Efficient methods for attachment of thiol specific probes to the 3'-ends of synthetic oligodeoxyribonucleotides

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ABSTRACT

Methodology is described for the synthesis of DNA oligomers containing a free 3'-thiol group which can be selectively crosslinked with a wide variety of probes. This chemistry is compatible with both phosphotriester and phosphoramidite solid phase chemistry. Moreover, the sulphydryl group is introduced into the 3'-nucleoside solid support linkage prior to oligonucleotide synthesis. Consequently, no additional coupling steps are required after oligonucleotide synthesis, and isolation of the 3'-thiol oligonucleotide requires only one additional deprotection step. Cross-linking of the thiol-containing oligonucleotide to a fluorescent probe was carried out with high selectivity, in high yield, and under mild conditions.

INTRODUCTION

Covalent adducts of oligodeoxyribonucleotides with organic molecules are becoming important tools in biochemistry and molecular biology. Biotin-labelled oligonucleotides are useful as gene probes, oligonucleotide fluorophore adducts have been used as primers in DNA sequence analysis, and oligonucleotides covalently modified with reactive chemical reagents are finding application in the selective chemical modification of DNA.

A number of chemical and enzymatic methods have been recently described for the selective incorporation of probes into synthetic oligonucleotides. Aliphatic amino groups have been incorporated at the 5'-terminus of oligonucleotides via the use of derivatized thymidine monomers [1,2], protected aminoethyl phosphoramidites or aminoethyl chlorophenyl phosphates [3,4], and by coupling 5'-phosphoramidazolides with amines [5-8]. The amino group was then derivatized in a second step with an electrophile to give the desired adduct. Derivatizable uridine monomers [9] and thiols (via S-tritylphosphoramidites) [10] have also been coupled to the 5'-terminus of oligodeoxyribonucleotides.

A number of enzymatic methods have also been reported for selectively incorporating probes into oligodeoxynucleotides. Analogues of UTP

containing a biotin tethered at the 5-position of the base have been incorporated into the 3'-terminus of oligonucleotides using the enzyme DNA polymerase I [11,12]. Biotinylated ADP analogues have been linked to oligonucleotides with T4 RNA ligase [13,14].

In our efforts to synthesize semisynthetic nucleases modified with oligodeoxyribonucleotide binding sites, we required a selective method to couple peptides and proteins to the 3'-terminus of oligonucleotides under mild chemical conditions. We report here a general method for selectively modifying the 3'-terminus of oligonucleotides in which the label is introduced in high yield, in one step, via a disulfide exchange reaction. The procedure differs from those described previously, in that the sulphydryl group is introduced into the 3'-nucleotide or nucleoside-support linkage as a disulfide bond, prior to automated oligonucleotide synthesis. This approach avoids any complications due to functionalities present in the final oligonucleotide. The oligonucleotide is synthesized from the thiolated 3'-terminal nucleoside (or nucleotide) using standard solid phase phosphotriester [15,16] or phosphoramidite [17,18] chemistry, deprotected by conventional methods, treated with dithiothreitol, and purified by reverse phase chromatography [19]. The thiolated oligonucleotide can then be activated with 2,2'-dithiodipyridine and cross-linked to a thiol containing probe. Alternatively, the 3'-thiol-containing oligonucleotide can be derivatized with an electrophile such as an α-haloacetyl or maleimidyl group conjugated to the probe.

EXPERIMENTAL SECTION

Melting points were determined by using a Mel-Temp melting point apparatus and are uncorrected. Infrared spectra were recorded on a Perkin-Elmer model 1320 spectrometer. UV-Spectra were recorded on a Perkin-Elmer Lambda 3A spectrophotometer. ^{1}H NMR were determined at 200 MHz or 250 MHz on fourier transform spectrometers at the UC Berkeley NMR laboratory. ^{13}C NMR spectra were measured at 50.78 MHz, and ^{31}P NMR were measured at 81.75 MHz, both on the UCB-200. Chemical shifts are reported in δ values, positive values indicating shifts downfield of the reference (internal Me_4Si for ^{1}H and ^{13}C, external 85% H_3PO_4 for ^{31}P). Significant ^{1}H NMR data are tabulated in order: multiplicity (s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet), number of protons, and coupling constants in Hertz. Mass spectra were recorded at the University of California, Berkeley, California.

Unless otherwise indicated, reactions were carried out under a positive pressure of dry nitrogen. 1-Hydroxybenzotriazole was dried for 3 days at 55°C in vacuo over P_2O_5 prior to use. Triethylammonium bicarbonate buffer (TEAB) was prepared by passing CO_2 gas through an aqueous 1M triethylamine solution until a pH of 7.5 was obtained. Pyridine and dioxane were dried by distillation from sodium/benzophenone under a nitrogen atmosphere, and N,N-dimethylformamide (DMF), was allowed to stand over molecular sieves (4Å) overnight, followed by distillation under reduced pressure. Acetonitrile was dried by heating with CaH_2 (5 g/L) at reflux for 2 hours, followed by distillation.

Oligonucleotide synthesis was carried out by the phosphoramidite method using a Biosearch Model 8600 DNA synthesizer with 2-cyanoethyl phosphoramidites (Biosearch), or by the phosphotriester method using a Biosearch Sam One DNA Synthesizer with 2-chlorophenyl phosphate monomers (Biosearch) as previously described [15-18]. Thymidine was obtained commercially from Sigma, and controlled pore glass from either Biosearch or Pierce Chemicals.

HPLC was carried out using a Waters gradient HPLC system consisting of two model 510 pumps, a Model 680 gradient controller, a Model 481 variable wavelength UV detector, and a Model U6K injector. Analytical HPLC was performed on a reverse phase Versapack C18 (10μM) column (4.1 x 250 mm). Preparative HPLC was performed on a Whatman Magnum 9 Partisil C18 (10μM) column (9.4 x 500 mm). Chromatography was carried out at ambient temperature at a flow rate of 2 mL min^{-1} (analytical) or 5 mL min^{-1} (preparative) with 0.1 M triethylammonium acetate, pH 7.5, and acetonitrile. A gradient of 15 to 34% (38% preparative) acetonitrile in 20 min was used for the purification of 5'-dimethoxytrityl protected oligonucleotides, and 5 to 30% (43% preparative) acetonitrile in 20 min (30 min preparative) was used for fully deprotected oligonucleotides.

TLC was performed on pre-coated silica gel plates (Merck 60-254) visualized with UV light and iodine staining. Silica gel was obtained from Merck (Kieselgel 60, 230-400 mesh).

3'-O-Methanesulfonyl-5'-O-(4,4'-dimethoxytrityl)thymidine (1)

Compound 1 was synthesized by a modification of the procedure of Lin [20]. To a stirring solution of 5'-O-(4,4'-dimethoxytrityl)thymidine (8.94 g, 16.4 mmol) in dry pyridine (30 mL) at 0°C was added dropwise methanesulfonyl chloride (5.90 g, 52 mmol). The resulting solution was allowed to stir overnight at 4°C. The reaction was quenched by the addition

of water (1 mL) followed by an additional 30 min of stirring. The reaction mixture was slowly poured into ice water (1.5 L) with strong stirring, and the resulting brown solid product was filtered and washed with water. The solid product was redissolved in 50 mL of acetone and again added to 1.5 L of ice water. The product was filtered and washed exhaustively with water. After drying in vacuo 9.21 g (90%) of a light brown amorphous solid was obtained: IR (KBr) 3150, 2920, 2840, 1680, 1605, 1590, 1450, 1365, 1275, 1235, 1185, 1015, 910, 815 cm^{-1}; ^1H NMR (DMSO-d_6) δ 1.39 (s, 3H), 2.42 (m, 2H), 3.18 (s, 3H), 3.44 (m, 2H), 3.66 (s, 6H), 4.14 (m, 1H), 5.23 (m, 1H), 6.13-6.19 (t, 1H, J=7Hz), 6.80 (d, 4H), 7.13-7.35 (m, 9H), 7.43 (s, 1H), 11.4 (s, 1H). Anal. Calcd. for $C_{32}H_{34}O_9N_2S$: C, 61.73%; H, 5.46%; N, 4.50%; S, 5.12%. Found: C, 61.41%; H, 5.10%; N, 4.65%; S, 4.85%.

5'-O-(4,4'-Dimethoxytrityl)-2,3'-anhydrothymidine (2)

To a stirring solution of potassium phthalimide (15.10 g, 82 mmol) in 140 mL of DMF and 40 mL of water was added (10.23 g, 16 mmol) of 1 [21]. The mixture was heated to 95°C over 20 min and then cooled to room temperature and slowly poured into 1.5 L of a vigorously stirring ice/water mixture. A whitish solid precipitated immediately and the mixture was stirred for 15 min. The product was filtered and then washed with water. After drying, 7.31 g (87%) of a white powder was obtained: IR (KBr) 3420, 2929, 1730, 1660, 1605, 1530, 1470, 1305, 1250, 1180, 1030, 880, 835, cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.82 (s, 3H), 2.45 (m, 1H), 2.67 (m, 1H), 3.30 (m, 2H), 3.79 (s, 6H), 4.30 (m, 1H), 5.26 (s, 1H), 5.50 (s, 1H), 6.78-7.43 (m, 13H); MS-FAB: 527 $(M+H)^+$, 303 (M-DMT). Anal. Calcd. for $C_{31}H_{30}O_6N_2$: C, 70.70%; H, 5.70%; N, 5.31%; Found C, 69.12%; H, 5.49%; N, 5.30%.

3',6-Hexanethiol-5'-O-(4,4'-dimethoxytrityl)thymidine (3a)

To a stirred solution of 1,3-hexanedithiol (4.90g, 32 mmol) and sodium ethoxide (10 mmol) in ethanol (10 mL) and DMF (30 mL) was added 5'-O-(4,4'-dimethoxytrityl)-2,3'-anhydrothymidine (4.02 g 7.6 mmol). The reaction was maintained at 75° C for 2.5 h; tlc in 8% methanol in methylene chloride showed complete conversion to a major UV absorbing product (R_f = 0.7). After cooling to room temperature, the reaction was neutralized by pouring into cold 1 M aqueous ammonium acetate buffer, pH 7, followed by addition of 10% aqueous acetic acid to bring the pH to 7.5. This solution was extracted with methylene chloride (3 x 200 mL). The combined organic layers were washed with water (100 mL) followed by brine (100 mL), dried over $MgSO_4$, and the solvent removed by rotary evaporation to give an oily brown foam. This was purified by column chromatography (5 x 20 cm) using a

step gradient of 0-5% methanol in methylene chloride, to give 3.52 g (58%) of a white foam. IR (KBr) 3185, 2840, 1685, 1605, 1505, 1470, 1400, 1250, 1175, 1030 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.30, (m, 5H), 1.38 (s, 3H), 1.55 (m, 4H), 2.18 (m, 2H), 2.50 (m, 4H), 3.35 (m, 1H), 3.65 (m, 2H), 3.78 (s, 6H), 3.93 (m, 1H), 6.05 (m, 1H), 6.71-7.35 (m, 13H), 7.53 (m, 1H), 9.53 (s, 1H); MS-FAB: 675 $(M-H)^-$, 373 (M-DMT).

3',3-Propanethiol-5'-O-(4,4'-dimethoxytrityl)thymidine (3b)

Compound 3b was prepared in a manner similar to 3',6-hexanethiol-5'-O-(4,4'-dimethoxytrityl)thymidine to give 2.85 g (65%) of a white foam: IR (KBr) 3185, 2840, 1690, 1605, 1505, 1470, 1400, 1250, 1175, 1030 cm^{-1}; ^1H NMR δ ($CDCl_3$) 1.16-1.27 (t, 1H, J=5), 1.35 (s, 3H), 1.69-1.78 (t, 2H, J=5), 2.65 (m, 2H), 2.25-2.75 (m, 4H), 3.25 (m, 1H), 3.60 (m,2H), 3.75 (s, 6H), 6.12 (m, 1H), 6.77-7.37 (m, 13H), 7.71 (s, 1H), 8.58 (s, 1H); (MS-FAB): 635 $(M+H)^+$, 333 (M-DMT).

3'-Hexanedithioethanol-5'-O-(4,4'-dimethoxytrityl)thymidine (4a)

To a stirred solution of 2,2-dithiodipyridine (5.25g, 23 mmol) in ethanol (15 mL) containing glacial acetic acid (0.7 mL) was added 2-mercaptoethanol (2.04g, 26 mmol) dropwise over 30 min. The mixture was allowed to stir for 2 hours, after which the solvent was removed by rotary evaporation to give a yellow oil. This oil was purified by chromatography on a silica gel column (2.0 x 40.0 cm) using a gradient of 0-10 % ethyl acetate in methylene chloride to afford 2.35 g (56%) of S-(2-thiopyridyl)-2-mercaptoethanol, a pale brown oil: IR (KBr) 3320, 2940, 2885, 1395, 1285, 1220, 1155, 1045, 935, 820 cm^{-1}; ^1H NMR ($CDCl_3$) δ 2.98 (t, 2H, J=2.6), 3.82 (t, 2H, J=5), 5.75 (s, 1H), 7.14-7.64 (m, 3H), 8.51-8.54 (m, 1H). To a stirred solution of S-(2-thiopyridyl)-2-mercaptoethanol (1.24 g, 6.60 mmol) in methanol (15 mL) containing triethylamine (1.5 mL) was added dropwise over 1 hour 3'-hexanedithiol-5'-O-(4,4'-dimethoxytrityl)thymidine (0.68g 1.02 mmol) in 90% methanol in chloroform (50 mL). After starting material had disappeared (tlc), 2-mercaptoethanol (1.0 mL, 14 mmol) was added. After removal of solvent under reduced pressure, the yellow oily residue was dissolved in methylene chloride (20 mL) and extracted with cold 10% aqueous citric acid (2 x 10 mL), followed by water (3 x 30 mL). The organic layer was dried under reduced pressure and purified on a silica gel column (2.0 x 25 cm) using 8% methanol in methylene chloride as eluent to yield 0.58 g (64%) of a white foam. IR (KBr) 3430, 3150, 1695, 1610, 1505, 1470, 1255, 1180, 1110, 1040, 835 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.34 (m, 4H), 1.40 (s, 3H), 1.52 (quintet, 2H, J=3.5), 1.65 (quintet, 2H, J=3.5), 2.42 (m, 4H), 2.50 (m

2H), 2.66 (t, 2H, J=5), 2.82 (t, 2H, J=12), 3.38 (m, 1H), 3.57 (quartet, 1H), 3.64 (d, 2H), 3.78 (s, 6H), 3.87 (m, 2H), 3.92 (m, 1H), 6.18 (d, 1H), 6.74-7.42 (m, 13H), 7.79 (m, 1H); MS-FAB: 751 $(M-H)^-$, 675 $(M-SCH_2CH_2OH)$, 373 (675-DMT). Anal. Calcd. for $C_{39}H_{48}N_2O_7S_3$: C, 62.23%; H, 6.38%; N, 3.72%; S, 12.76%. Found: C, 62.31%; H, 6.41%; N, 3.73%; S, 12.61%.

3'-Propanedithioethanol-5'-O-(4,4'-dimethoxytrityl)thymidine (4b)

Compound 4b was prepared in a manner similar to that for 3'-hexanedithioethanol-5'-O-(4,4'-dimethoxytrityl)thymidine to yield 170 mg (83%) of a white foam. IR (KBr) 3430, 3150, 1695, 1605, 1505, 1470, 1400, 1255, 1180, 1110, 1040, 830 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.41 (s, 3H), 1.93 (t, 2H, J=5), 2.39-2.51 (m, 6H), 2.60-2.68 (m, 2H), 2.75 (m, 2H), 3.32 (m, 1H), 3.60 (m, 2H), 3.75 (s, 6H), 4.0 (m, 2H), 6.12 (t, 1H, J=4), 6.77-7.37 (m, 13H), 7.71 (m, 1H), 8.58 (s, 1H); MS-FAB: 709 $(M-H)^-$, 633 $(M-SCH_2CH_2OH)$, 321 (633-DMT). Anal Calcd. for $C_{36}H_{42}N_2O_7S_3$: C, 60.84%; H, 5.91%; N, 3.94%; S, 13.52%. Found: C, 60.81%; H, 6.19%; N, 3.89% S, 13.29%.

3-[3-[5'-O-(4,4'-Dimethoxytrityl)-P-(2-chlorophenyl)-thymidylyl]-propyldithio]propanol (5)

5'-O-(4,4'-Dimethoxytrityl)thymidine (1.50 g, 2.8 mmol) was coevaporated twice with pyridine (5 mL). To the dried residue was added 2-chlorophenyl-O,O-bis-(1-benzotriazolyl) phosphate [22] (16.0 mL of a 0.2 M solution in dioxane, 3.2 mmol) via syringe, and the resulting solution was left to stir for 60 minutes. Meanwhile, 3,3'-dithiodipropanol [23] (2.51g, 13.8 mmol) was dissolved in pyridine (5 mL), evaporated to dryness, and redissolved in pyridine (15 mL). To this solution was transferred the above reaction mixture under nitrogen via a teflon tube, and the resulting solution was allowed to stir for 60 min. The mixture was then diluted with methylene chloride (250 mL), washed with 1 M TEAB pH 8.0 (100 mL), water (2 x 100 mL), dried over $MgSO_4$, and the solvent was removed by rotary evaporation to leave a light yellow oil. The product was purified by chromatography on a (7 x 50 mm) silica gel column eluted with 1 to 3% methanol in chloroform to yield 1.47 g (64%) of purified product: m.p. = 50°C (decomp.); IR (KBr) 3440, 2920, 1690, 1505, 1475, 1250, 1030 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.36 (s, 3H), 1.92 (m, 2H), 2.10 (quintet, 2H), 2.30-2.82 (m, 6H), 3.50 (m, 2H), 3.72 (m, 2H), 3.78 (s, 6H), 4.31 (quintet, 2H), 5.32 (t, 1H), 6.46 (m, 1H), 6.80-7.57 (m, 18H), 9.10 (d, 1H); ^{31}P NMR ($CDCl_3$) δ -7.18, -7.22; MS-FAB: $[M]^+$ = 898. Anal. Calcd. for $C_{43}H_{48}N_2O_{11}S_2PCl$: C, 57.42%; H, 5.38%; N, 3.11%; S, 7.13%; P, 3.44%; Cl, 3.94%. Found: C, 57.16%; H, 5.39%; N, 3.02%; S, 7.24%; P, 3.26%; Cl, 3.86%.

Derivation of solid support with 3'-thiol-containing nucleosides (nucleotide) (6a, 6b, 7)

To a stirring solution of either compound 4a, 4b or 5 (0.60 mmol) and 4-dimethylaminopyridine (0.036 g, 0.30 mmol) in pyridine (2.0 mL), was added succinic anhydride (0.048 g, 0.48 mmol) in one portion [15]. After stirring overnight, the mixture was concentrated to a gum under reduced pressure. The residual pyridine was removed by co-evaporation with toluene (3 x 3 mL). The residue was then dissolved in methylene chloride (10 mL), and washed with ice-cold 10% aqueous citric acid (2 x 10 mL) and water (2 x 10 mL). The organic phase was dried over sodium sulfate and the solvent removed under reduced pressure to leave an off-white solid foam. After dissolving this in methylene chloride (1.0 mL), the product was precipitated by adding the solution to rapidly stirring hexane (25 mL). The succinylated nucleoside was collected by vacuum filtration and dried in vacuo. To a stirring solution of the white solid in dioxane (2.0 mL) containing pyridine (1 drop) and 4-nitrophenol (0.070 g, 0.50 mmol), was added dicyclohexyl-carbodiimide (0.206 g, 1.00 mmol). The precipitated dicyclohexylurea was removed by vacuum filtration after 2.5 hours. To the stirring filtrate was added DMF (1.0 mL) and aminopropylated controlled pore glass (1.00 g) followed by triethylamine (0.20 mL, 1.4 mmol), which caused the mixture to turn bright yellow. After five to eight hours on a rotary shaker, the silica was collected by vacuum filtration, followed by washing with DMF (3 x 30 mL), methanol (3 x 30 mL), ether (3 x 30 mL), and drying in vacuo. A trityl assay [15] on a 2.0 mg portion indicated a loading of 48 μmol nucleoside per gram of support. Any underivatized amino groups on the support were capped by suspending the silica in a solution of pyridine (0.010 g, 0.083 mmol) and acetic anhydride (0.20 mL, 2.1 mmol) for 30 min. The support was collected by vacuum filtration, washed with methanol (3 x 50 mL), and ether (3 x 50 mL), and dried in vacuo.

Synthesis of 3'-thiopyridylated oligonucleotide (8c, 9c, 10c)

A 15mer 5'-TCC-CTA-CTC-TCG-CA(T)-3' was synthesized with the thymidine-disulfide controlled pore glass support 6a, 6b or 7 as prepared above using the phosphoramidite method with a Biosearch Model 8600 DNA synthesizer or by the phosphotriester method using a Biosearch Sam One DNA Synthesizer. 30 mg (~1 μmol) of support was used for each synthesis. The 5'-DMT group was left on, and the fully protected oligo was not cleaved from the support after the synthesis was completed. Average coupling yields as

determined by trityl release were the same as those obtained using standard 3'-succinylated thymidine.

The oligonucleotide-SS-support was treated with 80% pyridine in water (1 mL) containing 2-pyridine aldoxime (76 mg, 0.62 mmol) and tetramethylguanidine (89 µL, 0.62 mmol), at 37°C overnight. In the case of thymidine derivatives 6a and 6b this step is not required. To the above mixture was added 6 mL of concentrated NH_4OH. This mixture was tightly sealed in a screw-capped vial, and left at 55° for 6 hours. The ammonia solution was removed from the CPG and concentrated under reduced pressure to ~300 µL. The crude 5'-DMT oligo was desalted on a C18 Sep-Pak (Waters), by loading and washing the salts through with 10 mL 25 mM TEAB (pH 7-8) and eluting with 5 mL 30% CH_3CN, 50 mM TEAB (pH 7-8). The desalted solution was concentrated to 1.5 mL, and purified by reverse-phase HPLC.

The purified material was concentrated to dryness and 1 mL 80% HOAc was added. After 20 minutes the acetic acid solution was rapidly concentrated to near dryness under reduced pressure. To the residue was added 250 µL of 500 mM Tris·HCl, 2 mM EDTA, pH 8.0, and this solution was washed with ether (2 x 250 µL).

The detritylated oligonucleotide 8a, 9a or 10a was treated with dithiothreitol (5 mg, 0.032 mmol) dissolved in 0.50 mL ddH_2O, and the resulting solution was left to stir 18 hours at 37°C. The product 8b, 9b or 10b was then collected by preparative HPLC directly into 2.0 mL of a 2,2'-dithiodipyridine solution (2.00 mg in 2.0 mL of 100 mM phosphate buffer, pH 5.5, containing 15% acetonitrile). This mixture was allowed to react overnight at 25°C after which the product was purified by HPLC. The purified 3'-thiopyridyl oligonucleotide 8c, 9c or 10c was stored at -20°C, and showed no signs of degradation by analytical HPLC after several months. The yield based on the OD_{260} was 32 A_{260} units (21%), (OD_{260}/OD_{280} = 1.50). The yield based on the absorbance of the thiopyridyl anion at 343 nm (ε = 7060 M^{-1}) after cleavage with 10 mM DTT was 39 A_{260} units.

Sequencing of the 3'-thiopyridylated oligonucleotide

100 pmol of 3'-thiol-containing oligonucleotide 10a was end-labelled with γ-^{32}P ATP (6000 Ci/mmol) using T4 polynucleotide kinase [24]. The 5'-^{32}P end-labelled oligonucleotide was then desalted on Sephadex G-50, ethanol precipitated and sequenced by a modified version of the method of Maxam and Gilbert [25,26]. Electrophoresis was carried out on a 7M urea, 25% polyacrylamide denaturing gel.

Bis-(N-fluoresceinthioureido)-2,2'-dithiodiethylamine, tetra-triethyl-ammonium salt (11)

Fluorescein isothiocyanate isomer II (200 mg, 0.52 mmol, Sigma) was suspended in water (3.0 mL) and brought to pH 9.0 by the dropwise addition of 1M aqueous K_2CO_3. To this stirring solution was added a solution of 2,2'-dithiodiethylamine dihydrochloride (39 mg, 0.17 mmol) [23] in water (1.0 mL) over a 1 hour period at room temperature. The pH was maintained at 9.0 $\pm$ 0.1 throughout the reaction by the addition of aqueous 1M K_2CO_3 as necessary. The reaction was left to stir an additional hour. The major product was isolated by preparative HPLC in several injections. The combined fractions (50 mL) were brought to pH 4.0 with acetic acid, diluted with water (50 mL), and extracted with 1:1 chloroform in ethanol (3 x 75 mL). The combined organic layers were dried over $MgSO_4$, and the solvent was evaporated *in vacuo* to yield 140 mg (73%) of the tetra-triethylammonium salt. IR (KBr) 3200, 2980, 2940, 2680, 1610, 1470, 1400, 1260, 1170, 1110 cm^{-1}; ^1H NMR (DMSO-d_6) δ 1.18 (t, 27H), 2.82-3.05 (m, 32H), 3.70 (s, 4H), 6.50-6.80 (m, 6H), 7.70-8.00 (m, 2H), 8.85 (s,1H); MS-FAB: $[MH]^+$ = 931.

(N-Fluoreceinthioureido)-2-mercaptoethylamine (12)

The fluorescein dimer 11 (2.5 mg, 2.2 µmol) was dissolved in 0.5 M phosphate buffer, 2 mM EDTA, pH 8.0 (800 µL), and a 100 mM solution of dithiothreitol (200 µL, 20 µmol) was added. The reaction mixture was incubated at 37°C for 1 hour. Analytical HPLC showed 95% conversion of starting material to a product peak of shorter retention time. The product was purified by preparative HPLC and used immediately in subsequent disulfide exchange reactions, because oxidation of the product to the symmetrical disulfide was quite rapid in aqueous solution.

Attachment of Fluorescent Label to Thiol-Containing Oligonucleotides

To 7.8 A_{260} units of 3'-thiopyridylated oligonucleotide 10c in 2.0 mL 0.1 M triethylammonium acetate, pH 7.5, containing 20% acetonitrile, was added 800 µl of thiolated fluorescein 12 (79 mmol, 1.2 equivalents) in the same solvent. The reaction mixture was left at room temperature for 4 hours after which no additional thiopyridone was released as monitored by UV-VIS at 343 nm. Analytical HPLC showed 95% conversion of 10c to product which had a longer retention time (see figure 3). The solution was concentrated to 1.0 mL and purified by reverse-phase HPLC. UV spectra of 13 were carried out in water, and showed λ max values at 492 and 262 nm, 262/492 = 3.3. Fluorescence spectra in water showed an emission maximum at 521 nm when

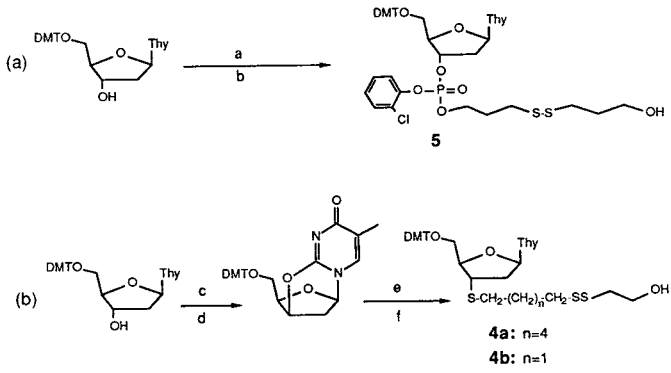
excited at 492 nm. Reaction of 0.26 A_{260} units of fluorescein-oligonucleotide adduct 13 with 33 mM dithiothreitol in 300 µl 100 mM phosphate buffer, pH 8.0, for 15 min at 37°C resulted in complete conversion to the free 3'-thiol oligonucleotide 10b as monitored by reverse phase HPLC retention times. To demonstrate the pH stability of the disulfide adduct, samples containing 0.26 A_{260} units of 13 and either 50 mM acetate buffer pH 4.0, or 10 mM sodium hydroxide pH 12.0, were incubated at 37°C. Analytical HPLC after 12 hours showed no signs of degradation.

RESULTS AND DISCUSSION

We report here a 3'-labelling method for synthetic oligonucleotides with the following features. The method is compatible with both solid phase phosphoramidite and phosphotriester methods. The procedure is applicable to labelling oligonucleotides with a wide variety of probes containing thiol specific functionalities. These include electrophilic reagents such as α-haloacetyl or maleimidyl groups, as well as probes containing a free thiol moiety. Importantly, disulfide exchange reactions and alkylation reactions can be carried out with high selectivity due to the high nucleophilicity of the sulphydryl group, and under mild conditions (pH, temperature) which are compatible with a wide number of probes. Moreover, this methodology requires no additional steps or coupling reactions in oligonucleotide synthesis, since the derivatizable thiol is incorporated into the linkage between the 3'-terminal nucleoside and the solid support as a disulfide. Consequently oligonucleotide synthesis and deprotection is carried out in the standard fashion with one additional deprotection step involving treatment with dithiothreitol.

The procedure involves derivatization of the 3'-nucleoside to the solid support via a disulfide linkage. The only requirement is that the 3'-terminal nucleoside contains a free thiol, which serves both as the site of attachment to the solid support, as well as to the requisite probe. We have developed two simple methods for introducing free sulphydryl groups into thymidine nucleosides (scheme I). In method (a) 5'-dimethoxytrityl-thymidine is first phosphorylated with 2-chorophenyl-0,0-bis(1-benzotriazolyl) phosphate [22]. The resulting activated nucleotide is coupled without isolation of product to 3,3'-dithiodipropanol to afford the sulphydryl containing thymidine nucleotide 5 in 64% isolated yield. This method can be generalized to protected 2'-deoxycytidine, 2'-deoxyadenosine

Scheme I



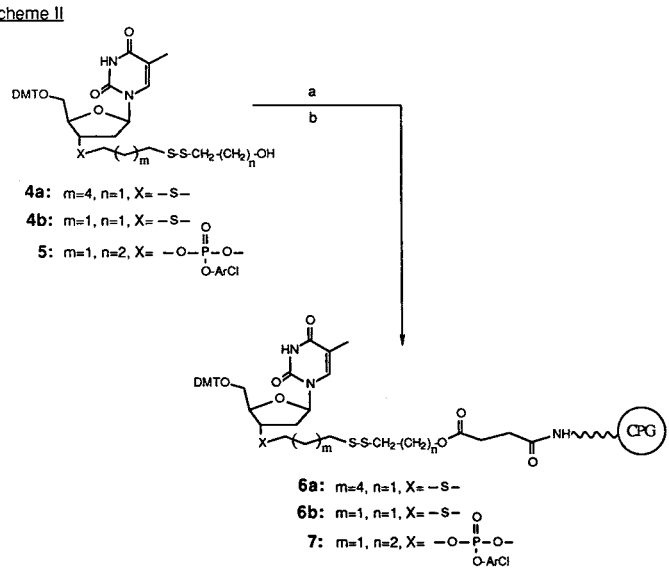
(a) 2-chlorophenyl-O,O-bis-(1-benzotriazolyl)phosphate, pyridine/dioxane, 1 hour, 25°C; (b) 3,3'-dithiodipropanol, pyridine, 1hour, 25°C; (c) methanesulfonyl chloride, pyridine, 12 hours, 4°C; (d) potassium phthalimide, DMF/water, 20 min., 95°C; (e) 1,3-propanedithiol or 1,6-hexanedithiol, NaOEt/EtOH, 2.5 hours, 75°C; (f) S-(2-thiopyridyl)- 2-mercaptoethanol, methanol, 30 min, 25°C.

and 2'-deoxyguanosine, and the thiol containing tethers can be of varying lengths. We, in addition to Connolly [10], have noted that the thymidine derivative with 2,2'-dithiodiethanol, on reduction of the disulfide, is unstable toward elimination of mercaptoethanol in aqueous buffered solutions higher than pH 7.5.

The 3'-position of thymidine can also be directly derivatized with sulphydryl tethers of the form $HS-(CH_2)_n-SH$. We have synthesized two such thiol-containing nucleosides via method (b) as outlined in scheme I. Dimethoxytrityl-2,-3'-anhydrothymidine 2, which is available in high yield in two steps from 5'-O-dimethoxytritylthymidine [20,21], was reacted with either 1,3-propanedithiol or 1,6-hexanedithiol and sodium ethoxide in ethanol to produce the 3'-thiolated nucleosides 3a and 3b, respectively, in 45% isolated yield. One should also be able to introduce thiols into the 3'-O-methanesulfonyl-5'-O-dimethoxytrityl-D-threo-pentofuranosyl derivatives of the other deoxynucleosides by similar chemistry [27]. The 3'-thiolated 5'-protected thymidine 3a or 3b was then coupled with S-(2-thiopyridyl)-2-mercaptoethanol to afford the thymidine derivatives 4a and 4b in 80% isolated yield.

The disulfide-containing thymidine derivative obtained via either method (a) or (b) was then coupled to aminopropyl controlled pore glass by

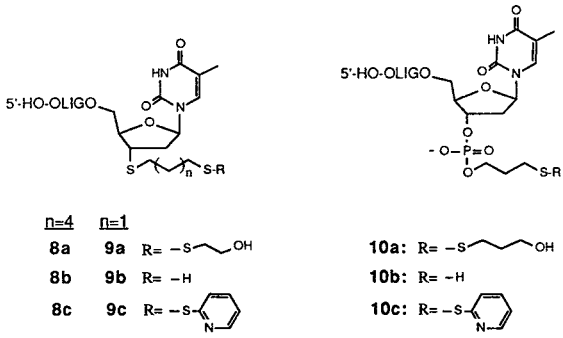
Scheme II



(a) succinic anhydride, pyridine, 12 hours, 25°C; (b) p-nitrophenol, DCC, dioxane, 2.5 hours, 25°C; followed by treatment with aminopropyl controlled pore glass, 5 hours, 25°C.

the conventional methods employed with 5'-dimethoxytrityl 3'-hydroxyl and base protected deoxyribonucleosides [15]. The nitrophenyl esters of the 5'-dimethoxytrityl-3'-0-succinate derivatives of 4a, 4b and 5 were synthesized and condensed with the primary amino groups of aminopropyl-derivatized controlled pore glass. Typically 20-50 μmol protected nucleoside were loaded per gram of support as determined by dimethoxytrityl cation release. Underivatized amino groups were capped by treatment with acetic anhydride in pyridine.

To demonstrate the utility of the thymidine disulfide containing controlled pore glass supports 6a, 6b and 7, the synthesis of the oligonucleotide 5'-TCC-CTA-CTC-TCG-CAT-SH-3' was carried out by both the phosphotriester and phosphoramidite routes. Phosphoramidite solid phase synthesis was carried out on a Biosearch 8600 DNA synthesizer using β-cyanoethylphosphoramidites (Biosearch). Phosphotriester solid phase synthesis was carried out on a Biosearch Sam One DNA synthesizer using 2-chlorophenylphosphate monomers (Biosearch). In each case the standard 5'-DMT-thymidine derivatized controlled pore glass was also used as a support to synthesize the underivatized 15mer. The coupling yields at each step



n=4 n=1

8a 9a R= -S-CH2-CH2-OH

8b 9b R= -H

8c 9c R= -S-(pyridyl)-N

10a: R= -S-CH2-CH2-CH2-OH

10b: R= -H

10c: R= -S-(pyridyl)-N

were determined by assay of trityl cation release, and were identical within experimental error for the disulfide-containing 15mer oligonucleotides 8a, 9a, 10a and the underivatized 15mer oligonucleotide. It was also demonstrated that 2,2'-dithiodiethanol was stable to the coupling, deprotection, capping and oxidative conditions used in either phosphoramidite or phosphotriester solid phase DNA synthesis.

The crude, fully protected oligonucleotides were then deprotected by standard methods [15,18], and purified by reverse phase HPLC to yield the 3'-disulfide containing oligonucleotides 8a, 9a and 10a typically in 5 - 20% overall yield. The elution times of the oligonucleotides 8a, 9a and 10a were slightly longer than that of the corresponding underivatized 15mer oligonucleotide. The disulfide was then reduced by treatment with 40 mM aqueous dithiothreitol for 18 hours at 37°C in 170 mM phosphate buffer, pH 8.0, and purified by preparative reverse phase HPLC (figure 1). The free 3'-thiol-containing oligonucleotides 8b, 9b and 10b have slightly shorter retention times on analytical reverse phase HPLC than the corresponding disulfides 8a, 9a and 10a. Compounds 8b, 9b and 10b must be stabilized with 1 mM DTT to prevent oxidation of the thiol to the symmetrical disulfide. When the 3'-thiopyridyl oligonucleotide was desired, the reduced 3'-disulfide-protected oligonucleotides 8b, 9b and 10b were collected directly by HPLC into 5 mM 2,2'-dithiodipyridine, 100 mM phosphate, pH 5.5 buffer containing 15% acetonitrile. The disulfide exchange reaction to produce the 3'-thiopyridyl oligonucleotides 8c, 9c and 10c can be conveniently followed by monitoring 2-thiopyridone formation (λ max = 343 nm, ε = 7,060 M^{-1} [28]) spectrophotometrically. The reaction times were typically 1-2 hours. The ratio of thiopyridone release to oligonucleotide (assayed by A_{260}) was 1.2, consistent with the conversion of the free 3'-thiol to the thiopyridyl

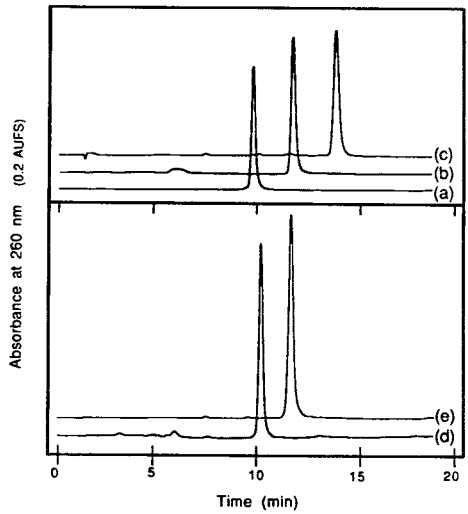


Figure 1. Reverse phase HPLC traces of (a) purified deoxynucleotide 15mer 5'-TCC-CTA-CTC-TCG-CAT-3', (b) 3'-mercapto 15mer 9b, (c) 3'-thiopyridyl 15mer 9c, (d) 3'-mercapto 15mer 10b, (e) 3'-thiopyridyl 15mer 10c. HPLC conditions: column, C18 Versapack 10 μm analytical; gradient, linear from to 30% solvent B in 20 minutes at a flow rate of 2.0 mL/min. Solvent A = 1 mM triethylammonium acetate, pH 7.5, solvent B = acetonitrile.

disulfide containing oligonucleotides. The thiol-containing 15mer oligonucleotide 10a was characterized by Maxam-Gilbert sequencing [25,26] of 5'-^{32}P end-labelled 10a on a 25% denaturing polyacrylamide gel which

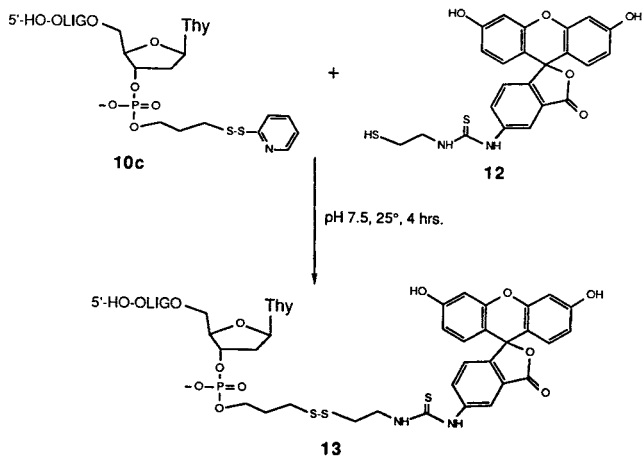


Figure 2. Attachment of thiolated fluorescein to the 3'-thiopyridyl oligonucleotide via disulfide exchange reaction.

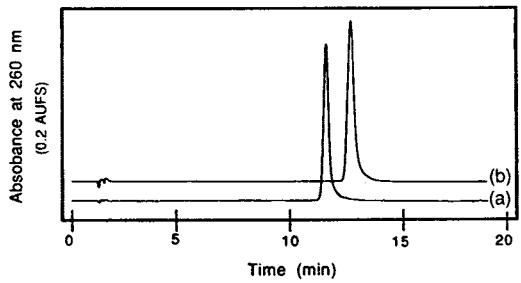


Figure 3. Reverse-phase HPLC traces of (a) purified 3'-thiopyridylated 15mer 10c, and (b) purified fluorescein-15mer adduct.

confirmed the sequence of the 15mer oligonucleotide.

The utility of this methodology for forming oligonucleotide adducts with a variety of probes was tested by crosslinking the thiopyridyl disulfide 10c to thiolated fluorescein 12 (figure 2). The oligonucleotide 10c was reacted with 1.2 equivalents of mercaptoethyl fluorescein 12 in 100 mM triethylammonium acetate buffer, pH 7.5, containing 20% acetonitrile for 4 hours at 25°C. Completion of reaction was determined by analytical HPLC, and by monitoring thiopyridone release spectrophotometrically at 343 nm. The product was isolated by reverse phase HPLC in 95% yield (figure 3). HPLC of the crude mixture revealed disappearance of the peak corresponding to thiopyridyl oligonucleotide 10c and appearance of one product peak. The adduct 13 is characterized by a longer retention time relative to the 3'-thiol 15mer 10b. The ratio of OD_{262}/OD_{492} is 3.3 for the product. Reduction of the adduct with 33 mM dithiothreitol in 100 mM buffer, pH 8.0 at 37°C for 15 min resulted in clean conversion to the 3'-thiol 15mer oligonucleotide 10b and mercaptoethyl fluorescein 12.

Importantly, the fluorescein oligonucleotide adduct is stable at 37°C for greater than 12 hours between pH 4.0 and pH 12.0. We have also recently crosslinked the 3'-thiopyridyl-containing 15mer oligonucleotide 10c to a site-directed mutant of staphylococcal nuclease containing a unique cysteine introduced at residue 116. These results will be described in detail elsewhere.

CONCLUSION

The methods described in this paper allow the synthesis of oligomers containing a free 3'-thiol group which can be selectively modified with a

wide variety of probes. The chemistry is compatible with both phosphotriester and phosphoramidite solid phase chemistry. Moreover, the thiol is introduced into the 3'-nucleoside-support linkage prior to oligonucleotide synthesis. Consequently, no additional coupling steps are necessary, and complications due to reactive functionalities in the completed oligonucleotide are avoided. Isolation of the 3'-thiol containing oligonucleotide requires only one additional deprotection step. Crosslinking of the oligonucleotide to the probe can be carried out with high selectivity under mild conditions.

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29. Abbreviations: CPG = controlled pore glass, DCC = N,N'-dicyclohexylcarbodiimide, DMF = N,N'-dimethylformamide, DMT = 4,4'-dimethoxytrityl, DTT = dithiothreitol.