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

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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 phosphorothioate 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 phospho diester 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 hydroxy 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 hydroxy group) being tethered to the conjugate group. Biotinyl (*5*, *10*), pyrenyl (*6*, *7*), dansyl (DNS) (*7*), carborane (*8*), dinitrophenyl (*9*), phosphotyrosinyl (*10*), or fluorescenyl (*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 postsynthetically 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 hydroxy 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-(benzyloxycarbonyl)ethyl]-1,3-propanediol (*15*), and 1-[(6-aminohexanoyl)amino]-2,4-propanediol (*4*). More recently, phosphoramidites from longer α,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-ethanediamine 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 intermolecular 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-

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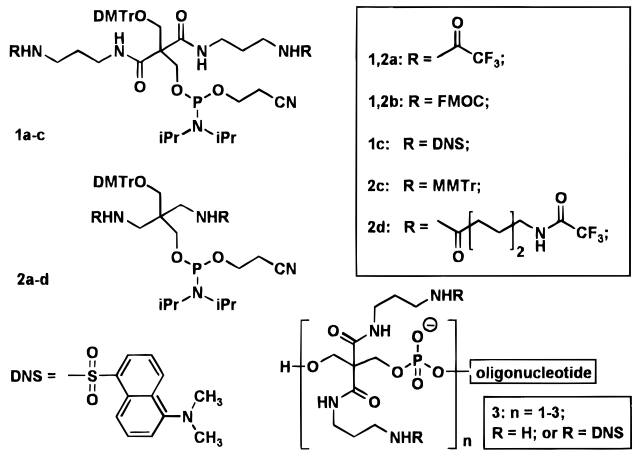
Scheme 1. Structures of Non-Nucleosidic Phosphoramidites 1 and 2 and Multilabeled Oligonucleotides 3



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 four primary amino functions or 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 399.8 and 161.9 MHz for ^{1}H and ^{31}P, respectively. Either $CDCl_3$ or DMSO-d_6 was used as the solvent, with either tetramethylsilane (TMS) as an internal (^{1}H) or H_3PO_4 as an external (^{31}P) standard.

Melting Experiments. A λ 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 NH_4OAc; buffer B, 0.05 M NH_4OAc 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 hexathymidylates 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 MeCN, with 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 "#14 (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 NH_3 (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 $NaHCO_3/Na_2CO_3$ 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 NH_4OAc (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*-diisopropylamine/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 dioxane (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(ethoxycarbonyl)-1,3-dioxane (6). Concentrated H_2SO_4 (0.2 mL) was added to a solution of diethyl 2,2-bis(hydroxymethyl)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 $NaHCO_3$ (5% aqueous, 300 mL). The product was extracted with

diethyl ether, washed with saturated aqueous NaCl, and dried over Na_2SO_4. The solvent was evaporated, and the residue was distilled *in vacuo* to give **6** (34.8 g, 84%): bp 135−137 °C (1 Torr); n_D^{20} 1.4416; ^{1}H NMR ($CDCl_3$) δ 5.36 (s, 1H, C^2-H), 4.49 (d, 2H, J^2_{AB} = 11.2 Hz, C^4-H^e, C^6-H^e), 4.26 (2H, q, J^3 = 7.0 Hz, CH_2OC^aO), 4.24 (d, 2H, J^2_{AB} = 11.2 Hz, C^4-H^a, C^6-H^a), 4.23 (2H, q, J^3 = 7.0 Hz, CH_2OC^eO), 3.64 (2H, q, J^3 = 7.0 Hz, CH_2OC^2), 1.28 (t, J^3 = 7.0 Hz), 1.27 (t, J^3 = 7.0 Hz), 1.25 (t, J^3 = 7.0 Hz, C***H***$_3$-CH_2OC^2, 2 × C***H***$_3CH_2OCO$); IR ν 1731 cm^{-1} ($\nu_{C=O}$); TLC R_f 0.3 (CH_2Cl_2), 0.8 (49/1 CH_2Cl_2/MeOH). Anal. ($C_{12}H_{20}O_7$) C, H.

2-Ethoxy-5,5-bis[*N*-[3-(trifluoroacetamido)propyl]-carboxamido]-1,3-dioxane (8a). Compound **6** (10.0 g, 36.2 mmol) was added to a mixture of 1,3-propanediamine (11.1 g, 150 mmol) and THF (50 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 *p*-xylene (5 × 100 mL) until no 1,3-propanediamine could be detected by TLC (1/1/1/5 concentrated aqueous ammonia/H_2O/MeOH/THF). 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)carboxamido]-1,3-dioxane (**7**): ^{1}H NMR (D_2O) δ 5.48 (s, 1H, C^2-H), 4.53 (d, 2H, J^2_{AB} = 12.4 Hz, C^4-H^e, C^6-H^e), 4.23 (d, 2H, J^2_{AB} = 12.4 Hz, C^4-H^a, C^6-H^a), 3.74 (q, 2H, J^3 = 7.0 Hz, CH_2O), 3.30 (t, 2H, J^3 = 6.8 Hz, CH_2NHC^aO), 3.25 (t, 2H, J^3 = 6.8 Hz, CH_2NHC^eO), 2.61 (t, 2H, J^3 = 7.0 Hz, $C^aH_2NH_2$), 2.58 (t, 2H, J^3 = 7.3 Hz, $C^eH_2NH_2$), 1.65 (m, 2H, C-C^aH_2-C), 1.62 (m, 2H, C-C^eH_2-C), 1.21 (t, 3H, J^3 = 7.0 Hz, CH_3). The content of **7** in the crude sample was more than 95%, and it was hence used without further purification for the preparation of **8a**−**c**. A mixture of crude **7** (4.75 g, 14.3 mmol), methyl trifluoroacetate (4.20 g, 32.8 mmol), triethylamine (4.05 g, 40 mmol), and dry pyridine (50 mL) was kept for 5 h at room temperature. The reaction mixture was evaporated to an oil, dissolved in CH_2Cl_2 (200 mL), washed with aqueous $NaHCO_3$ (5%, 2 × 50 mL), and dried over Na_2SO_4. The solvent was evaporated *in vacuo*, and the residue was recrystallized twice from benzene/ethyl acetate to give **8a** (5.76 g, 77%). An analytical sample was recrystallized once to give pure **8a**: mp 152.5−153 °C; ^{1}H NMR ($CDCl_3$) δ 7.79 (2H, t, NH), 7.66 (2H, t, NH), 5.39 (1H, s, C^2-H), 4.50 (2H, d, J^2_{AB} = 12.2 Hz, C^4-H^e, C^6-H^e), 4.19 (2H, d, J^2_{AB} = 12.2 Hz, C^4-H^a, C^6-H^a), 3.68 (2H, q, J^3 = 7.3 Hz, CH_2O), 3.38 (8H, m, CH_2NHCO), 1.78 (4H, m, C-CH_2-C), 1.28 (3H, t, J_3 = 7.3 Hz, CH_3); TLC R_f 0.51 (9/1 CH_2Cl_2/MeOH), 0.40 (19/1 CH_2Cl_2/MeOH). Anal. ($C_{18}H_{26}F_6N_4O_7$) C, H, N.

2-Ethoxy-5,5-bis[*N*-[3-[(9-fluorenylmethoxycarbonyl)amino]propyl]carboxamido]-1,3-dioxane (8b). FMOC-Cl (4.75 g, 16.5 mmol) was added in small portions to a stirred solution of crude **7** (2.5 g, 7.5 mmol) and $NaHCO_3$ (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 Na_2SO_4. After evaporation to dryness, the pure **8b** (4.3 g, 74%) was isolated by recrystallization (hexane/benzene). Two additional recrystallizations from the same mixture gave an analytical sample: mp 95−97 °C; ^{1}H NMR ($CDCl_3$) 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^2-H), 4.53 (2H, d, J^2_{AB} = 13.4 Hz, C^4-H^e, C^6-H^e), 4.39 (4H, d, J^3 = 6.8 Hz, CHC***H***$_2$O-C═O in FMOC), 4.21 (2H, d, J^2_{AB} = 13.4 Hz, C^4-H^a, C^6-H^a), 4.19 (2H, t, J^3 = 6.8 Hz, C***H***CH_2O-C═O in FMOC), 3.65 (2H, q, J^3 = 7.1 Hz, CH_3C***H***$_2$O), 3.32 (4H, m, CH_2NHCO), 3.21 (4H, m, CH_2NH-FMOC), 1.67 (4H, m, C-CH_2-C), 1.26 (3H, t, J^3 = 7.1 Hz, C***H***$_3$-CH_2O); TLC R_f 0.70 (9/1 CH_2Cl_2/MeOH), 0.45 (19/1 CH_2Cl_2/MeOH). Anal. ($C_{44}H_{48}N_4O_9$) C, H, N.

2-Ethoxy-5,5-bis[*N*-[3-[5-(dimethylamino)-1-naphthalenesulfonamido]propyl]carboxamido]-1,3-dioxane (8c). DNS-Cl (1350 mg, 5.0 mmol) was added portionwise to a solution of crude **7** (660 mg, 2.0 mmol) in dry pyridine (25 mL) during 2 h. The mixture was left overnight at room temperature and the reaction quenched by addition of MeOH (0.5 mL). The reaction mixture was evaporated to an oil, dissolved in CH_2Cl_2 (200 mL), washed with aqueous $NaHCO_3$ (5%, 2 × 50 mL), and dried over Na_2SO_4. The solvent was evaporated *in vacuo*, and the pure compound was isolated on a silica gel column, eluting with a gradient of MeOH (0 to 7%) in CH_2Cl_2 to give **8c** (1295 mg, 81%): ^{1}H NMR ($CDCl_3$) δ 8.51 (2H, dd, J = 0.5 and 8.5 Hz), 8.33 (2H, dd, J = 3.9 and 8.5 Hz), 8.22 (2H, dt, J = 1.2 and 7.3 Hz), 7.58−7.50 (4H, m), 7.16 (2H, dt, J = <1 and 7.6 Hz (aromatic)), 7.36 (2H, s, NH), 5.77 (2H, t, NH), 5.24 (1H, s, C^2-H), 4.28 (2H, d, J^2_{AB} = 12.0 Hz, C^4-H^e, C^6-H^e), 3.93 (2H, d, J^2_{AB} = 12.0 Hz, C^4-H^a, C^6-H^a), 3.61 (2H, q, J^3 = 7.1 Hz, CH_2O), 3.28 (4H, m, CH_2NHCO), 2.93−2.86 (4H, m, CH_2NHSO_2), 2.87 (12H, s, $N(CH_3)_2$), 1.64−1.56 (4H, m, C-CH_2-C), 1.22 (3H, t, J^3 = 7.3 Hz, CH_3); TLC R_f 0.52 (9/1 CH_2Cl_2/MeOH) 0.68 (19/1 CH_2Cl_2/MeOH). Anal. ($C_{38}H_{50}N_6O_9S_2$) C, H, N, S.

***N,N′*-Bis[3-(trifluoroacetamido)propyl]-2,2-bis-(hydroxymethyl)malonodiamide (9a).** Compound **8a** (2.20 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 monoformate. The deprotection was completed by treating the material with MeOH/Et_3N (90/10, 50 mL) for 20 min. *The use of alkalies 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 CH_2Cl_2 gave **9a** (1.75 g, 89%): mp 101−102 °C (benzene/MeOH); ^{1}H NMR (DMSO-d_6/$CDCl_3$) δ 9.30 (2H, t, NH), 7.76 (2H, t, NH), 4.86 (2H, t, J^3 = 4.9 Hz, OH), 3.81 (4H, d, J^3 = 4.9 Hz, CH_2O), 3.18 (4H, m), 3.11 (4H, m, CH_2NHCO), 1.62 (4H, tt, 2 × J^3 = 6.6 Hz, C-CH_2-C); TLC R_f 0.32 (9/1 CH_2Cl_2/MeOH), 0.12 (19/1 CH_2Cl_2/MeOH). Anal. ($C_{15}H_{22}F_6N_4O_6$) C, H, N.

N,N′*-Bis[3-[(9-fluorenylmethoxycarbonyl)amino]-propyl]-2,2-bis(hydroxymethyl)malonodiamide (9b)** was obtained from **8b** (932 mg, 1.2 mmol) by a procedure analogous to that described for **9a**. After the removal of MeOH and Et_3N, the product was dissolved in CH_2Cl_2, washed with saturated aqueous NaCl, and dried over Na_2SO_4. The solvent was evaporated *in vacuo*, and the pure compound was isolated on a silica gel column, eluting with a gradient of MeOH (0 to 12%) in CH_2Cl_2 to give **9b** (700 mg, 81%) as a colorless foam: ^{1}H NMR ($CDCl_3$) δ 7.77−7.73 (4H, m, aromatic), 7.73−7.70 (2H, br m, NH), 7.62−7.56 (4H, m, aromatic), 7.41−7.35 (4H, m, aromatic), 7.32−7.27 (4H, m, aromatic), 5.27 (2H, br, m, NH), 4.38 (4H, d, J^3 = 6.3 Hz, CHCH***$_2$O-C═O in FMOC), 4.18 (2H, t, J^3 = 6.3 Hz, C***H***CH_2O-C═O in FMOC), 3.99 (2H, br t, OH), 3.91 (4H, d, J^3 = 5.5 Hz, C***H***$_2$OH), 3.32 (4H, m, CH_2NHCO), 3.20 (4H, m, CH_2NH-FMOC), 1.68 (4H, m, C-CH_2-C); TLC R_f 0.50 (9/1 CH_2Cl_2/MeOH), 0.35 (19/1 CH_2Cl_2/MeOH). Anal. ($C_{41}H_{44}N_4O_8$) C, H, N.

***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_2Cl_2 gave **9c** (667 mg, 90%) as a green foam: ^{1}H NMR ($CDCl_3$) δ 8.52 (2H, dd, J = 0.7 and 8.5 Hz), 8.31 (2H, dd, J = 0.6 and 8.6 Hz), 8.22 (2H, dd, J = 1.2 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_2OH), 3.22 (4H, m, CH_2-NHCO), 2.88 (12 H, s, $N(CH_3)_2$), 2.81 (4H, m, CH_2-$NHSO_2$), 1.50 (4H, m, C-CH_2-C); UV (MeCN) λ_{max} = 260 (ϵ 26 500), 340 nm (ϵ = 9700); TLC R_f 0.54 (9/1 CH_2Cl_2/MeOH), 0.36 (19/1 CH_2Cl_2/MeOH). Anal. ($C_{35}H_{46}N_6O_8S_2$) C, H, N, S.

General Procedure for the Selective Dimethoxytritylation of Diols: 3-[(4,4′-dimethoxytrityl)oxy]-2,2-bis(azidomethyl)propanol (12). A solution of 4,4′-dimethoxytrityl chloride (880 mg, 2.60 mmol) in dry dioxane (40 mL) was added dropwise to 2,2-bis(azidomethyl)-1,3-propanediol, **11** (*22*) (700 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_2Cl_2 (100 mL), washed with aqueous saturated NaCl (2 × 50 mL), and dried over Na_2SO_4. The solvent was removed *in vacuo*, and the residue was separated on a silica gel column (30 × 100 mm), eluting with a CH_2Cl_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 DMTr-Cl) as a colorless oil: ^{1}H NMR ($CDCl_3$) δ 7.42−7.20 (9H, m, aromatic), 6.88−6.80 (4H, m, aromatic), 3.80 (6H, s, CH_3O), 3.53 (2H, d, J^3 = 6.3 Hz, C***H***$_2$-OH), 3.43 (2H, s), 3.42 (2H, s, 2 × CH_2N_3), 3.08 (2H, s, C-O-CH_2), 1.79 (1 H, t, J^3 = 6.3 Hz, ***H***O-CH_2); IR ν 3510 (O-H), 2102 cm^{-1} (N_3); TLC R_f 0.2 (100/1 $CHCl_3$/EtOH). Anal. ($C_{26}H_{28}N_6O_4$) C, H, N. Further elution of the column with a mixture of MeOH and CH_2Cl_2 (3/97) gave about 10% of the starting material, **11**.

***N,N′*-Bis[3-(trifluoroacetamido)propyl]-2-[[(4,4′-dimethoxytrityl)oxy]methyl]-2-(hydroxymethyl)malonodiamide (10a)** was prepared analogously from **9a** (1400 mg, 3.0 mmol) and DMTr-Cl (1020 mg, 3.0 mmol). Isolation on a silica gel column, eluting with a gradient of MeOH (0 to 10%) in CH_2Cl_2, gave first pure **10a** (1660 mg, 72%) along with unreacted **9a** (20%): ^{1}H NMR ($CDCl_3$) δ 7.58 (2H, t, J^3 = 6 Hz, NH), 7.52 (2H, t, J^3 = 6.2 Hz, NH), 7.38−7.13 (9H, m, aromatic), 6.84−6.80 (4H, m, aromatic), 4.13 (2H, d, J^3 = 6.0 Hz, CH_2-OH), 3.79 (6H, s, OCH_3), 3.59 (1H, t, J^3 = 6.0 Hz, OH), 3.50 (2H, s, CH_2O), 3.39−3.22 (8H, m, CH_2NHCO), 1.67 (4H, m, C-CH_2-C); TLC R_f 0.42 (19/1 CH_2Cl_2/MeOH, 0.15 (49/1 CH_2Cl_2/MeOH). Anal. ($C_{36}H_{40}F_6N_4O_8$) C, H, N.

N,N′*-Bis[3-[(9-fluorenylmethoxycarbonyl)amino]propyl]-2-[[(4,4′-dimethoxytrityl)oxy]methyl]-2-(hydroxymethyl)malonodiamide (10b)** was prepared analogously from **9b** (577 mg, 0.8 mmol) and DMTr-Cl (271 mg, 0.8 mmol). Isolation by using a gradient of MeOH (0 to 8%) in CH_2Cl_2 gave first pure **10b** (614 mg, 75%) as a white foam along with unreacted **9b** (18%). ^{1}H NMR ($CDCl_3$) δ 7.75−7.73 (4H, m, aromatic), 7.58−7.56 (4H, m, aromatic), 7.53−7.46 (2H, br 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^3 = 6.8 Hz, CHCH***$_2$O-C═O), 4.18 (2H, t, J^3 = 6.8 Hz, C***H***CH$_2$O-C═O), 4.08 (4H, d, J^3 = 6.8 Hz, C***H***$_2$OH), 3.75 (6H, s, OCH_3), 3.50 (2H, s, CH_2ODMTr), 3.29 (2H, m), 3.26 (2H, m, 2 × CH_2NHCO), 3.14 (4H, m, CH_2NHCOO), 1.68−1.57 (4H, m, C-CH_2-C); IR ν 3500−3250 (O-H, N-H), 1698 ($\nu_{C=O}$, amid I), 1539 cm^{-1} (N-H, amid II); TLC R_f 0.50 (19/1 CH_2Cl_2/MeOH), 0.2 (49/1 CH_2Cl_2/MeOH). Anal. ($C_{62}H_{62}N_4O_{10}$) C, H, N.

***N,N′*-Bis[3-[5-(dimethylamino)-1-naphthalenesulfonamido]propyl]-2-[[(4,4′-dimethoxytrityl)oxy]-methyl]-2-(hydroxymethyl)malonodiamide (10c)** was prepared analogously from **9c** (525 mg, 0.7 mmol) and DMTr-Cl (237 mg, 0.7 mmol). Isolation by using a gradient of MeOH (0 to 9%) in CH_2Cl_2 gave first pure **10b** (600 mg, 82%) along with unreacted **9b** (12%): ^{1}H NMR ($CDCl_3$) δ 8.49 (2H, d, J = 8.5 Hz, DNS), 8.28 (2H, d, J = 8.5 Hz, DNS), 8.19 (2H, dd, J = 1.0 and 7.3 Hz, DNS), 7.49−7.44 (4H, m, DNS), 7.32 (2H, m), 7.26−7.11 (9H, m, DMTr and NH), 7.12 (2H, d, J = 7.6 Hz, DNS), 6.83−6.80 (4H, m, DMTr), 5.94 (2H, t, NH), 3.98 (2H, d, J^3 = 6.0 Hz, CH_2OH), 3.79 (6H, s, OCH_3), 3.51 (1H, t, J^3 = 6.0 Hz, OH), 3.39 (2H, s, CH_2ODMTr), 3.07 (4H, m, CH_2NHCO), 2.87 (12H, s, $N(CH_3)_2$), 2.71 (4H, m, CH_2-$NHSO_2$), 1.33 (4H, m, C-CH_2-C); TLC R_f 0.60 (19/1 CH_2Cl_2/MeOH), 0.25 (49/1 CH_2Cl_2/MeOH). Anal. ($C_{56}H_{64}N_6O_{10}S_2$) C, H, N, S.

3-[(4,4′-Dimethoxytrityl)oxy]-2,2-bis[(trifluoroacetamido)methyl]propanol (14a). (*a*) *Reduction with 1,3-Propanedithiol/$NaBH_4$.* Compound **12** (966 mg, 2.0 mmol), 1,3-propanedithiol (20 μL, 0.2 mmol), and Et_3N (0.6 mL, 4.0 mmol) were dissolved in *i*-PrOH (20 mL). $NaBH_4$ (380 mg, 10 mmol) was added under vigorous stirring in several portions, and the mixture was stirred overnight at room temperature. The solvent was evaporated, and the residue was dissolved in CH_2Cl_2 (150 mL), washed with 10% NaOH in saturated aqueous NaCl (25 mL) and saturated aqueous NaCl (5 × 25 mL), and dried over Na_2SO_4. The solvent was evaporated, and the resulting oil was separated on a silica gel column, eluting with a gradient of MeOH (2 to 20%) in CH_2Cl_2/Et_3N (95/5). The combined fractions were evaporated to dryness, and the resulting oil was redissolved in CH_2Cl_2/hexane (7/3, 25 mL). The turbid solution was filtered through a paper filter and evaporated to give 3-[(4,4′-dimethoxytrityl)oxy]-2,2-bis(aminomethyl)propanol (**13**) (688 mg, 80%) as a colorless foam which was used without further purification: ^{1}H NMR ($CDCl_3$) δ 7.42−7.20 (9H, m, aromatic), 6.85−6.80 (4H, m, aromatic), 3.79 (6H, s, CH_3O), 3.71 (2H, s, C***H***$_2$-OH), 3.04 (2H, s, C-O-CH_2), 2.91 (2H, d, J^2 = 10.0 Hz, 2 × C***H***^{A}H^BNH_2), 2.88 (2H, d, J^2 = 10.0 Hz, 2 × CH^A***H***^{B}NH_2), 2.47 (4H, br s, NH_2), 1.27 (1H, s, HO); TLC R_f 0.6 (85/10/5 CH_2Cl_2/MeOH/Et_3N).

(b) Reduction with Ph_3P. Ph_3P (2070 mg, 7.9 mmol) was added to a solution of **12** (1820 mg, 3.8 mmol) in pyridine (15 mL) for 30 min. The mixture was stirred for 3 h, treated with concentrated aqueous ammonia (3 mL), and left overnight. After evaporation *in vacuo* and coevaporation with toluene (3 × 20 mL), the resulting gum was separated on a silica gel column to give **13** (605 mg, 37%) which was identical to that synthesized by reduction with 1,3-propanedithiol/$NaBH_4$ (TLC and ^{1}H NMR).

A solution of **13** (646 mg, 1.5 mmol) and methyl trifluoroacetate (0.45 mL, 4.5 mmol) in dry pyridine (5 mL) was kept overnight at ambient temperature. Aqueous $NaHCO_3$ (5%, 3 mL) was added, and the mixture was evaporated *in vacuo* to one-third of the initial volume. Water (10 mL) was added to the residue, and the product was extracted with CH_2Cl_2 (2 × 50 mL), washed with saturated aqueous NaCl, and dried over Na_2SO_4. The solvent was evaporated, and the product was isolated by silica gel column chromatography, eluting with a gradient of MeOH in CH_2Cl_2 (0 to 4%) to obtain 820 mg (87%) of

14a as a white foam: ^{1}H NMR ($CDCl_3$) δ 7.43−7.20 (9H, m, (aromatic), 6.90 (2H, br m, NH), 6.88−6.84 (4H, m, aromatic), 3.80 (6H, s, CH_3O), 3.47 (2H, br s, C***H***$_2$-OH), 3.43 (2H, dd, $J^2 = 14.2$ Hz, $J^3 = 7.8$ Hz, 2 × C***H***^{A}H^{B}NH), 3.08 (2H, s, C-O-CH_2), 2.97 2H, dd, $J^2 = 14.2$ Hz, $J^3 = 5.9$ Hz, 2 × CH^A***H***^{B}NH), 1.58 (1H, br, s, HO); IR ν 3600−3300 (O-H, N-H), 1714 ($\nu_{C=O}$, amid I), 1556 (N-H, amid II), 1216 and 1177 cm^{-1} (ν_{C-F}); TLC R_f 0.7 (19:1 CH_2Cl_2/MeOH). Anal. ($C_{30}H_{30}F_6N_2O_6$) C, H, N.

3-[(4,4′-Dimethoxytrityl)oxy]-2,2-bis[[*N*-(9-fluorenylmethoxycarbonyl)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 10% MeOH in CH_2Cl_2), the pure **14b** (350 mg, 57%) was obtained as a colorless foam. ^{1}H NMR ($CDCl_3$) δ 7.8−7.7, 7.6−7.15 (25H, m, aromatic), 6.9−6.8 (4H, m, aromatic), 5.45 (1H, m, OH), 5.06 (2H, br m, NH), 4.46 (2H, d, $J^3 = 6.3$ Hz, C***H***$_2$OH), 4.38 (2H, dd, $J^3 = 7.3$ Hz, $J^2 = 10.5$ Hz, CHC***H***^{A}H^{B}O-C═O), 4.34 (2H, dd, $J^3 = 7.3$ Hz, $J^3 = 10.5$ Hz, CHCH^A***H***^{B}O-C═O), 4.19, (2H, t, $J^3 = 7.3$ Hz, C***H***CH$_2$O-C═O), 3.72 (6H, s, OCH_3), 3.24 (2H, s, CH_2ODMTr), 3.02 (4H, m, 2 × CH_2-NHCO); IR ν 3500−3300 (O-H, N-H), 1704 ($\nu_{C=O}$, amid I), 1509 cm^{-1} (N-H, amid II); TLC R_f 0.8 (19/1 CH_2Cl_2/MeOH). Anal. ($C_{56}H_{52}N_2O_8$) C, H, N.

3-[(4,4′-Dimethoxytrityl)oxy]-2,2-bis[[*N*-(4-methoxytrityl)amino]methyl]propanol (14c). Trimethylsilyl chloride (0.5 mL, 4.2 mmol) was added to a solution of crude **13** (600 mg, 1.4 mmol) in dry pyridine (10 mL). After 2 h, MMTr-Cl (1.73 g, 5.6 mmol) in dry pyridine (10 mL) was added to the mixture, the mixture was stirred overnight at room temperature, and the reaction was quenched with MeOH (10 mL). The resulting solution was evaporated to an oil, and the residue was treated with 5% aqueous $NaHCO_3$ (50 mL) and extracted with CH_2Cl_2 (2 × 50 mL). The organic phase was washed in succession with 5% aqueous $NaHCO_3$ (50 mL) and saturated aqueous NaCl (2 × 50 mL), dried over Na_2SO_4, and evaporated. The residue was separated on a silica gel column, eluting with a gradient from 1/1 hexane/CH_2Cl_2 to 95/5 CH_2Cl_2/MeOH to give **14c** (950 mg, 70%) as a yellow foam: ^{1}H NMR ($CDCl_3$) δ 7.35−7.10 (33H, m, aromatic + NH), 7.01 (3H), 6.83 (1H, m), 6.75 (3H, m), 6.66 (3H, m, aromatic), 3.77 (6H, s), 3.74 (6H, s, CH_3O), 3.68 (2H, s, C***H***$_2$-OH), 2.84 (2H, s, C-O-CH_2), 2.50 (2H, d, $J^2 = 15.9$ Hz, 2 × C***H***^{A}H^{B}NH), 2.41 (2H, d, $J^2 = 15.9$ Hz, 2 × CH^A***H***^{B}NH); TLC R_f 0.5 (99/1 CH_2Cl_2/MeOH), 0.1 (CH_2Cl_2). Anal. ($C_{66}H_{64}N_2O_6$) C, H, N.

3-[(4,4′-Dimethoxytrityl)oxy]-2,2-bis[[*N*-[6-(trifluoroacetamido)hexanoyl]amino]methyl]propanol (14d). Crude diamine **13** (1.6 g, 3.7 mmol) and *N*-oxysuccinimido 6-(trifluoroacetamido)hexanoate (*23*) (2.65 g, 8.2 mmol) in dry pyridine (50 mL) were stirred for 3 h at room temperature and treated with 5% aqueous $NaHCO_3$ (25 mL). The resulting mixture was concentrated to one-third of the initial volume, diluted with ethyl acetate (100 mL), washed with water (2 × 50 mL) and saturated aqueous NaCl (2 × 50 mL), and dried over Na_2SO_4. The solvent was evaporated, and the product was isolated by silica gel column chromatography, eluting with a gradient of MeOH (0 to 6%) in CH_2Cl_2/pyridine (99/1) to obtain **14d** (1.20 g, 38%) as a colorless oil: ^{1}H NMR ($CDCl_3$) δ 7.46−7.15 (9H, m, aromatic), 6.90−6.83 (4H, m, aromatic), 6.78 (4H, br m, NH), 5.95 (4H, br m, NH), 4.41 (1H, br, m, OH), 3.80 (6H, s, CH_3O), 3.42−3.30 (8H, m, C***H***$_2$-OH, 2 × C***H***$_2$NHCOCF$_3$, 2 × C***H***^{A}H^B-NHCO), 3.04 (2H, s, C-O-CH_2), 2.07 (4H, t, $J^3 = 6.8$ Hz, 2 × C***H***$_2$CONH), 2.50 (2H, dd, $J^2 = 14.2$ Hz, $J^3 = 7.3$ Hz, 2 × CH^A***H***^{B}NH), 1.61−1.53 (8H, m), 1.39−1.28 (4H, m, 6 × C-C***H***$_2$-C); IR ν 3600−3300 (O-H, N-H), 1710, 1690 ($\nu_{C=O}$, amid I), 1550 (N-H, amid II), 1218 and 1170 cm^{-1} (ν_{C-F}); TLC R_f 0.6 (9/1 CH_2Cl_2/MeOH). Anal. ($C_{42}H_{52}F_6N_4O_8$) C, H, N.

General Procedure for the Preparation of Phosphoramidites: *2-Cyanoethyl 3-[(4,4′-Dimethoxytrityl)-oxy]-2,2-bis[N-[(3-trifluoroacetamido)propyl]carboxamido]-propyl (N,N-Diisopropylamino)phosphoramidite* (**1a**). Dry 1*H*-tetrazole (0.46 M in MeCN, 1.30 mmol, 2.9 mL) was added to a solution of **10a** (960 mg, 1.25 mmol) predried by coevaporation with MeCN (3 × 20 mL) and 2-cyanoethyl *N,N,N′,N′*-tetraisopropylphosphorodiamidite (*24*) (490 mg, 1.6 mmol) in dry MeCN (5 mL). The reaction mixture was left at ambient temperature, and the reaction was found to be completed in 30 min (TLC, 19/1 CH_2Cl_2/MeOH). The reaction was stopped by addition of 5% aqueous $NaHCO_3$ (10 mL). The viscous product was extracted with CH_2Cl_2 (2 × 20 mL), washed with saturated aqueous NaCl, and dried over Na_2SO_4. The solvent was removed under reduced pressure, and the residue was dried on an oil pump and dissolved in dry toluene (3 mL). The solution was added dropwise to well-stirred hexane (100 mL) at room temperature. The precipitate was collected on a sintered glass filter and washed with hexane (2 × 20 mL). Final drying *in vacuo* gave **1a** (1163 mg, 95%) as a white powder: ^{31}P NMR ($CDCl_3$) δ 149.2; TLC R_f 0.70 (19/1 CH_2Cl_2/MeOH), 0.45 (49/1 CH_2Cl_2/MeOH). Phosphoramidites **1b**,**c** and **2a**−**d** were prepared and isolated in 90−97% yield by a similar procedure.

Phosphoramidite ***1b:*** ^{31}P NMR ($CDCl_3$) δ 147.2; TLC R_f 0.85 (19/1 CH_2Cl_2/MeOH), 0.55 (49/1 CH_2Cl_2/MeOH).

Phosphoramidite ***1c:*** ^{31}P NMR ($CDCl_3$) δ 147.0; TLC R_f 0.9 (19/1 CH_2Cl_2/MeOH), 0.6 (49/1 CH_2Cl_2/MeOH).

Phosphoramidite ***2a:*** ^{31}P NMR ($CDCl_3$) δ 149.5; TLC R_f 0.85 (19/1 CH_2Cl_2/MeOH), 0.5 (49/1 CH_2Cl_2/MeOH).

Phosphoramidite ***2b:*** ^{31}P NMR ($CDCl_3$) δ 147.4; TLC R_f 0.9 (19/1 CH_2Cl_2/MeOH), 0.6 (49/1 CH_2Cl_2/MeOH).

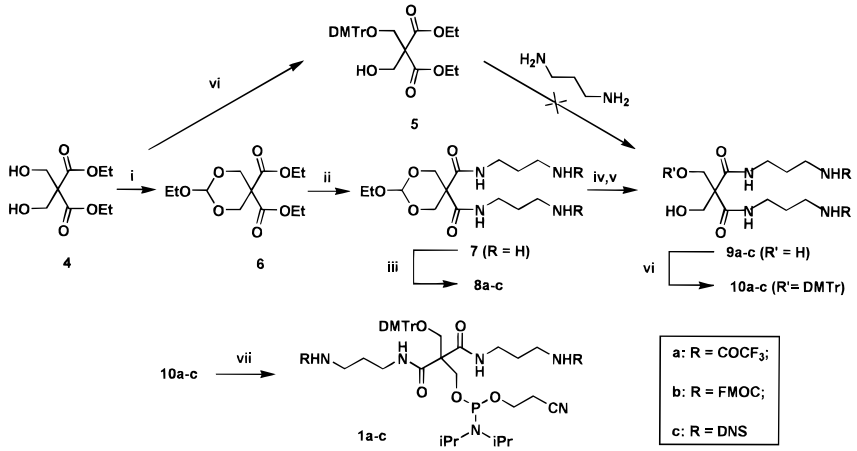
Phosphoramidite ***2c:*** ^{31}P NMR ($CDCl_3$) δ 146.9; TLC R_f 0.95 (19/1 CH_2Cl_2/MeOH), 0.65 (49/1 CH_2Cl_2/MeOH).

Phosphoramidite ***2d:*** ^{31}P NMR ($CDCl_3$) δ 148.6; TLC R_f 0.8 (19/1 CH_2Cl_2/MeOH), 0.55 (49/1 CH_2Cl_2/MeOH).

RESULTS AND DISCUSSION

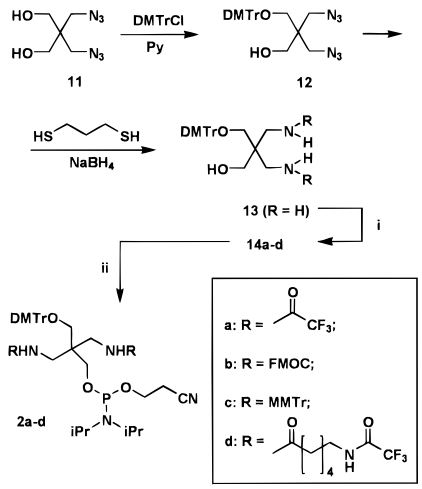
Synthesis of Phosphoramidites 1 and 2. Building blocks **1a**−**c** were synthesized from commercially available diethyl 2,2-bis(hydroxymethyl)malonate (**4**) (*21*), as depicted in Scheme 2. The synthetic use of **4** is hampered by its rapid degradation in basic media, in particular in the presence 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 aminoalkyl 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]



[a] (i) $(EtO)_3CH/H_2SO_4$; (ii) 1,3-diaminopropane/THF; (iii) for **8a**, methyl trifluoroacetate/Py; for **8b**, FMOC-Cl/$NaHCO_3$/aqueous dioxane; for **8c**, DNS-Cl/Py; (iv) 80% aqueous AcOH; (v) Et_3N/EtOH; (vi) DMTr-Cl/Py; (vii) 2-cyanoethyl *N*,*N*,*N*′,*N*′-tetraisopropylphosphorodiamidite/1*H*-tetrazole. DNS, 5-(dimethylamino)-1-naphthalenesulfonyl.

Scheme 3[a]



[a] (i) for **14a**, methyl trifluoroacetate/Py; for **14b**, FMOC-Cl/$NaHCO_3$/aqueous dioxane; for **14c**, MMTr-Cl/Py; for **14d**, *N*-oxysuccinimido 6-(trifluoroacetamido)hexanoate; (ii) 2-cyanoethyl *N*,*N*,*N*′,*N*′-tetraisopropylphosphorodiamidite/1*H*-tetrazole. DMTr, 4,4′-dimethoxytrityl; MMTr, 4-monomethoxytrityl; FMOC, 9-fluorenylmethoxycarbonyl.

tetraisopropylphosphorodiamidite (*24*) in the presence of 1*H*-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_2S (*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 FMOC-Cl 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.

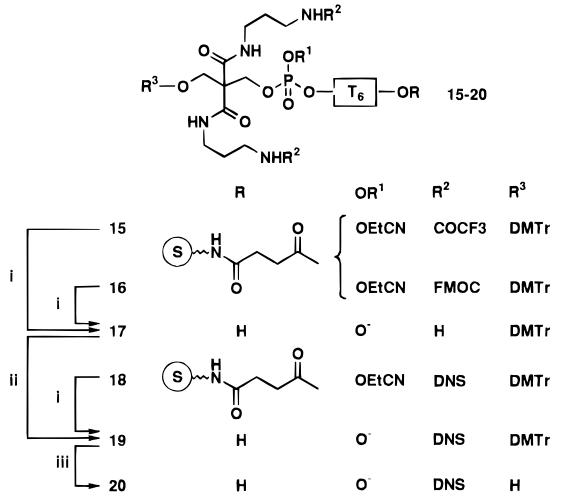
Synthesis of Oligonucleotide Conjugates. The compatibility of phosphoramidites **1** and **2** with oligonucleotide synthesis by phosphoramidite strategy was first examined by assembling model oligonucleotides 5′-XT_6, where X stands for the non-nucleosidic unit. For reference purposes, a small part 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 hexathymidylate 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 hexathymidylyl 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**.

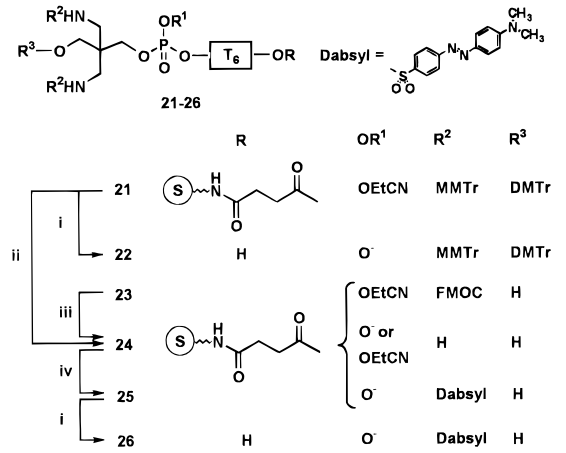
The same problem concerns the 3′-labeled oligonucleotides, which may be obtained by conventional attachment

Scheme 4[a]



[a] (i) concentrated aqueous NH_3; (ii) DNS-Cl; (iii) 80% aqueous AcOH.

Scheme 5[a]

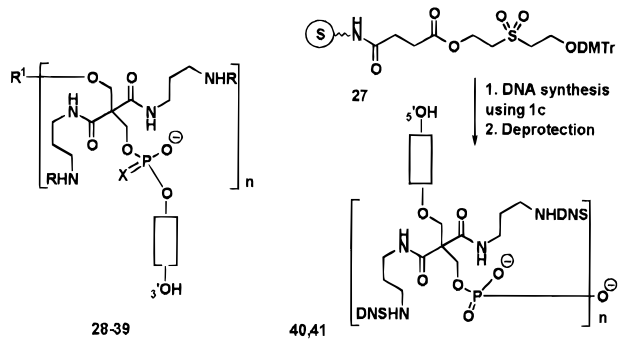


[a] (i) concentrated aqueous NH_3; (ii) 3% Cl_2HCCO_2H/CH_2Cl_2; (iii) 10% piperidine/DMF; (iv) dabsyl chloride.

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 phosphate group. 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 ammonolyzed in the DMTr-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

Scheme 6. Solid Support 27 and Structures of Polylabeled Oligonucleotides 28−41[a]



Compd.	28	29	30	31	32	33	34	35	36	37	38	39	40	41
R	DNS	DNS	DNS	H	H	DNS	DNS	DNS	DNS	DNS	DNS	DNS	–	–
R^1	DMT	DMT	DMT	DMT	DMT	H	H	H	DMT	DMT	H	H	–	–
X	O	O	O	O	O	O	O	O	S	S	S	S	–	–
n	1	2	3	1	2	1	2	3	2	3	2	3	2	3

[a] The DNA fragments are marked as rectangles.

upon ammonolysis. This is somewhat unexpected, since the 5′-terminal tails consisting of several mono(aminoalkyl) 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 DMTr-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

Table 1. RP HPLC Retention Times (RT)[a] of the Multilabeled Oligonucleotides and Melting Temperatures (T_m) of Their Complexes with the 27-Nucleotide Complement

oligonucleotide	**28**	**29**	**30**	**31**	**32**	**33**	**34**	**35**	**38**	**39**	**40**	**41**
RT, min	23.0	31.0	37.6	21.7	21.8	13.0	24.6	31.0	24.7	30.2	24.56	31.01
T_m, °C	–	–	–	–	–	58.0	57.5	59.5	–	50.3	–	55.1

[a] For conditions, see Experimental Procedures.

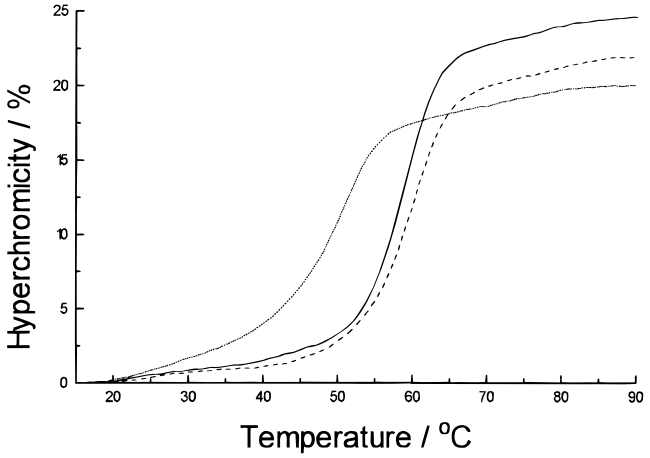


Figure 1. Melting curves for the complexes of unmodified (solid line) 5′-hexalabeled **35** (dashed line), and 5′-hexalabeled phosphorothioate **39** (dotted line) oligonucleotides with complementary oligonucleotide sequence. For structures, consult Scheme 6 and the text.

DNS-Cl 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 polylabeled 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 ACG 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 heavily labeled non-nucleosidic tails intercalate 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 T_m values for the multilabeled oligonucleotides **33**−**35** remain practically unchanged compared to that of the underivatized oligonucleotide (T_m = 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 (T_m = 50.3 °C). Expectedly, the low melting point results from the modified phosphate backbone (*30*) rather than from the non-nucleosidic moiety.

CONCLUSIONS

Among the phosphoramidites **1** and **2** synthesized, the derivatives of *N*,*N*′-bis(3-aminopropyl)-2,2-bis(hydroxymethyl)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.

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