Novel Non-Nucleosidic Building Blocks for the Preparation of Multilabeled Oligonucleotides

Andrei Guzaev, Harri Salo, Alex Azhayev, and Harri Lönberg

Department of Chemistry, University of Turku, FIN-20014 Turku, Finland. Received September 27, 1995. Synthesis of new non-nucleosidic phosphoramidites 1 and 2 derived from achiral precursors containing two reporter groups or amino functions is described. Among them, only phosphoramidites 1a–c allow multiple derivatization of synthetic oligonucleotides. The usefulness of building blocks 1a–c is demonstrated by introduction of several amino functions or fluorescent dansyl reporters at the 3′- and 5′-termini of synthetic oligonucleotides and their phosphothioate analogues. It is demonstrated that multilabeled non-nucleosidic tether has no or only minor influence on the hybridization properties of the oligonucleotides.

INTRODUCTION

Oligodeoxynucleotides bearing either reporter groups, bioaffinity groups, or additional functional groups have proven to be potent tools in molecular biology, finding applications in hybridization diagnostics, sequencing, chemical tailoring, and molecular recognition studies of nucleic acids (1). Related structural modifications have also been exploited to increase the cellular uptake of oligonucleotides and to improve their stability toward nucleases. The methods elaborated to introduce various conjugate groups into synthetic oligonucleotides fall into two categories. The first alternative is derivatization of nucleotide units at either the base, sugar, or phosphodiester moiety (1). All these modifications change, sometimes even dramatically, the ionic, tautomeric, and/or steric properties of the oligonucleotide. In particular, introduction of several conjugate groups within the sense sequence that is engaged in the hybrid formation may cause problems. The second alternative is to extend the sense sequence with a non-nucleosidic tail designed to bear the desired functionalities. Obviously, this approach is the method of choice for multilabeling.

The non-nucleosidic tails used for multilabeling of oligonucleotides include both branched and linear structures. Branched dendrimeric structures have, for example, been assembled on the 5′-terminus of the oligonucleotide by using bis(4,4′-dimethoxytrityl)alkanetriol phosphoramidite as a building block (2–4). Each condensation step thus doubles the number of 5′-terminal hydroxyl functions. At the end of the chain assembly, all the terminal hydroxyl functions are reacted with a phosphoramidite building block derived from the desired conjugate group, such as a biotinyl (2–4), dinitrophenyl (3), or protected aminoalkyl (3, 4) group. Polyamino oligonucleotides may finally be labeled in solution, e.g., with fluorescein, digoxigenin, or biotin (3, 4).

Linear tails have, in turn, been prepared by using non-nucleosidic phosphoramidites derived from substituted diols that carry either a protected amino function or a reporter group (4–11). Among them, derivatives of 3-amino-1,2-propanediol (5–9) and glycerol (9–11) have been used most extensively. The 1,2-diol system has been used to construct the non-nucleosidic backbone, the remaining functionality (either amino or the third hydroxyl group) being tethered to the conjugate group. Biotinyl (5, 10), pyrenyl (6, 7), dansyl (DNS) (7), carbocarane (8), dinitrophenyl (9), phosphotyrosinyl (10), or fluoresceinyl (11) residues have been introduced in this manner. It is also possible to keep the remaining functionality appropriately protected during the chain assembly and label it post-synthetically after deprotection (5). The 4,4′-dimethoxytrityl (DMTr) group has, however, been observed to retard sterically the coupling reaction of the vicinal phosphoramidite function (6, 10, 11). Moreover, the presence of two chemically different hydroxyl groups (primary and secondary) results in phosphate migration at the terminal moiety (12).

In order to improve the efficiency of synthesis, as well as to mimic the stereochemical properties of the natural polynucleotide backbone, building blocks derived from 1,3-diols have been used instead of their 1,2-diol counterparts. The compounds employed include 2-amino-1,3-butanediol (13), 2-(4-aminobutyl)-1,3-propanediol (14), 2-methyl-2-[2-(benzyloxy carbonyl)ethyl]-1,3-propanediol (15), and 1-[(6-aminohexanoyl)amino]-2,4-propanediol (4). More recently, phosphoramidites from longer a,ω-diols have also been used for the same purpose (8, 16, 17).

The non-nucleosidic building blocks discussed above suffer from two common shortcomings. Except for 5-(aminomethyl)-1,3-benzenedimethanol-based phosphoramidite (17), they are derived from chiral compounds giving upon chain assembly a family of diastereomeric oligonucleotide conjugates. In few cases, a pure enantiomer has been employed as a starting material (8, 13, 16). Furthermore, they allow introduction of only one functionality per one building block added. Both of these drawbacks have been avoided in one case. The phosphoramidite of achiral N,N′-bis(2-hydroxyethyl)-N,N′-bis(2,4-dinitrophenyl)-1,2-ethanediylamine has been used to prepare oligonucleotide conjugates containing up to 10 labels (18). The reporter groups can hardly be introduced postsynthetically, since the secondary amino functions are less reactive than primary ones. With fluorescent reporter groups, attachment via longer linkers would also be desirable to reduce intramolecular quenching.

These data clearly indicate that, regardless of the nature of the reporter (functional) group, the structure of the linker tethering the conjugate group to the oligonucleotide is of major importance for the properties of the entire conjugate. It seems to be likely that a non-
nucleosidic building block optimal for preparation of multilabeled oligonucleotides should meet the following requirements. (i) The parent structure should be 1,3-diol. (ii) This structure should contain neither chiral nor prochiral centers. (iii) It should bear at least two functional groups for labeling, situated sufficiently far from each other and the groups engaged in the oligomer backbone. As an attempt to fulfill these requirements, we now report on preparation of several novel phosphoramidites, 1 and 2, that are derived from achiral precursors and that possess two primary amino groups (either protected or labeled with reporter groups). Among them, building blocks 1a–c bearing two N-(3-aminopropyl)carboxamido linkers were found to meet all the requirements of oligonucleotide synthesis, deprotection, and labeling (Scheme 1). Their usefulness is demonstrated by preparation of oligonucleotide conjugates (3) and their phosphorothioate analogues that bear up to six fluorescent DNS labels at either the 3'- or 5'-terminus. Melting point measurements showed that the bulky non-nucleosidic tether only slightly retards hybridization of the oligonucleotide moiety (15-mer) with a longer DNA target (27-mer).

**EXPERIMENTAL PROCEDURES**

**General.** Diethyl 2,2-bis(hydroxyethyl)malonate was purchased from Aldrich. The reagents for oligonucleotide synthesis were from Cruachem. Adsorption column chromatography was performed on silica gel 60 (Merck). NMR spectra were recorded on a JEOL GX-400 spectrometer operating at 398.16 and 161.9 MHz for 1H and 31P, respectively. Either CDCl3 or DMSO-d6 was used as the solvent, with either tetramethylsilane (TMS) as an internal (1H) or H3PO4 as an external (31P) standard.

**Melting Experiments.** A 2 UV/vis spectrophotometer equipped with a PTP-6 temperature programmer, comprising an electronic control unit and a Peltier cell holder (Perkin-Elmer), was used. The temperature was increased at a rate of 1 °C/min. The melting curves were recorded at 260 nm in a buffer containing 20 mM Tris-HCl and 0.1 M NaCl (pH 7.0).

**High-Performance Liquid Chromatography (HPLC) Techniques.** The oligonucleotides were analyzed and isolated by reversed phase (RP) chromatography [column, Nucleosil 300-5C18, 4.0 × 250 mm, Macherey-Nagel; buffer A, 0.05 M NH4OAc; buffer B, 0.05 M NH4OAc in 65% MeCN; flow rate, 1.0 mL min⁻¹; a linear gradient from 5 to 60% B in 30 min was applied for dimethoxytrityl-protected hexathymidylylates and for 31 and 32; otherwise (28–30 and 33–41) from 30 to 100% B in 35 min and then 100% B for 5 min].

**Oligodeoxyribonucleotide Synthesis.** The protected oligonucleotides were assembled on an Applied Biosystems 392 DNA synthesizer in 0.2 and 1.0 µmol scales using either commercial solid supports or 27 (19) and phosphoramidite chemistry. Phosphoramidites 1a–c and 2a–d were used as 0.1 M solutions in dry 1,4-DCN, with an unaltered coupling time. In the synthetic protocol for the attachment of non-nucleosidic units derived from 1a–c, the capping subroutine was excluded, and the detritylation step was carried out by using two subsequent "#4 (acid solution) to column" steps (2 × 60 s), separated by a trityl flush step (3 s). In other cases, the recommended protocols were employed. The oligonucleotides bearing 5'-terminal non-nucleosidic moieties were subjected to the final deprotection in DMT-on mode. The support-bound material was deprotected with concentrated aqueous NH3 (2 h at room temperature, then 7 h at 55 °C), and products were isolated by RP HPLC. The 5'-DMTr group was cleaved with 80% aqueous AcOH for 20 min at ambient temperature, followed by analysis on both RP and ion exchange columns, RP isolation, and final desalting of the target oligonucleotides.

**Labeling of the Amino-Derivatized Oligonucleotides in Solution.** DNS-Cl [solution in dimethylformamide (DMF), 10 mg/mL, 120 µL] was added to the oligonucleotides 17, 31, or 32 (triethylammonium form, desalted, ca. 4 OD) dissolved in 0.1 M NaHCO3-Na2CO3 buffer (pH 10.3, 80 µL). The mixture was incubated in the dark for 3 h, the second portion of the DNS-Cl solution (50 µL) was added, and the reaction mixture was incubated for another 3 h. The reaction mixture was treated with 0.05 M aqueous NH4OAc (800 µL), and the excess of label was extracted with ethyl acetate (2 × 200 µL). The organic layer was discarded, and the labeled oligonucleotides 19, 28, and 29 were isolated from aqueous phase by RP HPLC. The fractions were evaporated in vacuo and detritylated with 80% aqueous AcOH (3 mL) for 20 min. After the removal of AcOH by evaporation, the material was separated by RP HPLC, and the fractions collected were finally desalted to give the DNS-labeled oligonucleotides 20, 33, or 34.

**Labeling of the Amino-Derivatized Oligonucleotides on the Solid Support.** The oligonucleotide-derivatized solid supports 21 and 23 (4 mg, ca. 0.1 µmol) were placed in a synthetic column. For 21, the standard detritylation step was carried out. For 23, the column was treated with a mixture of DMF/piperidine (1.5 mL, 9/1 v/v) for 10 min by a two-syringe method (20). In both cases, the deprotection was completed by extensive washing with MeCN. The solid supports 24 obtained by both routes were reacted with dabsyl chloride in N,N-dimethylformamide/pyridine (500 µL, 1/20/80 w/v/v) for 12 h in the dark. The liquid phases were withdrawn, and the solid supports, 25, were washed with dichloromethane (5 × 2 mL) and treated with concentrated ammonia for 2 h. The solutions obtained were evaporated, and the residues were separated by RP HPLC to give 26 in 85 and 60% yields, respectively.

**2-Ethoxy-5,5-bis(ethoxy carbonyl)-1,3-dioxane (6).** Concentrated H2SO4 (0.2 mL) was added to a solution of diethyl 2,2-bis(hydroxyethyl)malonate 4 (21) (33.0 g, 150 mmol), and triethyl orthoformate (33.4 g, 225 mmol) in dry tetrahydrofuran (THF) (50 mL). The reaction mixture was left overnight and then slowly poured into a magnetically stirred ice-cold solution of NaHCO3 (5% aqueous, 300 mL). The product was extracted with...
diethyl ether, washed with saturated aqueous NaCl, and dried over Na2SO4. The solvent was evaporated, and the residue was distilled in vacuo to give 6 (34.8 g, 84%): bp 135 – 137°C (1 Torr); nD20 1.4416; 1H NMR (CDCl3) δ 5.36 (s, 1H, C=C-H), 4.49 (2H, Jαβ = 12.2 Hz, C4-H6, C6-H4), 4.26 (2H, q, J = 7.0 Hz, CH2-O-CO), 4.24 (2H, d, Jαβ = 11.2 Hz, C4-H6, C6-H4), 4.24 (2H, q, J = 7.0 Hz, C4-H6, Jβγ = 7.1 Hz, C6-H4); TLC Rf 0.3 (CH2Cl2); 0.8 (49/1 CH2Cl2/MeOH). Anal. (C32H31NO8) C, H, N.

2-Ethoxy-5,5-bis[N-(3-trifluoroacetamido)propylcarbamido]-1,3-dioxane (8a). Compound 6 (10.0 g, 36.2 mmol) was added to a mixture of NaHCO3 (2.5 g, 30 mmol) in 40% aqueous dioxane (100 mL), and the mixture was coevaporated with water (3 × 100 mL) and stirred overnight at ambient temperature. The colorless solution obtained was evaporated in vacuo, and the residue was coevaporated with water (3 × 100 mL) and xylene (5 × 100 mL) until no 1,3-propanediamine could be detected by TLC (1/1/1/5 concentrated aqueous ammonia/H2O/MeOH/TFA). The viscous residue was dried on an oil pump at 50 °C to give 12 g (100%) of crude 2-ethoxy-5,5-bis[N-(3-aminopropyl)carbamido]-1,3-dioxane (7): 1H NMR (CDCl3) δ 5.48 (s, 1H, C=C-H), 4.53 (2H, Jαβ = 12.4 Hz, C4-H6, C6-H4), 4.23 (2H, d, Jαβ = 12.4 Hz, C4-H6, C6-H4), 3.74 (q, 2H, J = 7.0 Hz, CH2O), 3.30 (t, 2H, J = 6.8 Hz, CH2NHCO), 3.25 (t, 2H, J = 6.8 Hz, CH2NHCO), 2.61 (t, 2H, J = 7.0 Hz, CH2NH2), 2.58 (t, 2H, J = 7.3 Hz, CH2NH2), 1.65 (m, 2H, C-CH2-C), 1.62 (m, 2H, C-CH2-C); TLC Rf 0.1 (49/1 CH2Cl2/MeOH). Anal. (C22H26N2O7) C, H, N.

N,N-Bis[3-(trifluoroacetamido)propyl]-2,2-bis(hydroxymethyl)malonodiamide (9a). Compound 8a (2.0 g, 4.2 mmol) was dissolved in 80% aqueous AcOH (50 mL) and left for 2 h at room temperature. The solution was evaporated to an oil and coevaporated with water (3 × 25 mL). At this step, the reaction mixture consisted of 9a and its monoformal. The deprotection was completed by treating the material with MeOH/Et3N (90/10, 50 mL) for 20 min. The use of alcohols or primary or secondary amines causes degradation of 9a. The solution was evaporated, and the residue was coevaporated with toluene (2 × 50 mL). Chromatography on a short silica gel column eluted with a gradient of MeOH (3 to 20%) in CH2Cl2 gave 9a (1.75 g, 89%): mp 101 – 102 °C (benzene/MeOH); 1H NMR (DMF-d6) δ 9.30 (2H, t, J = 7.0 Hz, CH2O), 3.38 (8H, m, CH2NHCO), 1.78 (4H, m, C-CH2-C), 1.28 (3H, t, J = 7.3 Hz, CH3); TLC Rf 0.39 (5/1 CH2Cl2/MeOH). Anal. (C38H50N6O9) C, H, N.

N,N'-Bis[3-(9-fluorenylmethoxycarbonyl)laminio]propyl]carbamido]-1,3-dioxane (8b). Compound 6 (4.57 g, 16.5 mmol) was added in small portions to a stirred solution of crude 7 (2.5 g, 7.5 mmol) and NaHCO3 (2.5 g, 30 mmol) in 40% aqueous dioxane (100 mL) on ice bath. The stirring was continued for 2 h and then for 4 h at room temperature. The suspension was evaporated to one-third of the initial volume and diluted with water (100 mL). The precipitate was extracted with ethyl acetate (3 × 100 mL), and the extracts were washed with water (3 × 50 mL) and dried over Na2SO4. After evaporation to dryness, the pure 8b (4.3 g, 74%) was isolated by recrystallization from hexane/benzene. Two additional recrystallizations from the same mixture gave an analytical sample: mp 95 – 97 °C; 1H NMR (CDCl3) δ 7.75 (4H, m, aromatic), 7.58 (4H, m, aromatic), 7.39 (4H, m, aromatic), 7.30 (4H, m, aromatic), 5.38 (1H, s, C=C-H), 4.53 (2H, d, Jαβ = 13.4 Hz, C4-H6, C6-H4), 4.39 (4H, d, J = 6.8 Hz, CH2CH2O-C=O in FMOC), 4.21 (2H, d, Jαβ = 13.4 Hz, C4-H6, C6-H4), 4.19 (2H, t, J = 6.8 Hz, CH2CH2O-C=O in FMOC), 3.65 (2H, q, J = 7.1 Hz, CH2CH2O), 3.32 (4H, m, CH2NHCO), 3.21 (4H, m, CH2NH-FMOC), 1.67 (4H, m, C-CH2-C), 1.26 (3H, t, J = 7.1 Hz, CH2CH2O); TLC Rf 0.70 (9/1 CH2Cl2/MeOH), 0.45 (9/1 CH2Cl2/MeOH). Anal. (C47H44N10O12) C, H, N.
**Building Blocks for Oligonucleotide Multilabeling**

*N,N′-Bis[3-[[5-(dimethylamino)-1-naphthalenesulfonamido]propyl]-2,2-bis(hydroxymethyl)malonodiamide (9c)* was obtained from 8c (800 mg, 1.0 mmol) by a procedure analogous to that described for 9a. Chromatography on a short silica gel column eluted with a gradient of MeOH (0 to 12%) in CH$_2$Cl$_2$ gave 9c (667 mg, 90%) as a green foam. 1H NMR (CDCl$_3$) $\delta$ 7.52 (2H, dd, $J = 0.6$ and 8.6 Hz), 8.31 (2H, dd, $J = 0.6$ and 7.3 Hz), 7.57–7.49 (4H, m), 7.17 (2H, $d$, $J = 7.5$ Hz (aromatic)), 7.59 (2H, m, NH), 5.97 (2H, t, NH), 3.74 (4H, s, CH$_2$OH), 3.22 (4H, m, CH$_2$NHC), 2.88 (12H, s, N(CH$_3$)$_2$), 2.81 (4H, m, CH$_2$NHSO$_3$), 1.50 (4H, m, C-CH$_2$-C); UV (MeCN) $\lambda_{max} = 260$ (ε 2500), 340 (ε = 9700); TLC R$_f$ 0.54 (9/1 CH$_2$Cl$_2$/MeOH), 0.36 (19/1 CH$_2$Cl$_2$/MeOH). Anal. (C$_{35}$H$_{44}$N$_{10}$O$_{6}$S) C, H, N.

**General Procedure for the Selective Dimethoxytritylation of Diols: 3-[[[4,4-dimethoxytrityloxy]yl]-2,2-bis(azidomethyl)propanol (12).** A solution of 4,4-dimethoxytrityl chloride (880 mg, 2.60 mmol) in dry pyridine (15 mL) was added dropwise to 2,2-bis(azidomethyl)-1,3-propanediol, 12 (720 mg, 3.75 mmol), in dry pyridine (15 mL). The reaction mixture was left overnight at ambient temperature and the reaction stopped by addition of methanol (0.5 mL). Upon neutralization with NaHCO$_3$ (5% in water, 10 mL), the mixture was evaporated in vacuo to an oil, dissolved in CH$_2$Cl$_2$ (100 mL), washed with aqueous saturated NaCl (2 × 50 mL), and dried over Na$_2$SO$_4$. The solvent was removed in vacuo, and the residue was separated on a silica gel column (30 × 100 mm), eluting with a CH$_2$Cl$_2$/hexane mixture (1/3). Fractions containing pure product were pooled, evaporated, and dried on an oil pump to give 12 (1040 mg, 83% on DMTM-Cl) as a colorless oil: 1H NMR (CDCl$_3$) $\delta$ 7.42–7.20 (9H, m, aromatic), 6.88–6.80 (4H, m, aromatic), 3.86 (16H, s, CH$_2$O), 3.53 (2H, d, $J = 6.3$ Hz, CH$_2$OH), 3.43 (2H, s, CH$_2$N), 3.08 (2H, s, C-CH$_2$O); 1H NMR (CD$_2$D$_2$) $\delta$ 7.19 (1H, t, $J = 6.3$ Hz, HO-CH$_3$); IR (KBr) ν$_{max}$ = 3510 (1H, CH$_2$OH), 2012 cm$^{-1}$ (N$_2$); TLC R$_f$ 0.2 (100/1 CH$_2$Cl$_2$/EtOH). Anal. (C$_{35}$H$_{44}$N$_{10}$O$_{6}$S) C, H, N. Further elution of the column with a mixture of MeOH and CH$_2$Cl$_2$ (3/97) gave about 10% of the starting material, 11.

*N,N′-Bis[3-[(trifluoroacetamidomethyl)oxy]ethyl]-2-(hydroxymethyl)malonodiamide (10a) was prepared analogously from 9a (1400 mg, 3.0 mmol) and DMTM-Cl (1020 mg, 2.60 mmol). Isolation on a silica gel column, eluting with a gradient of MeOH (0 to 10%) in CH$_2$Cl$_2$, gave first pure 10a (1660 mg, 72%) along with unreacted 9a (20%): 1H NMR (CDCl$_3$) $\delta$ 7.58 (2H, t, $J = 6$ Hz, NH), 7.52 (2H, t, $J = 6.2$ Hz, NH), 7.38–7.13 (9H, m, aromatic), 6.84–6.80 (4H, m, aromatic), 4.13 (2H, d, $J = 6.0$ Hz, CH$_2$OH), 3.79 (6H, s, OCH$_3$), 3.59 (1H, t, $J = 6.0$ Hz, OH), 3.50 (2H, s, CH$_2$O), 3.39–3.22 (8H, m, CH$_2$NHSO$_3$), 1.67 (4H, m, C-CH$_2$-C); TLC R$_f$ 0.42 (19/1 CH$_2$Cl$_2$/MeOH, 0.15 (49/1 CH$_2$Cl$_2$/MeOH). Anal. (C$_{29}$H$_{32}$F$_{6}$N$_{2}$O$_{10}$) C, H, N.

**Building Blocks for Oligonucleotide Multilabeling**


*N,N′-Bis[3-[[5-(dimethylamino)-1-naphthalenesulfonamido]propyl]-2-[(4,4-dimethoxytrityloxy)yl]ethyl]-2-(hydroxymethyl)malonodiamide (10b) was prepared analogously from 9b (577 mg, 0.8 mmol) and DMTM-Cl (271 mg, 0.8 mmol). Isolation by using a gradient of MeOH (0 to 8%) in CH$_2$Cl$_2$ gave first pure 10b (614 mg, 75%) as a white foam along with unreacted 9b (18%): 1H NMR (CDCl$_3$) $\delta$ 7.75–7.73 (4H, m, aromatic), 7.58–7.56 (4H, m, aromatic), 7.53–7.46 (2H, m, NH), 7.40–7.35 (6H, m, aromatic), 7.29–7.16 (11H, m, aromatic), 6.83–6.80 (4H, m, aromatic), 5.29 (2H, br m, NH), 4.36 (4H, d, $J = 6.8$ Hz, CH$_2$OH), 3.97 (6H, s, OCH$_3$), 3.50 (2H, s, CH$_2$ODMTM), 3.29 (2H, m, 3.26 (2H, m, 2 × CH$_2$NHCO), 3.14 (4H, m, CH$_2$NHCOC), 1.68–1.57 (4H, m, C-CH$_2$-C); IR ν$_{max}$ = 3500–3250 (O-H, N-H), 1698 (υC=O, amid I), 1539 cm$^{-1}$ (N-H, amid II); TLC R$_f$ 0.50 (19/1 CH$_2$Cl$_2$/MeOH), 0.49 (9/1 CH$_2$Cl$_2$/MeOH). Anal. (C$_{41}$H$_{48}$N$_{12}$O$_{12}$) C, H, N.
14a as a white foam: \( ^{1}H\) NMR (CDCl3) \( \delta 7.43-7.20 \) (9H, m, aromatic), 6.90 (2H, br m, NH), 6.88-6.84 (4H, aromatic), 3.80 (6H, s, CH3O), 3.47 (2H, br s, C\( \text{-} \text{O}\)H), 3.43 (2H, dd, \( \delta J = 14.2 \) Hz, \( \delta J = 7.8 \) Hz, 2 \( \times \) CH\( ^{3} \)H\( ^{1} \)NH), 3.08 (2H, s, C\( \text{-} \text{O}\)C\( \text{H}_{2}\)), 2.97 2H, dd, \( \delta J = 14.2 \) Hz, \( \delta J = 5.9 \) Hz, 2 \( \times \) CH\( ^{3} \)H\( ^{1} \)NH), 1.58 (1H, br s, HOH); IR \( \nu \) 3600, 3300 (O-H, N-H), 1716 (C\( \text{-} \text{O}\)H), 1414 (CONH), 1363 (C\( \text{-} \text{O}\)H), 1216 and 1171 cm\(^{-1}\) (N-H); TLC \( R_{f} \) 0.7 (19:1 CH\(_{2}\)Cl\(_{2}\)/MeOH). Anal. (C\(_{30}\)H\(_{30}\)F\(_{6}\)N\(_{2}\)O\(_{6}\)) C, H, N.

General Procedure for the Preparation of Phosphoramidites: 2-Cyanoethyl \( 3\{4,4\text{-Dimethoxytrityloxy}\}\)ox,2,2-bis[N-(9-fluorenlymethoxycarbonyl)amino]methyl]propanol (14b) was prepared from crude 13 (300 mg, 0.7 mmol) and Fmoc-Cl (520 mg, 2.0 mmol) by a procedure analogous to that for 8b. Upon isolation by silica gel chromatography (gradient from 0 to 100% MeOH in CH\(_{2}\)Cl\(_{2}\)), the pure 14b (350 mg, 57%) was obtained as a colorless foam. \( ^{1}H\) NMR (CDCl3) \( \delta 7.8-7.7, 7.6-7.15 \) (25H, aromatic), 6.9-6.8 (4H, aromatic), 5.45 (1H, m, OH), 5.06 (2H, br m, NH), 4.46 (2H, d, \( \delta J = 6.3 \) Hz, CH\( _{2}\)OH), 4.38 (2H, dd, \( \delta J = 7.3 \) Hz, \( \delta J = 10.5 \) Hz, CH\( _{2}\)C\( ^{3} \)H\( ^{1} \)CONH), 3.74 (6H, s, CH\(_{3}\)O), 3.68 (2H, s, C\( \text{-} \text{O}\)H), 3.24 (2H, s, CH\( _{2}\)ODMTr), 3.02 (4H, m, 2 \( \times \) CH\(_{2}\)NH-C\( \text{-} \text{O}\)H), 2.50 (2H, d, \( \delta J = 14.2 \) Hz, 2 \( \times \) CH\(_{2}\)CONH), 2.20 (2H, t, \( \delta J = 7.3 \) Hz, 2 \( \times \) CH\(_{2}\)CH\( ^{3} \)H\( ^{1} \)NCO), 2.07 (4H, t, \( \delta J = 6.8 \) Hz, 2 \( \times \) CH\(_{2}\)OH). Anal. (C\(_{56}\)H\(_{52}\)N\(_{2}\)O\(_{8}\)) C, H, N.

RESULTS AND DISCUSSION

Synthesis of Phosphoramidites 1 and 2. Building blocks 1a-c were synthesized from commercially available diethyl 2,2-bis(hydroxymethyl)malonate (4) (21), as depicted in Scheme 2. The synthetic use of 4 is hampered by its rapid degradation in basic media, in particular in the presence of primary amines. We have recently found that the mono-O-DMTr derivative of 4 (5) when treated with a catalytic amount of aqueous ammonia or butylamine is decomposed, and a stoichiometric amount of DMTr-OH is released (25). This precludes straightforward preparation of any aminooxyl derivatives from 5. To overcome this problem, 4 was converted to its ethoxymethylene derivative 6, which gave the bis[N-(3-aminopropyl)amide] 7 upon treatment with 1,3-propanediamine in THF. The primary amino groups were subsequently acylated with methyl trifluoroacetate, Fmoc-Cl, or Dns-Cl to obtain 8a-c, respectively, and the ethoxymethylene protection was removed by successive acid-catalyzed hydrolysis and triethylamine-catalyzed methanolysis. The use of a tertiary amine as a catalyst is crucial, since ammonia and primary and secondary amines degrade the deprotected products 9a-c. Selective protection of one of the hydroxy functions of 9a-c with DMTr-Cl gave 10a-c. They were finally converted into corresponding phosphoramidites 1a-c with 2-cyanoethyl N,N,N',N'-

**Scheme 2**

\[ a \] (i) \((\text{EtO})_2\text{CH}/\text{H}_2\text{SO}_4\); (ii) 1,3-diaminopropane/THF; (iii) for 8a, methyl trifluoroacetate/Py; for 8b, FMOCl-Cl/NaHCO\(_3\)/aqueous dioxane; for 8c, DNS-Cl/Py; (iv) 80% aqueous AcOH; (v) Et\(_3\)N/ EtOH; (vi) DMTr-Cl/Py; (vii) 2-cyanoethyl N,N,N′,N′-tetraisopropylphosphoramidite/1H-tetrazole. DNS, 5-(dimethylamino)-1-naphthalenesulfonyle.

**Scheme 3**

\[ a \] (i) for 14a, methyl trifluoroacetate/Py; for 14b, FMOCl-Cl/NaHCO\(_3\)/aqueous dioxane; for 14c, MMTr-Cl/Py; for 14d, N-oxysuccinimido 6-(trifluoroacetamido)hexanoate; (ii) 2-cyanoethyl N,N,N′,N′-tetraisopropylphosphoramidite/1H-tetrazole. DMTr, 4,4′-dimethoxytrityl; MMTr, 4-monomethoxytrityl; FMOCl, 9-fluorenylmethoxy carbonyl.

tetraisopropylphosphoramidite (24) in the presence of 1H-tetrazole, followed by aqueous workup and precipitation from toluene solution to hexane.

Building blocks 2a–d were prepared from easily available 2,2-bis(azidomethyl)-1,3-propanediol (11) (22) as outlined in Scheme 3. Treatment of 11 with a deficient amount of 4,4′-dimethoxytrityl chloride afforded 12 which was isolated by column chromatography in a moderate yield. The azido groups of 12 were subsequently reduced to amino functions with a mixture of NaBH\(_4\) and 1,3-propanedithiol (26), yielding 85% of 13. Reduction with H\(_2\)S (27) and 2-mercaptoethanol was unsuccessful, and reaction with triphenylphosphine followed by hydrolysis or ammonolysis gave 13 only in 30–40% yield.

Diamine 13 was N-acylated with either methyl trifluoroacetate or FMOCl to give 14a and 14b in a high yield, respectively. Compound 14c, having an acid-labile 4-monomethoxytrityl (MMTr) protection at the amino functions instead of N-acyl groups, was obtained by treating 13 with MMTr-Cl. Compound 14d, bearing N-[6-(trifluoroacetamido)hexanoyl] side arms, was prepared by N-acylation with N-oxysuccinimido 6-(trifluoroacetamido)hexanoate (23). Compounds 14a–d were finally converted into corresponding phosphoramidites 2a–d as described above.

**Synthesis of Oligonucleotide Conjugates.** The compatibility of phosphoramidites 1 and 2 with oligonucleotide synthesis by phosphoramidite strategy was first examined by assembling model oligonucleotides 5′-XT\(_6\) where X stands for the non-nucleosidic unit. For reference purposes, a small part of the solid support bearing the T\(_6\) sequence (20 nmol) was withdrawn from each synthesis column before the attachment of the non-nucleosidic unit. After conventional deprotection and cleavage from the solid support, the high yield of T\(_6\) was verified by HPLC. All non-nucleosidic building blocks were attached at the final step of chain elongation by using 0.1 M solutions of 1 and 2 and unaltered coupling time (Applied Biosystems 392 DNA synthesizer, 0.2 µmol scale). The yield of the last coupling measured by DMTr assay was found to be 95–96% with 2a, 97–98% with 1c, and more than 98% with other phosphoramidites, indicating an acceptable efficiency.

Phosphoramidites 1a–c were shown to be compatible with the standard protocol of oligonucleotide synthesis and ammonolytic deprotection. No side reactions were detected when the oligonucleotide conjugates prepared from 1a (15) or 1b (16) were subjected to ammonia treatment (Scheme 4). Both conjugates (15 and 16) gave the same deprotected oligomer (17), as indicated by ion exchange and RP HPLC. Labeling 17 with DNS-Cl gave the fluorescent oligonucleotide, 19. The identity of 19 was verified by insertion of a DNS-labeled building block (1c) into the 5′-terminus of a hexamidylate on a solid support. The oligonucleotide conjugate obtained (18) gave 19 upon ammonolysis. Finally, 19 was detritylated in solution to 20 and isolated by RP HPLC.

It is essential to note that the 5′-DMTr group may be removed only after the deprotection and labeling of the amino functions. While the deprotected oligonucleotide conjugate 20 is stable for several weeks at pH < 8.5, i.e., a period long enough for most diagnostic purposes, it is at higher pH converted to hexamidylidy 5′-phosphate. We have shown previously that the unsubstituted hydroxymethyl group undergoes retrograde aldol reaction under alkaline conditions, and this leads to elimination of the non-nucleosidic structure (25). For example, in the presence of 0.01 M butylamine, the half-life of this reaction was observed to be less than 30 min with 20.

The same problem concerns the 3′-labeled oligonucleotides, which may be obtained by conventional attachment
of compounds 14a–c to aminoalkylated controlled pore glass via a succinyl linker and subsequent oligonucleotide synthesis. Cleavage from the solid support with aqueous ammonia creates a free hydroxymethyl function, and this leads to formation of oligonucleotide 3′-monophosphate instead of the desired 3′-labeled oligonucleotide conjugate. Therefore, the synthesis must be carried out on a modified support, from which the 3′-terminal hydroxy group is released in a protected form upon ammonolysis. For example, the hydroxy function of the non-nucleosidic moiety derived from 14a–c may be kept protected with a phosphategroup. This is readily achieved by carrying out the synthesis on the solid support 27 (Scheme 6) described previously (19).

Apparently, the phosphoramidites, 2a–d, are also compatible with the conventional oligonucleotide synthesis. However, ammonolysis of the hexathymidylates derivatized with 2a, b, or d, i.e., building blocks having base-labile amino protections, resulted in rather complicated reaction mixtures that contained a great number of truncated oligonucleotides, even when ammonolysis in the DMT-Tr-on mode. Only the conjugate of 2c, having a base stable MMTr protection at the primary amino functions, gave a single product, 22. Accordingly, oligonucleotide conjugates derived from non-nucleosidic building blocks that contain two unprotected primary amino functions appear to undergo an efficient self-cleavage upon ammonolysis. This is somewhat unexpected, since the 5′-terminal tails consisting of several mono(ami-noalkyl) units, such as 3-amino-1,2-propanediol, do not result in a similar instability (14).

For the reasons indicated above, building blocks 2a–d may be used for multilabeling of oligonucleotides only if the amino protections are removed under mild conditions and the oligonucleotide conjugate is labeled when still attached to the solid support (Scheme 5). To demonstrate the feasibility of this approach, the oligonucleotide conjugate derived from 2c (21) was converted to 24 by treatment with 3% dichloroacetic acid in acetonitrile, and the amino functions released were labeled with dabsyl chloride in organic medium to give 25. Ammonolysis then led to a bis-labeled oligonucleotide 26 in a high yield. Similarly, the oligonucleotide conjugate of 2b (23) was treated with 10% piperidine (20) to remove the FMOC protections. After labeling with dabsyl chloride and ammonolysis was complete, a bis-labeled product (26) was again obtained in a moderate yield, along with monolabeled oligonucleotides. The latter are probably formed by partial cleavage of the diamino oligonucleotide (24) during the removal of the FMOC groups and the subsequent labeling.

In summary, while 1a–c may be conveniently used for multilabeling of oligonucleotides, 2b,c suffer from serious limitations, and 2a,d turned out to be useless for this purpose.

Synthesis of Polylabeled Oligonucleotide Conjugates by Building Blocks 1a–c. Since 1a–c were observed to allow an efficient introduction of either amino or DNS groups, they were applied to preparation of both 3′- and 5′-multiderivatized oligonucleotides and their phosphorothioate analogues. The oligonucleotide part of these structures, 5′-GAA CAT CAT GGT CGT-3′, is known to be an efficient antisense sequence against c-fos mRNA (28).

5′-Labeled oligonucleotides 28–32 were assembled as outlined in Scheme 6. The standard synthetic protocol was only slightly modified. A longer detritylation step (2 min, 3% dichloroacetic acid in dichloromethane) was used to detritylate the non-nucleosidic units. After DMT-Tr-on ammonolysis, 28–32 were isolated in a yield typical for a routine synthesis (16–20 AU from 0.2 µmol column). The di- and tetraamino-derivatized oligomers obtained (31 and 32) were subsequently labeled with

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**Scheme 4**

![Scheme 4](image)

- (i) concentrated aqueous NH₃; (ii) DNS-Cl; (iii) 80% aqueous AcOH.

**Scheme 5**

![Scheme 5](image)

(a) concentrated aqueous NH₃; (ii) 3% Cl₂HCCO₂H/CH₂Cl₂; (iii) 10% piperidine/DMF; (iv) dabsyl chloride.

**Scheme 6. Solid Support 27 and Structures of Polylabeled Oligonucleotides 28–41**

- The DNA fragments are marked as rectangles.
DNS-CI to give 28 and 29 by an independent route. The synthesis was completed by conventional cleavage of the 5'-terminal DMTr group (80% aqueous AcOH, 20 min). This contrasts with the enhanced resistance of the DMTr group of non-nucleosidic units toward acidic treatment under nonaqueous conditions. The deprotected poly-labeled oligonucleotides 33–35 were isolated by RP HPLC (Table 1).

When the iodine oxidation step within the DNA synthetic cycle was replaced by treatment with tetraethylthiuram disulfide (29), the same methodology allowed an efficient preparation of the 5'-polylabeled phosphorothioate oligonucleotide analogues, 36–39 (Table 1).

Oligonucleotides labeled at the 3'-terminus with either four or six DNS groups (40 and 41) were analogously synthesized on support 27 with the aid of 1c (Table 1).

Hybridization Properties of the Polylabeled Oligonucleotides. In order to characterize the hybridization properties of the labeled oligonucleotides, melting experiments were carried out for the 5'-dansylated oligonucleotides, 33–35, phosphorothioate analogue, 39, and the 3'-labeled oligomer, 41. A longer, 27-nucleotide, complementary synthetic oligonucleotide, 5'-CAG TCT ACC ACC ATG ATG TTC GTT CAG-3', was used as a hybridization target. The noncomplementary sites (six nucleotides at both termini) were included in its structure to find out whether the newly labeled non-nucleosidic tails interactate or, vice versa, destabilize the base pairing by creating steric hindrances. The melting points observed are listed in Table 1, and the thermal denaturation curves for the unmodified oligonucleotide and its hexalabeled analogues 35 and 39 are presented in Figure 1. As seen, the Tm values for the multilabeled oligonucleotides 33–35 remain practically unchanged compared to that of the underivatized oligonucleotide (Tm = 58.4 °C), whereas 3'-hexalabeled 41 exhibits a decrease of the melting point (Δ = -3.3 °C). For hexalabeled phosphorothioate analogue 39, the melting point is lower (Tm = 50.3 °C). Expectedly, the low melting point results from the modified phosphate backbone (30) rather than from the non-nucleosidic moieties.

CONCLUSIONS

Among the phosphoramidites 1 and 2 synthesized, the derivatives of N,N'-bis-(3-aminopropyl)-2,2-bis(hydroxy-methyl)malonodiamide 1a–c allow the preparation of polyamino- and polydansyl-labeled oligonucleotides and their phosphorothioate analogues. The labeling may be accomplished via routine synthetic cycles at both the 5'- and 3'-termini of the oligonucleotide. In the 3'-labeling, a solid support that caps the 3'-terminal hydroxy group must, however, be used. The introduction of the reporter groups has no or only minor effect on the hybridization properties of the multilabeled oligonucleotides.

ACKNOWLEDGMENT

Financial support from the Research Council for Natural Sciences, the Academy of Finland, is gratefully acknowledged. A.G. is indebted to the International Science and Technical Center (the U.S. and E.C. financial bodies) for partial financial support.

LITERATURE CITED


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