxytrityl)thymidine] *O*-Methyl Phosphorothioate. Either **3** or **4** was treated with diisopropylaminomethoxychlorophosphine by methods similar to those reported in the literature.[12] In a typical reaction, **3** (131 mg, 0.132 mmol) was dissolved in 3 mL of dry dichloromethane, and diisopropylethylamine (100 μL) was added to the solution. Diisopropylaminomethoxychlorophosphine (60 μL) was added to the solution with stirring at 0 °C under argon, and the reaction was maintained at 0 °C for 60 min. Six milliliters of ethyl acetate was added and washed with 5% aqueous sodium bicarbonate solution and saturated aqueous sodium chloride solution. The organic layer was added directly to a column of silica gel 60 (ca. 10 g) packed with ethyl acetate–triethylamine (99:1). Products were eluted with ethyl acetate–acetonitrile–triethylamine (80:19:1). Fractions containing the desired product were combined and evaporated to dryness. The residue was redissolved with dry dichloromethane, the solution was precipitated into petroleum ether, and the resulting white precipitate was collected on a glass filter. The product (**5**) obtained by this procedure was over 95% pure as judged by ^{31}P NMR: typical yield, 99 mg (65%); "fast" (*Sp*) isomer, ^{31}P NMR ($CDCl_3$) δ = 67.4, 147.1, 147.5 ppm; "slow" (*Rp*) isomer, ^{31}P NMR ($CDCl_3$) δ 66.9, 147.1, 147.4 ppm.

Enzymatic Hydrolysis of the Dimer and Corresponding Oligomer. For enzymatic digestion, fully protected dimer blocks were deprotected by treatment with thiophenol–triethylamine–dioxane (1:2:2, room temperature, 90 min),[16b] 28% aqueous ammonia solution (50 °C, 5 h), and 80% acetic acid (room temperature, 1 h). Nuclease P1 digestion of the dimer was carried out for 1 h at 37 °C in 50 mM sodium acetate solution (pH 5.3) containing 4 units of nuclease P1. Snake venom phosphodiesterase digestion of the dimer was carried out for 1 h at 37 °C in 25 mM Tris-HCl (pH 7.0) containing 10 mM magnesium chloride and 3 μL of snake venom phosphodiesterase solution.

Digestion of oligodeoxynucleotides containing a single phosphorothioate diester was performed with snake venom phosphodiesterase and alkaline phosphatase in 30 μL of 20 mM Tris-HCl (pH 8.0), 40 mM $MgCl_2$ for 1 h at 37 °C. Each reaction mixture was analyzed by reversed-phase HPLC.

Synthesis of Oligodeoxynucleotides Containing a Single Phosphorothioate Diester. Method A. Oligodeoxynucleotides containing a diastereomeric mixture of phosphorothioate linkages was prepared by altering the oxidation step of the synthetic cycle to use a sulfur oxidation procedure employing 2.5 M sulfur in carbon disulfide/2,6-lutidine (1:1) as previously described in the literature.[5e,12a,14b] Assembly of the sequence employed the 2′-deoxynucleoside β-cyanoethylphosphoramidites. Sequences prepared in this manner were partially deprotected by treatment with 28% aqueous ammonia for 6 h at 50 °C. The dimethoxytrityl-containing sequences were isolated as described below.

Method B. Stereochemically pure fragments containing a single phosphorothioate diester were prepared using normal phosphoramidite coupling procedures. At the appropriate position in the sequence, the R_p or S_p phosphorothioate dimer phosphoramidite building block was incorporated into the sequence by using the same coupling cycle and reaction time as employed for the "common" nucleoside phosphoramidite derivatives. These sequences were prepared using 2′-deoxynucleoside methoxyphosphoramidites. Sequences prepared in this manner were partially deprotected in two steps: (i) thiophenol/triethylamine/dioxane[16b] for 60 min ambient temperature and (ii) 28% aqueous ammonia for 6 h at 50 °C. Isolation then proceeded as described below.

Purification of the Phosphorothioate Diester-Containing Oligodeoxynucleotides. Purification of the modified oligodeoxynucleotides was performed with sequences containing the 5′-terminal dimethoxytrityl group by reversed-phase HPLC on MOS-Hypersil (9.4 × 250 mm) using 50 mM triethylammonium acetate (pH 7.0) and a gradient of acetonitrile (14.0–45.5% in 40 min).[13] Isolated strands were treated with 80% acetic acid solution for 20 min to remove the dimethoxytrityl group and desalted over a Sephadex G-10 column.

Labeling of the Phosphorothioate Diester-Containing Oligodeoxynucleotides. Oligodeoxynucleotides containing a single phosphorothioate diester can be site-specifically labeled in buffered solutions over a wide range of pH values. We have typically labeled the DNA fragments of interest within the pH range of 5.0–8.0. The labeling reaction will function at ambient temperature but the rates are accelerated with increasing temperature. The labeling of self-complementary sequences typically requires temperatures of 50 °C in order to remove secondary structure that appears to inhibit the labeling at phosphorus. Four example reactions are given below.

Labeling of the S_p Diastereomer of a 24-mer with 5-Acetamidofluorescein (5-IAF). A 63 μL reaction mixture containing 0.725 mM of S_p-5′-d[CTTGCGTACT(s)AGTTAACTAGTTCG], 16 mM of 5-IAF in 20 mM potassium phosphate buffer (pH 8.0), and 32% dimethylformamide was sealed in an eppendorf tube and submerged in a water bath at 50 °C for 13 h. After this time, HPLC analysis (see below) indicated that the reaction was approximately 86% complete. The product was isolated by HPLC as described below.

Labeling of a Dodecamer with *N*-((3-Bromo-2-propene)carbonyl)-2-(methoxycarbonyl)-1,2-dihydro-3*H*-pyrrolo[3,2-*e*]indole-2 (CDPI-Br). An 850 μL reaction mixture containing 0.19 mM of d[CGCA(s)AAAAAGCG], 3.7 mM CDPI-Br, 10 mM potassium phosphate (pH 6.0) and 30% dimethylformamide was sealed in an eppendorf tube and submerged in a water bath at 50 °C for 12 h. After this time period, HPLC analysis (see below) indicated that the reaction was approximately 85% complete. The product was isolated by HPLC as described below.

Labeling of a Self-Complementary Eicosomer with *N*-{[2-(Iodoacetoxy)ethyl]-*N*-methyl]amino}-7-nitrobenz-2-oxa-1,3-diazole (IANBD). A 112 μL reaction mixture containing 0.24 mM of d[CGTACT(s)AGTTACTAGTACG], 4.7 mM of IANBD, 20 mM potassium phosphate (pH 6.0), and 36% dimethyl formamide was sealed in an eppendorf tube and submerged in a water bath at 50 °C for 9 h. After this time period, HPLC analysis (see below) indicated that the reaction was approximately 90% complete. The product was isolated by HPLC as described below.

Labeling of a 30-mer with 5-Iodoacetamidoeosin (5-IAE). A 100 μL reaction mixture containing 0.323 mM of the phosphorothioate-containing 30-mer, d[CCCGTCCTAGCAAGCCGCTGCTACCGG(s)AGG], 6.67 mM of 5-IAE in 20 mM potassium phosphate buffer (pH 8.0) and 13% dimethylformamide was incubated for 30 h at 50 °C. After 30 h, HPLC analysis (see below) indicated that the reaction was approximately 70% complete. The product was isolated by HPLC as described below.

Purification of the Labeled Oligodeoxynucleotides. Labeled oligodeoxynucleotides were purified by HPLC using 4.6 × 250 mm column

of 5 μm ODS-Hypersil and 20 mM potassium phosphate (pH 5.5) with a gradient of methanol. Alternatively, the buffer system described above for the isolation of phosphorothioate-containing oligodeoxynucleotides could be employed. We have more commonly used the phosphate buffer system since the lower pH tends to prevent hydrolysis of the labeled triesters. After HPLC isolation, the labeled fragments were desalted (Sephadex G-10) and lyophylized to dryness. Purified products were stored at −20 °C.

Enzymatic Analysis of a Selected Labeled Oligodeoxynucleotide. To 0.25 A_{260} unit of diastereomeric d[CGCT(s)TTTTTGCG] labeled with the iodoacetamido PROXYL spin label in 5 μL of 50 mM sodium acetate pH 5.3 was added 2 units of nuclease P1. After a 6-h incubation at 37 °C, the reaction mixture was rebuffered by the addition of 10 μL 200 mM Tris·HCl (pH 8.0), 20 mM $MgCl_2$. To this mixture was added 2 units of snake venom phosphodiesterase and 2 units of bacterial alkaline phosphatase, and the mixture incubated an additional 18 h at ambient temperature. The hydrolysate was analyzed by reversed-phase HPLC using a 4.6 × 250 mm column of 5 μm ODS-Hypersil and 20 mM potassium phosphate (pH 5.5) with a gradient of methanol (0–70% in 60 min).

Hydrolytic Stability of the Labeled Phosphorothioate Diesters. The labeled oligodeoxynucleotides were incubated in 25–50 mM buffer at ambient temperature in the dark, and aliquots were removed at various time periods and analyzed by HPLC using a 4.6 × 250 mm column of 5 μm ODS-Hypersil and 20 mM potassium phosphate (pH 5.5) with a gradient of methanol (0–70% in 60 min). The buffers used for the respective studies were potassium phosphate pH 7.0, Tris·HCl pH 8.0, and 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS), pH 10.0.

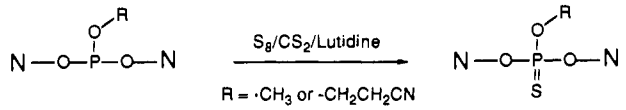
T_m Measurements. T_m values were obtained in 10 mM sodium phosphate (pH 7.0) and 150 mM or 1.0 M sodium chloride at duplex concentrations in the low micromolar range (see details in Tables II and IV). Absorbance values were measured with a Perkin-Elmer Lambda 3B UV–visible spectrophotometer equipped 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). T_m 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 5-nm excitation and emission slit widths.

The phosphorothioate labeled oligomers were isolated directly from the HPLC in the phosphate/methanol buffers, and the fluorescence scans to confirm the presence of the fluorophore were performed in this solvent without further treatment.

Results

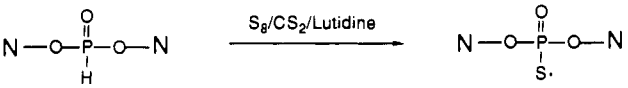
The synthesis of an oligodeoxynucleotide containing internucleotidic phosphorothioate diesters can be accomplished by both chemical[14] and enzymatic (for a review see Eckstein[15]) means, but the chemical approach is more versatile for the specific placement of a single residue within a relatively short sequence. In the present study, a single phosphorothioate triester was introduced at a preselected site within a sequence during the assembly of an oligodeoxynucleotide by oxidation of the intermediate phosphite triester obtained during standard phosphoramidite chemistry[16] with elemental sulfur in carbon disulfide/2,6-lutidine, similar to previously reported methods.[12a,14b] Oxidation generates a pentavalent phosphorus at this site in which the phosphorothioate triester exists as a thione:



The site-specific placement of a phosphorothioate in this manner requires that the triester be stable during the remaining synthetic steps necessary to complete the assembly of the sequence. To test the stability of the phosphorothioate triester to subsequent steps of the synthetic cycle, particularly further oxidations with iodine in water, we prepared the two dimers DMT-Tp(=S)(-OCH_3)A^{Bz} and DMT-Tp(=S)(OCH_2CH_2CN)A^{Bz} each containing a phosphorothioate triester. The purified materials, containing the methoxy or β-cyanoethoxy protecting group, were dissolved in the oxidizing solution (I_2/D_2O/THF/pyridine) and each exhibited two ^{31}P NMR ($CDCl_3$) resonances at 70.5, 70.3 and 67.5, 67.3 ppm, respectively. After incubation for a number of hours under these conditions, we were unable to detect any new signals.

After assembly of sequence containing a single phosphorothioate triester, deprotection of the oligodeoxynucleotide with aqueous ammonia (or thiophenol/triethylamine/dioxane followed by aqueous ammonia) removed the phosphorus protecting groups and generated an oligodeoxynucleotide containing a single phosphorothioate diester. Oligodeoxynucleotides containing a single phosphorothioate diester could be isolated by HPLC methods (see Experimental Section) under conditions that were identical to those used for unmodified sequences.

We should note that phosphorothioates can also be formed from the reaction of an internucleotidic H-phosphonate with elemental sulfur.[7] However, in this approach the H-phosphonate is oxidized directly to the phosphorothioate *diester*. The diester species is



not compatible with subsequent oxidations employing I_2/H_2O/THF/lutidine as the phosphorothioate diesters react quickly with iodine resulting in desulfurization and conversion to the phosphate diester.[17] While the H-phosphonate method has been employed efficiently to introduce phosphorothioates at all positions within a sequence,[7a,18] it is less useful for the introduction of a single internal phosphorothioate diester.

Synthesis of Oligodeoxynucleotides Containing a Single Phosphorothioate Diester Diastereomer. The chemical oxidation of the intermediate phosphite triester with sulfur resulted in roughly an equal mixture of the R_p and S_p diastereomers of dTp(s)A based upon the ^{31}P NMR spectra (data not shown). Reaction of this mixture with the desired label or agent yielded a corresponding isomeric mixture of labeled DNA fragments. With short DNA fragments it was sometimes possible to chromatographically resolve the two diastereomers after labeling with a reporter group, but this was not an effective general approach; with longer fragments there was often little difference in HPLC retention times between the labeled R_p and labeled S_p isomer. In order to effectively generate R_p or S_p labeled DNA, it was most efficient to first generate the isomerically pure phosphorothioate dinucleotide building block using procedures similar to those previously reported.[12] In this study, the two dimers **1** and **2** (Figure 1) were prepared from 5′-*O*-(dimethoxytrityl)thymidine 3′-*O*-diiso-

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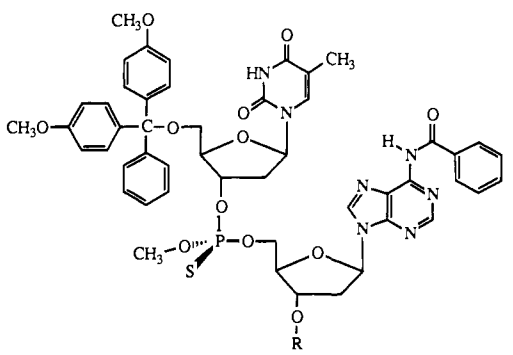
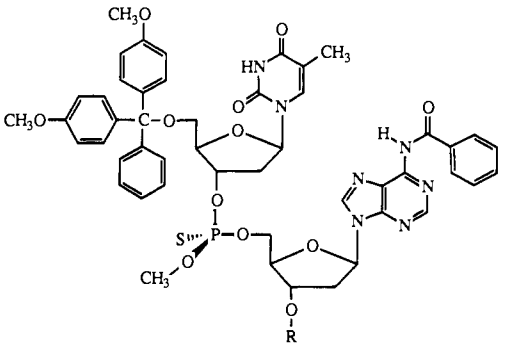




Rp Diastereomer		^{31}P-NMR
1	R = -(C=O)CH_2OCH_3	–
3	R = -H	66.9 ppm
5	R = -$P(OCH_3)N(iPr)_2$	66.9 147.1, 147.4

Sp Diastereomer		^{31}P-NMR
2	R = -(C=O)CH_2OCH_3	–
4	R = -H	67.5 ppm
6	R = -$P(OCH_3)N(iPr)_2$	67.4 147.1, 147.5

Figure 1. Structures of the partially and fully protected dimers of R_p and S_p-dTp(s)A.

Table I. Analytical Data for the dTp(s)A Dinucleotide Phosphorothioate

	σ/ppm ^{31}P NMR[a]	retention time (min)[b]	nuclease P1 digestion[c]	SVPD digestion[c]	absolute configuration
fast	57.6	27.7	+	-	S_p
slow	58.4	26.0	-	+	R_p

[a] ^{31}P NMR spectra were measured in D_2O relative to 85% phosphoric acid (external). [b] HPLC conditions: 4.6 × 250 mm ODS-Hypersil, 50 mM triethylammonium acetate (pH 7.0), acetonitrile 0–21% (42 min), 1.0 mL/min. [c] (+) indicates that the phosphorothioate diester was cleaved, and (-) indicates that the phosphorothioate diester was not cleaved by the nuclease.

propylaminomethoxyphosphine and N^6-benzoyl-3′-(methoxyacetyl)-2′-deoxyadenosine. The diastereomers resulting from this synthesis were separated by flash chromatography using a relatively long silica gel column and slowly increasing the gradient of methanol in chloroform. In our hands, the two diastereomers could be only separated effectively prior to deprotection of 3′-hydroxyl group (present as a methoxyacetyl ester). After deprotection of the 3′-terminal hydroxyl, the ^{31}P NMR spectra of the isolated diastereomers **3** and **4** (Figure 1) indicated that each dimer was ≥98% pure. After complete deprotection of each dinucleoside phosphorothioate, the absolute configurations were determined from their sensitivities to nuclease hydrolysis. Under standard assay conditions, the fast isomer was a substrate for nuclease P1 but was not hydrolyzed by snake venom phosphodiesterase (Table I). The slow isomer exhibited the reversed nuclease sensitivities. Upon the basis of previous studies,[14a] the relative nuclease sensitivities suggested that the fast isomer has the S_p configuration, while the slow isomer has the R_p configuration. HPLC analysis of the fully deprotected dimers using a reversed-phase column indicated that the fast isomer had a longer retention time than slow isomer, and this observation is consistent with the results of several reports.[12,14a,c] Additionally, for both the fully protected and deprotected dimers, the S_p diastereomer resonated at higher field (^{31}P NMR) in agreement with other work.[12a]

The fully protected dinucleoside phosphorothioates were then converted to the phosphoramidite building blocks **5** and **6** (Figure 1) by the reaction with diisopropylaminomethoxychlorophosphine. The isolated products were ≥95% pure as judged from ^{31}P NMR spectra. Each dimer phosphoramidite was used in automated solid-phase based DNA synthesis without modification of the reaction cycle. Upon the basis of the color of the solutions containing the dimethoxytrityl cation, and HPLC profiles of crude products obtained after deprotection of the sequence, the dimers could be incorporated into sequences without any change in coupling efficiency. Oligodeoxynucleotide products were cleaved from support, deprotected by the normal method, and purified by reversed-phase HPLC.[13]

Oligodeoxynucleotides containing a single diastereomeric phosphorothioate linkage were also analyzed by nuclease digestion. A typical chromatogram is shown in Figure 2 for the analysis of both the S_p and R_p diastereomers of 5′-d[CTTGCGTACT(s)AGTTAACTAGTTCG]. Each fragment was treated with snake venom phosphodiesterase and alkaline phosphatase. HPLC analysis of the reaction containing the S_p diastereomer resulted in four peaks corresponding to dC, dG, dT, dA, and a fifth peak for S_p-dTp(s)A (Figure 2a). The dimer was refractory to snake venom phosphodiesterase activity under the described conditions. By comparison, HPLC analysis of the reaction containing the R_p

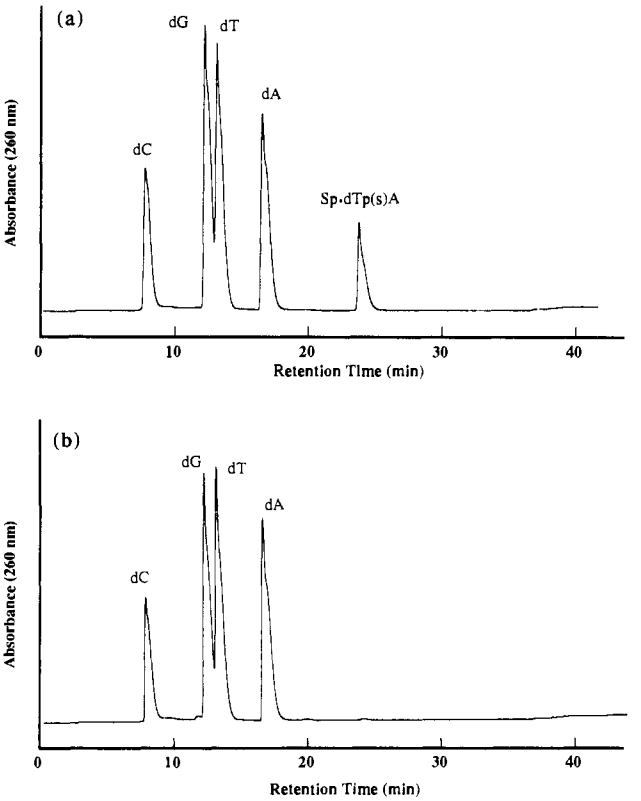
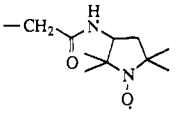
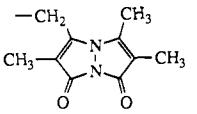
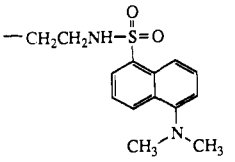
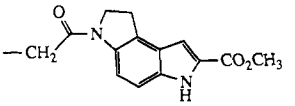
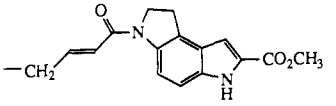


Figure 2. HPLC analysis of the hydrolysate resulting from the treatment of (a) the S_p diastereomer and (b) the R_p diastereomer of 5′-d[CTTGCGTACT(s)AGTTAACTAGTTCG] with snake venom phosphodiesterase and bacterial alkaline phosphatase. HPLC conditions: column, 4.6 × 250 mm ODS-Hypersil; buffer, 20 mM KH_2PO_4, pH 5.5; gradient, 0–70% methanol in 60 min.

Table II. Retention Times and T_m Values for Oligodeoxynucleotides Containing a Reporter Group Covalently-Bound to a Phosphorothioate Diester

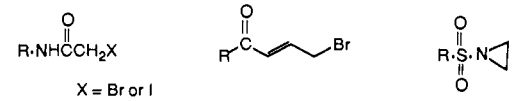
compd no.	reporter group (R)	retention time[a] (min)[d] 5′-d[CGCA(s-R)-AAAAAGCG]	T_m[b] (°C)/[total strands] (μM) 3′-d(GCGT-TTTTTCGC) 5′-d[CGCA(s-R)AAAAAGCG]
7	no substituent	15.6[d]	55.1/6
8		18.6, 19.4[c]	54.6/9
9		19.2, 20.1	52.0/5
10		24.1, 25.4[c]	51.0/5
11		22.2[d]	53.8/13
12		24.9[d]	54.1/11

[a] HPLC conditions: 4.6 × 250 mm ODS-Hypersil, 20 mM KH_2PO_4 (pH 5.5), 0–70% methanol, 60 min. [b] T_m values were obtained in 10 mM NaH_2PO_4 (pH 7.0) and 1.0 M NaCl. Estimated error is ±0.5 °C. [c] The PROXYL label has a chiral site, but only two of the four possible diastereomers are resolved. [d] Both phosphorothioate diastereomers elute as a single peak.

isomer produced only the four "common" 2′-deoxynucleosides and no phosphorothioate dimer (Figure 2b). These results confirmed that the former sequence contained the S_p phosphorothioate diastereomer while the latter sequence was of the R_p configuration.

Labeling of the Phosphorothioate-Containing Oligodeoxynucleotides. The phosphorothioate diester is ionized throughout a wide pH range, and we have examined labeling reactions over the pH range 5–8. We did not examine the labeling of sequences at values below pH 5 since we were concerned that competing acid catalyzed depurination would begin to introduce impurities into the reaction mixture: above pH 8, base catalyzed hydrolysis of the product phosphorothioate triester reduced overall yields (see below). Some labeling occurred at ambient temperature, particularly with small fragments such as dimers, but higher yields resulted when the reactions were performed at 50 °C. Higher temperatures appeared to enhance yields by removing secondary structure (particularly for self-complementary sequences).

Three functional groups could be employed to covalently alkylate the phosphorothioate diester. Reagents containing γ-bromo-α,β-unsaturated carbonyls, iodo(or bromo)acetamides, and aziridinylsulfonamides, all functioned effectively to label the sulfur residue and produce the corresponding phosphorothioate triester:



Both the R_p and S_p diastereomers exhibited equal reactivity in all reactions monitored to date.

The concentration and quantity of label required for an efficient reaction with the phosphorothioate diester varied significantly depending upon the functional group employed for the alkylation since the rates of competing hydrolytic reactions of the agents themselves also varied. Aqueous/dimethylformamide solutions were employed to solvate both the oligodeoxynucleotide and the label or agent of interest. In general, the reporter group was dissolved in dimethylformamide and then added to the oligodeoxynucleotide in aqueous buffer. The amount of dimethylformamide required for a given reaction mixture was dependent upon the solubility of the appropriate reagent. Reaction solutions could contain dimethylformamide concentrations up to 55%, but precipitation of the oligodeoxynucleotide became problematic with higher concentrations.

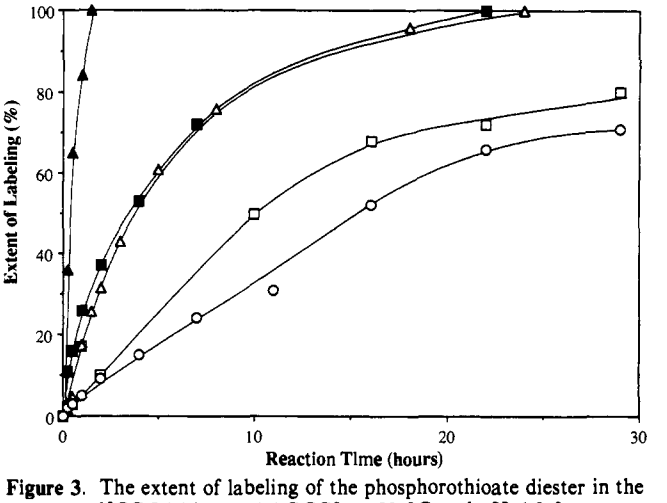


Figure 3. The extent of labeling of the phosphorothioate diester in the sequence d[CGCA(s)AAAAAGCG] at 50 °C and pH 6.0 for monobromobimane (▲) forming **9**, the spin label 3-[2-(iodoacetamido)]-2,2,5,5-tetramethyl-1-pyrrolidinyloxy free radical (PROXYL) (△) forming **8**, the bromoacetamido dihydropyrroloindole derivative (■) forming **11**, *N*-dansylaziridine (□) forming **10**, and γ-bromo-α,β-unsaturated carbonyl dihydropyrroloindole derivative (○) forming **12**.

The rates of labeling for different reagents varied significantly. For example, the extents of reaction at various times between the dodecamer 5′-d[CGCA(s)AAAAAGCG] and the iodoacetamido PROXYL spin label (yielding **8**, Table II), monobromobimane (yielding **9**, Table II), dansyl aziridine (yielding **10**, Table II), bromoacetamido-CDPI (yielding **11**, Table II), or the corresponding γ-bromo-α,β-unsaturated amide derivative (yielding **12**,

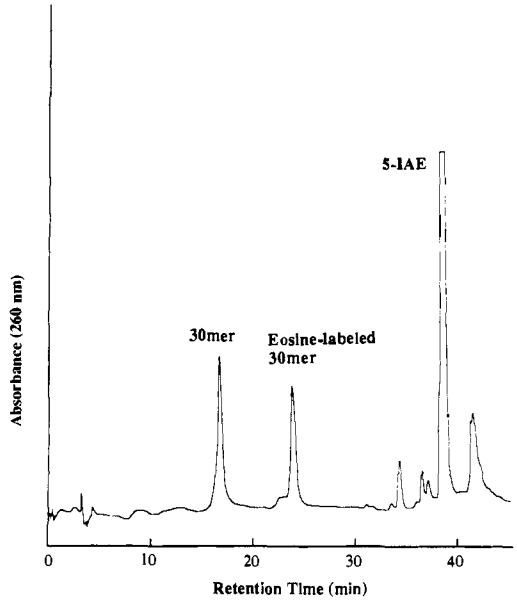


Figure 4. HPLC analysis of the reaction between the 30-mer d-[CCCGTCCTAGCAAGCCGCTGCTACCGG(s)AGG] and iodoacetamidoeosin. HPLC conditions: column, 4.6 × 250 mm ODS-Hypersil; buffer, 20 mM KH_2PO_4, pH 5.5; gradient, 0–70% methanol in 60 min.

Table II) at pH 6.0 and 50 °C are illustrated in Figure 3. Of the reporter groups examined, the reaction with monobromobimane was most rapid and was complete within 2 h. The reactions using either of the haloacetamido derivatives (bromo or iodo) gave similar results but required 24 h for completion. The least reactive derivatives were the aziridine sulfonamide (dansyl aziridine) and the γ-bromo-α,β-unsaturated carbonyl derivative; in these cases the extent of labeling was 70–80% complete after 30 h of reaction. We were unable to detect any significant reaction between the reporter groups and DNA sequences lacking a phosphorothioate diester in the case of monobromobimane or any of the haloacetamide or γ-bromo-α,β-unsaturated carbonyl derivatives. The analysis of these reactions often indicated the presence of a number of small peaks (see Figure 4), but these reflected impurities in the fluorophore preparation or associated hydrolysis products. Reactions employing dansylaziridine often contained a number of small additional unidentified products. These peaks were minor in character and may reflect the presence of nonspecific base modification, but alkylation of the phosphorothioate remained the major product of the reaction.

Self-complementary sequences up to 20 residues in length could be labeled at a single phosphorothioate residue, generally with high yields, after incubation overnight at 50 °C. For example, the four 20-mers 5′-[CGTACTAGTT(s)AACTAGTACG], 5′-[CGTACTAGT(s)TAACTAGTACG], 5′-[CGTACTAG(s)-TTAACTAGTACG], and 5′-[CGTACT(s)AGTTAACTAG-TACG] all differing in the location of the phosphorothioate diester were labeled with *N*-{{[2-(iodoacetoxy)ethyl]-*N*-methyl}amino}-7-nitrobenz-2-oxa-1,3-diazole (IANBD), the iodoacetamido PROXYL spin label or monobromobimane. After an overnight incubation at pH 6.0 and 50 °C, the labeling reactions were 80–90% complete.

Sequence length (<30 residues) appeared to effect the reaction rate only marginally. For example, HPLC analysis indicated that a series of single-stranded 24-mers could be labeled with 5-iodoacetamidofluorescein or 5-iodoacetamidoeosin with yields greater than 79% after reaction times of 12–16 h at 50 °C (data not shown). Additionally, the reaction between 5-iodoacetamidoeosin and the 30-mer 5′-d[CCCGTCCTAGCA-AGCCGCTGCTACCGG(s)AGG] was 70% complete after a 30-h incubation at 50 °C.

Isolation of the Labeled Phosphorothioate Diesters. The labeled oligodeoxynucleotides were purified using HPLC and reversed-phase columns. With relatively short sequences (~12 residue or less) containing diastereomeric phosphorothioate diesters, the

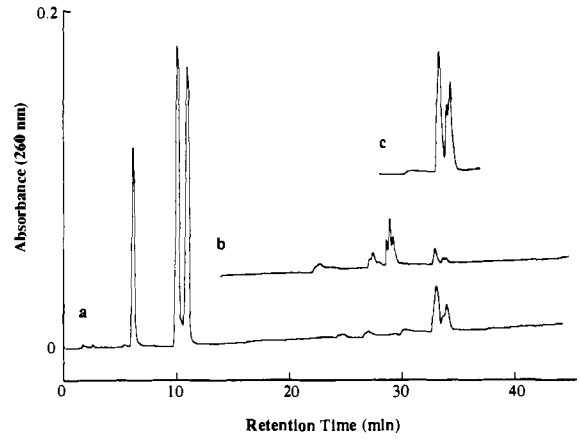


Figure 5. (a) HPLC analysis of the hydrolysate produced from the reaction of racemic d[CGCT(s)TTTTTGCG] (labeled with the iodoacetamido PROXYL) with nuclease P1, snake venom phosphodiesterase, and bacterial alkaline phosphatase for a total incubation time of 24 h. (b) HPLC analysis of the reaction mixture in (a) after a total incubation time of 2 h. (c) Retention times for diastereomeric dTp(s)T labeled with the iodoacetamido PROXYL spin label. HPLC conditions for all analyses: column, 4.6 × 250 mm ODS-Hypersil; buffer, 20 mM KH_2PO_4, pH 5.5; gradient, 0–70% methanol in 60 min.

existence of two isomeric sequences was often evident from the presence of two closely eluting peaks in the HPLC chromatogram. However, the resolution of such diastereomers was often lost with longer sequences.

Alkylation of a phosphorothioate diester converts a negatively charged diester into a neutral triester. Additionally, the reporter groups of interest are often hydrophobic in nature. Both of these characteristics result in the labeled product eluting from the reversed-phase matrix with a longer retention time than the unlabeled material (see Table II). This separation was usually sufficient to ensure that no unlabeled material was collected with the isolated product. Using this approach, it was quite simple to resolve an eosin-containing 30-mer from the unlabeled sequence using HPLC (Figure 4). In rare instances, for example, the product of the reaction between a dodecamer and 5-((2-((iodoacetyl)amino)ethyl)amino)naphthalene-1-sulfonic acid, purification was more difficult. In this case alkylation of the phosphorothioate diester generated the neutral triester, but the dansyl group itself carries a negatively charged sulfonic acid residue that tended to reduce differences in polarity between the labeled and unlabeled sequences. With the use of a shallow methanol gradient, small quantities of the dansyl labeled dodecamer could be suitably resolved, but we were unable to purify longer dansyl-labeled sequences.

Analysis of the Labeled Oligodeoxynucleotides. The phosphorothioate triesters were labile to base catalyzed hydrolysis (see below), and this property was often used to assist in identifying labeled sequences. After incubation of a labeled dodecamer in 50 mM 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS), pH 10.0, for 2–3 h at 50 °C, HPLC analysis indicated that the peak corresponding to the labeled material was reduced in size, and a new peak corresponding to the unlabeled phosphodiester-containing sequence was present. However, the most direct analysis of the labeled materials involved their digestion by nucleases and subsequent HPLC resolution of the hydrolysate. For example, after labeling of R_p- and S_p-5′-d[CGCT(s)TTTTTGCG] with the PROXYL spin label, enzymatic hydrolysis with nuclease P1 (1 h), followed by treatment with snake venom phosphodiesterase and bacterial alkaline phosphatase (1 h) (conditions that would completely hydrolyze the unlabeled sequence), HPLC analysis resulted in three peaks corresponding to the "common" 2′-deoxynucleosides dG, dC, and dT with additional peaks at later elution times (Figure 5b). The final group of peaks had the same elution times as those obtained for the dimer dTp(s)T after reaction with the iodoacetamido PROXYL spin label (Figure 5c). The remaining peaks were assumed to result from incomplete hydrolysis of the sequence. Attachment of the spin label to the

Table III. Hydrolytic Stability of a Labeled Dimer, ss Dodecamer, and ds Eicosomer[a]

complex	% hydrolysis after 24 h at ambient temp[b] pH 7.0	pH 8.0	pH 10.0
PROXYL-labeled dTp(s)T	10	29	100
PROXYL-labeled 5′-d[CGCA(s)AAAAAGCG]	7	31	100
PROXYL-labeled 5′-d[CGTACTAGTT(s)AACTAGTACG] 3′-d[GCATGATCAA(s)TTGATCATGC]	<1	<3	63

[a] All three complexes were formed by the reaction of iodoacetamido-PROXYL with the phosphorothioate dimer, dodecamer or eicosomer. [b] Accuracy is estimated to be ±5%.

dimer incorporates a second stereogenic site, and HPLC analysis of the labeled dimer could resolve three of the four diastereomers present (Figure 5c, also present in Figure 5b). It was necessary to increase the total incubation time of this reaction to 24 h before the partial hydrolysis products could largely be eliminated from the analysis (Figure 5a). After the extended incubation, in addition to the common nucleosides, the only remaining peaks present in the chromatogram corresponded to the labeled dTp(s)T dimer (Figure 5a). This analysis indicated that the labeling had occurred at the site of the phosphorothioate and that the phosphorothioate triester formed in this manner was itself refractory to nuclease hydrolysis. This analysis could not be performed in all cases since often the hydrophobic nature of the fluorophore precluded elution of the labeled dimer from the column, or significant hydrolysis of the labeled dimer occurred during the extended incubation time under the pH 8 conditions of the nuclease/phosphatase digest.

The oligodeoxynucleotides containing fluorophores were all analyzed spectroscopically to confirm that they exhibited the UV absorption properties of DNA and the fluorescent properties of the label in question (data not shown).

Hydrolytic Stability of Labeled Oligodeoxynucleotides. In order for the oligodeoxynucleotides labeled by these procedures to be valuable for further studies, including those of structure and dynamics, it is necessary that the phosphorothioate triesters be reasonably stable in aqueous solutions. In our hands, hydrolysis of the phosphorothioate triester proceeded almost exclusively by P–S bond cleavage to yield the native oligodeoxynucleotide with an internucleotidic phosphodiester in place of the phosphorothioate triester (minor amounts of chain cleavage can also occur and this phenomenon has been the basis for phosphorothioate-based DNA sequencing[9a]). We have not examined the fate of the reporter group in these experiments but have monitored the conversion of phosphorothioate triester to phosphate diester.

Initial experiments had indicated that the triester formed from the dimer, dTp(s)T, was stable for long periods of time at pH values below 7 but was rapidly hydrolyzed at values above pH 7. To compare the stability of a simple dimer with that of a single-stranded (ss) dodecamer and a double-stranded (ds) eicosomer (20-mer) the appropriate PROXYL-labeled derivatives were incubated at pH values of 7.0, 8.0, and 10.0 and ambient temperature over a 24-h time period. Under these conditions the extent of hydrolysis of the phosphorothioate triester present in either the dimer or a single-stranded dodecamer was quite similar. However, little hydrolysis was observed when the label was present in a double-stranded fragment (Table III). The hydrolytic stability of the labeled oligodeoxynucleotides also varied with the character of the label itself, as illustrated for a three labeled ss dodecamers in Figure 6. The bimane derivative was most easily hydrolyzed with significant quantities of label lost even at pH 7.0. By comparison, derivatives resulting from bromo(iodo)acetamides exhibited little hydrolysis at pH 7.0. In general, the triester resulting from reaction with the γ-bromo-α,β-unsaturated carbonyl exhibited stability similar to that of the haloacetamido labeled derivatives, while that resulting from reaction with the aziridinyl sulfonamide was significantly more stable (Figure 6). In all cases the extent of hydrolysis increased with a corresponding increase in pH value.

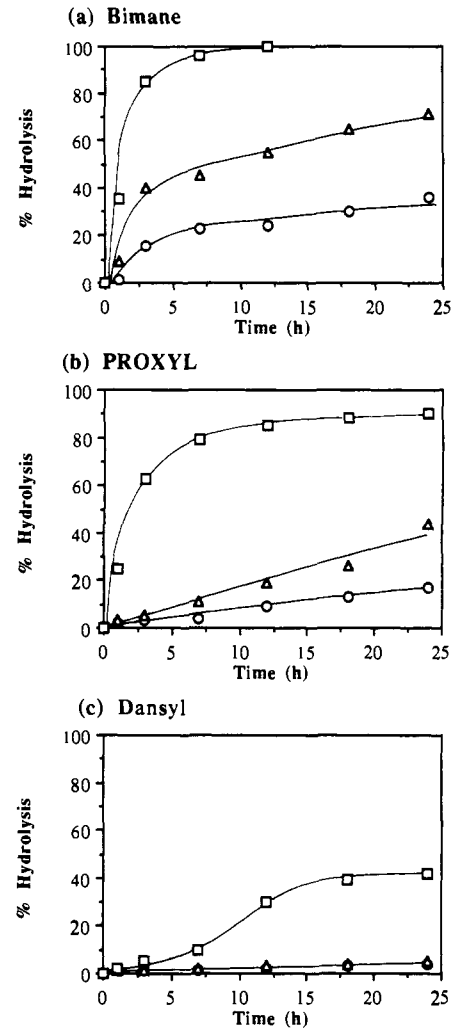


Figure 6. Extent of hydrolysis phosphorothioate triester-containing dodecamer d[CGCA(s-R)AAAAAGCG] labeled with (a) monobromobimane **9**, (b) iodoacetamido PROXYL **8**, and (c) *N*-dansylaziridine **10** at ambient temperature and pH values of 7.0 (○), 8.0 (△), and 10.0 (□).

Thermal Stability of Labeled Phosphorothioate Triester Containing DNA. It was necessary to consider the structural implications and possible perturbation of the structure which might occur with the introduction of reporter groups to the DNA backbone by the present procedures. Attachment of a label to the outer surface of the biomolecule, in this case to the phosphorus residue, may be of minimal structural consequence, but a large group may still interfere with proper Watson–Crick base pairing or base stacking. A number of modified sequences were investigated to determine if backbone modification generally destabilizes the duplex structure. The T_m values, obtained from absorbance vs temperature plots (helix-to-coil transitions), for a series of labeled dodecamers are shown in Table II. The unlabeled dodecamer (**7**) exhibited a T_m of 55.1 °C, and most of the labeled sequences (**8**, **11**, **12**) exhibited relatively little change in this value. However, both the bimane derivative (**9**) and the dansyl derivative (**10**) exhibited a slight decrease in duplex stability.

We also examined the effects of both the position and chirality of the labels on the stability of longer duplexes. The presence of diastereomeric fluorescein labels (F) near the terminus of the 24-mer duplex 5′-d[CGAACT(s)AGTTAACTAGTACGCAAG]·5′-d[CT(s-F)TGCGTACTAGTTAACTAGTTCG] resulted in a T_m (57.2 °C) virtually unchanged from the native unlabeled sequence (57.1 °C). However, the presence of a fluorescein label near the center of the duplex resulted in greater helix destabilization. For example, the S_p-5′-d[CGAACT(s)AGTTAACTAGTACGCAAG]·S_p-5′-d[CTTGCGTACT(s-F)AGTTAACTAGTTCG] duplex exhibited a T_m value of 55.5 °C only slightly below that of the unlabeled

Table IV. T_m Values for Labeled 24-mers

complex[a]	T_m[b] (°C) unlabeled	T_m[b] (°C) labeled	ΔT_m (°C)
M 5′-d[CGAACT(s)AGTTAACTAGTACGCAAG]			
M 3′-d[GCTTGATCAATTGATCATGCGT(s-F)TC]	57.1	57.2	0.1
S_p-5′-d[CGAACT(s)AGTTAACTAGTACGCAAG]			
S_p-3′-d[GCTTGATCAATTGA(s-F)TCATGCGTTC]	56.8	55.5	1.3
S_p-5′-d[CGAACT(s)AGTTAACTAGTACGCAAG]			
R_p-3′-d[GCTTGATCAATTGA(s-F)TCATGCGTTC]	57.6	54.0	3.6

[a] M indicates a diastereomeric mixture; S_p and R_p indicate absolute configurations. Of the two phosphorothioates present in the duplex, only the one marked with "F" has been labeled with fluorescein. [b] T_m values were obtained in 10 mM sodium phosphate (pH 7.0) and 150 mM sodium chloride. Estimated accuracy is ±0.5 °C.

S_p–S_p duplex (Table IV), while the R_p duplex exhibited a T_m value some 3.6 °C lower than the corresponding unlabeled duplex (Table IV).

Discussion

Phosphorothioate diesters can be placed at preselected sites within a DNA sequence using essentially standard DNA synthesis protocols. This provides a rapid a simple procedure for the introduction of a nucleophilic site into the DNA sequence. The triester formed in this manner is stable to further oxidation steps during the elongation of the oligodeoxynucleotide.

Labeling of the Phosphorothioate Diesters. Haloacetamides, γ-bromo-α,β-unsaturated carbonyls, and aziridine sulfonamides can all be used to efficiently alkylate phosphorothioate diesters and generate the corresponding triester carrying the reporter groups of interest. Labeling occurs over the pH range 5.0–8.0 with little noticeable differences in efficiency. However, with some derivatives, the product triesters undergo significant hydrolysis near pH 8.0, and this reduces overall yields. In these cases, effective labeling without competing product hydrolysis, was best performed at the lower end of this pH range (from pH 5.0–6.0). With the exception of monobromobimane, the conditions necessary for labeling with yields of 70–90% require reactions at 50 °C for a number of hours. With reactions employing haloacetamides or γ-bromo-α,β-unsaturated carbonyls, we were unable to observe any significant side products reflecting reactions at other sites. Alkylation of DNA bases by haloacetamido moieties has been reported,[19] but these reactions have required that the haloacetamido alkylating agent be bound tightly to the DNA by hybridization[19b] or other noncovalent interactions.[19a] Proximity effects will then enhance alkylation reactions that might not be observed in simple bimolecular reactions. Although we cannot completely exclude the presence of nonspecific alkylation of the DNA bases in these cases, we have been unable to observe such reactions to any significant extent. Conversely, the dansylaziridine derivative did appear to result in some nonspecific alkylation reactions, and this was not surprising since aziridines are known to alkylate DNA bases.[20] However, in all the reactions examined, alkylation of the phosphorothioate accounted for the major product.

Hydrolytic Stability of Labeled Oligodeoxynucleotides. Hydrolysis of the triester formed during the labeling reactions increases with increasing pH as would be expected. Hydrolysis results in loss of the label from the DNA with little observable cleavage of the DNA sequence. This is in agreement with previous observations indicating that hydrolysis of such triesters occurs primarily by cleavage of the P–S bond.[9a] Extended incubation of the labeled materials, even at pH 7.0, results in some hydrolysis of the phosphorothioate triester. This instability is more significant with single-stranded sequences than with double-stranded complexes (Table III). Hydrolysis also varied with the nature of the linking group. The bimane label was lost from the sequence to a significant extent after a few hours at pH 7.0 and ambient temperature, while the acetamide, unsaturated carbonyls or dansyl aziridine derivatives were generally stable under the same conditions for a number of hours. In all cases the hydrolysis could be enhanced by increasing the pH value. These results suggest that sequences labeled in this fashion can be used for study in solutions buffered near neutral pH values.

Thermal Stability of Labeled Phosphorothioate Triester Containing DNA. In general, the labeling of a single phosphorothioate diester did not alter dramatically the stability of a dodecamer duplex. With the diastereomeric dodecamers studied, the bimane and dansyl label resulted in a small 3–4 °C decrease in T_m value compared with the unlabeled duplex. This may reflect some local structure disruption near the site of the labeling, but it is difficult to gauge the extent of such structural modulation. It is unclear whether the observed decrease in T_m values is directly a result of the site of attachment or reflects some interaction between the DNA and the labels. Placement of the label near the terminus of a duplex did not alter the T_m in a significant manner. Labeling of the phosphorothioate diester as the R_p diastereomer results in more duplex destabilization than observed with the S_p diastereomer (Table IV). This is consistent with the expected orientation of the labels. The S_p derivative will tend to direct the label out away from the DNA helix, while the R_p derivative directs the label toward the major groove.

Conclusions

The introduction of a phosphorothioate diester site-specifically into a DNA sequence provides a nucleophilic site that is amenable to reaction with haloacetamides, γ-bromo-α,β-unsaturated carbonyls, and aziridine sulfonamides. Reactions require a number of hours at 50 °C in order to result in high yields (70 to >90%) of labeled oligodeoxynucleotide. The labeled materials are generally stable near neutral pH but undergo significant hydrolysis in solutions of increasing pH. In some cases the presence of the labeled phosphorothioate results in minor changes in T_m values, but in general the presence of a single label tethered to the DNA backbone does not alter helix stability significantly.

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