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REAGENTS FOR MULTIPLE NON-RADIOACTIVE LABELLING OF OLIGONUCLEOTIDES

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Abstract: Novel phosphoramidite reagents for nonradioactive polylabelling of oligonucleotides have been developed, including two branching reagents, **4** and **8**, and a biotin-containing phosphoramidite **19** which can be used to incorporate a biotin moiety into any position of the oligonucleotide.

During the past decade considerable advance has been made in the synthesis of modified oligonucleotides for nonradioactive labelling and DNA-DNA or DNA-protein interaction studies[1]. Nonradioactive labelling becomes more and more common, especially in clinics, as it does not require protection from radiation, and labelled species possess practically unlimited shelf-life time. Automated methods using modifying phosphoramidite reagents show a considerable promise for synthesis of

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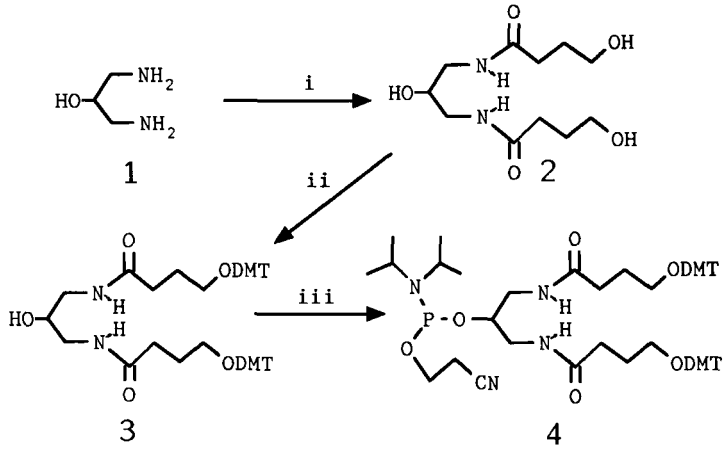
nonradioisotopically labelled oligonucleotides. Thus, there is a number of phosphoramidites that allow 5′-monobiotinylation of synthetic oligomers[2-4] as well as incorporation of one or several biotin residues, attached to a nucleic base moiety[5,6] or to a non-nucleotide backbone[7,8], into any position of oligonucleotides. Some of these phosphoramidites are commercially available (Clontech, Amersham, Pharmacia).

As a way to enhance the detection sensitivity, introduction of multiple labels in the oligonucleotide chain is tempting. Such an approach gave good results for biotin[7,9], phosphotyrosine[7], and Eu(III)-chelates[10] terminally attached to oligo-nucleotides but proved non-efficacious in the case of fluorescein[11], probably because of the fluorophore's self-quenching. In the case of biotin, due to a large size of the avidin molecule, an increase in the distance between the biotin residues allowed reaching proportionality between the number of the introduced labels and the signal level.

A plausible way to obtain oligos with spacially unhindered labels is to arrange them non-linearly. Reagents for the synthesis of oligos with a branched chain of labels at the 5′-terminus were proposed earlier[12,13], and later a simple reagent employing a 1,2,6-hexanetriol backbone was applied to the synthesis of biotinylated oligoribonucleotides[14]. A recent publication describing the peparation of synthons based on butane-1,3-diol and 1-aminobutane-2,4-diol which allow introduction of ramifications into 5' and/or 3' termini of oligonucleotides should also be noted[15]. However, the syntheses of the non-nucleotide[12] and nucleotide branching reagents[13] are too cumbersome, whereas the data on the high coupling yield of the 1,2,6-hexanetriol-derived reagent[14] are consistent neither with the earlier results[12] nor with our observations.

Here we describe a convenient synthesis of two reagents with linker arms of various lengths for branching oligos at the 5′-terminus and consequently labelling them during the automated synthesis. With such reagents at hand, we designed a novel easily available biotin-containing phosphoramidite for the synthesis of branched polybiotinylated oligonucleotide conjugates.

As a backbone unit for the branching site, commercially available 1,3-diaminopropanol-2 (**1**) was selected. Acylation of its amino groups with

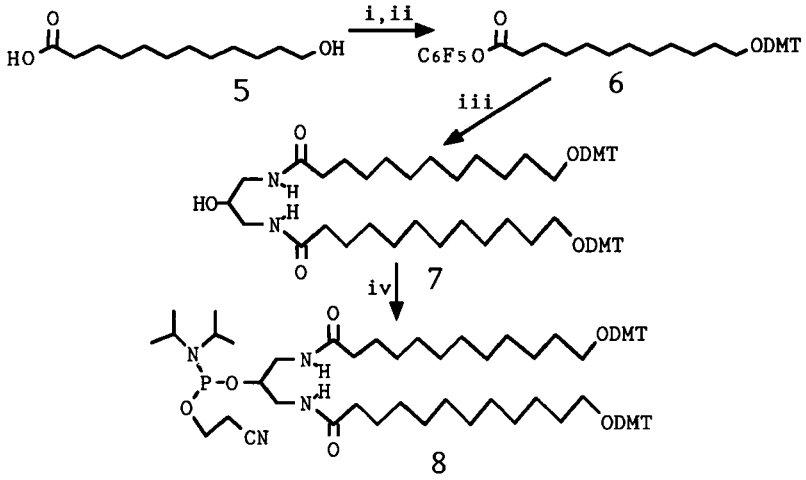


i: γ-butyrolactone, DMAP in MeOH, reflux 6 h; ii: DMTCl in pyridine, 0°C; iii: bis(diisopropylamino)-2-cyano-ethoxyphosphine, diisopropylammonium tetrazolide in MeCN.

Scheme 1

ω-hydroxy carboxylic acids would yield derivatives with two primary hydroxyls to be used for the further chain extension, whereas its secondary hydroxyl group is suitable for introducing a phosphoramidite function. The corresponding synthetic pathway is depicted in Scheme 1. Diaminopropanol **1** was first acylated with γ-butyrolactone in a boiling methanol solution in the presence of catalytic amounts of 4-(N,N-dimethylamino)pyridine (DMAP). Under these conditions, only nitrogen atoms were acetylated to give diamidotriol **2**. Then its primary hydroxyl groups were dimethoxytritylated, and the resulting compound **3** was converted, by phosphitylation with bis(diisopropylamino)-2-cyanoethoxyphosphine and diisopropylammonium tetrazolide as a catalyst in acetonitrile, to the branching phosphoramidite **4** with a 64% overall yield.

A somewhat different scheme was used to synthesize phosphoramidite **8** with longer linker arms (Scheme 2). 12-Hydroxy-dodecanoic acid **5** was O-protected with a dimethoxytrityl residue, and

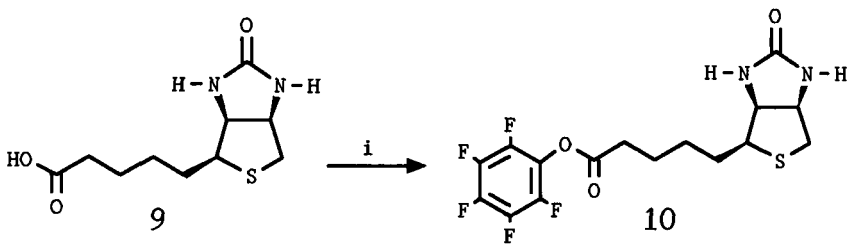


i: DMTCl in pyridine, 0°C; ii: pentafluorophenol, DCC in CH_2Cl_2 and pyridine, 0°C; iii: 1 in pyridine and THF; iv: bis(diisopropylamino)-2-cyanoethoxyphosphine, diisopropylammonium tetrazolide in CH_2Cl_2 and acetonitrile.

Scheme 2

the product, treated *in situ* with pentafluorophenol and dicyclohexylcarbodiimide (DCC), gave activated ester **6**. This ester acylated amino alcohol **1** with the quantitative formation of bis-DMT-amidotriol **7** (a structural analogue of **3**), which was phosphitylated like **3** but, due to a higher lipophilicity, in a CH_2Cl_2-MeCN (1:1 v/v) solution instead of pure acetonitrile; the overall yield with regard to hydroxy acid **5** was 53%.

Both phosphoramidites, **4** and **8**, are fit to be used as branching reagents to modify oligo- and polynucleotides, thus preparing them for polylabelling. After **n** coupling cycles with any of these reagents, up to 2^n free hydroxyl groups can be introduced at the nucleotide's 5' end to serve as acceptors for reporter groups, e.g. for biotin. To this goal, we have synthesized a novel biotin-containing phosphoramidite **19** from a non-chiral pseudosugar unit comprising diethanolamine and ε-aminohexanoic acid moiety as a linker. At first, biotin **(9)** was converted into its activated



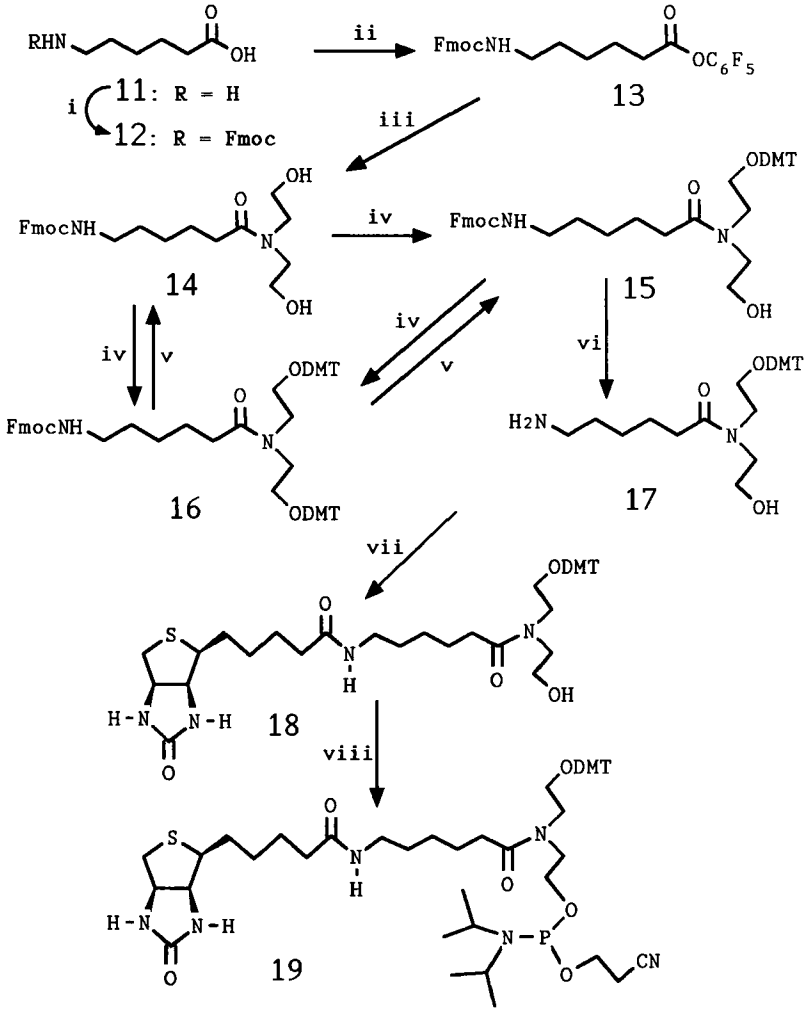
9 10

i: pentafluorophenol, DCC in pyridine, 5°C.

Scheme 3

ester **10** (Scheme 3) by treatment in a pyridine oversaturated solution with pentafluorophenol/DCC (cf.[16,17]). On the other hand, pentafluorophenyl N-Fmoc-ε-aminohexanoate (**13**)[18] was condensed with diethanolamine, and the reaction mixture, after treatment with 4,4'-dimethoxytrityl chloride (DMTCl), gave products varying in the tritylation degree and separated by chromatography on silica gel. Mono-DMT-derivative **15** was transformed into phosphoramidite **19** by the removal of the Fmoc-protection with pyrrolidine followed by biotinylation with **10** and phosphitylation as described above. In this synthesis, the overall yield of phosphoramidite **19** with regard to biotin was 64% (Scheme 4).

This reagent can be applied to obtaining oligonucleotides biotinylated not only at the 5' terminus but also at any other position of oligonucleotide chain. Though its five-atom backbone is not isosteric to the three-carbon 5′-3′ chain of natural nucleosides, incorporation of this moiety must not hinder hybridization, because the flexible backbone with an attached biotin residue can easily bulge out. It is noteworthy that biotin-containing reagents produced by Clontech[8] possess a prochiral carbon atom (carrying the biotin residue) that becomes chiral in the course of the oligonucleotide synthesis, thus leading to a number of diastereomers depending upon the number of the non-nucleotide units included. On the contrary, reagent **19**, in which the biotin residue is attached to a nitrogen atom, does not engender chirality after conjugation, thus providing stereohomogeneity of the conjugate.



i: FmocCl, Na_2CO_3 in dioxane and H_2O, 0°C; ii: pentafluorophenol, DCC in THF, 0°C, iii: diethanolamine in pyridine, 0°C, iv: DMTCl in pyridine, 0°C; v: CF_3CO_2H in $CHCl_3$; vi: pyrrolidine; vii: 10 in pyridine; viii: bis(diisopropylamino)-2-cyanoethoxyphosphine, diisopropylammonium tetrazolide in acetonitrile.

Scheme 4

The modifying amidites obtained, **4**, **8**, and **19**, are well soluble in acetonitrile and therefore can be used in the automated synthesis of labelled conjugates in a 0.1 M acetonitrile solution like standard nucleoside amidites. Amidites **4** and **8**, derived from secondary alcohols, are more stable than compound **19** and can be used even after a week's storage in an acetonitrile solution; in this respect they are only slightly inferior to the standard nucleoside phosphoramidites. As to amidite **19** from a primary alcohol, it should be used within 2-3 days after being dissolved, the condensation yields later on decreasing dramatically. At the same time, reagents **4**, **8** and **19** in solid state proved to be stable at -20°C under argon during at least 1.5 years. It should be noted that in sequential condensations of non-nucleotide reagents **4** or **8** to increase the branching degree, yields of the products of branching were lower with **4** than in the case of **8** (to be published). It is apparently due to the steric hindrances that are created by two bulky DMT-substituents of the introduced moiety after the condensation at one of the OH-groups, thus impeding the attack of another molecule of the reagent at the second OH-group. The higher yields with the use of reagent **8**, containing longer spacers than reagent **4**, are consistent with data on the reactivity of some other phosphitylating reagents with linkers of a varying length[12].

EXPERIMENTAL

DCC, DMAP and solvents were obtained from Merck (FRG). THF was distilled from $LiAlH_4$, pyridine and acetonitrile were distilled from CaH_2 and stored over 3A molecular sieves. Diethanolamine, 1,3-diamino-propanol-2, pentafluorophenol, (+)-biotin, diisopropylethylamine and triethylamine (distilled over Na before use), and 9-fluorenylmethoxy-carbonyl chloride (FmocCl) were purchased from Fluka (Switzerland); 12-hydroxydodecanoic acid, 1*H*-tetrazole (sublimed at 110°C/0.02 mm before use), and 4,4′-dimethoxytrityl chloride were obtained from Aldrich (USA); pyrrolidine was from Ferak (FRG). Diisopropylammonium tetrazolide and bis(diisopropylamino)-2-cyanoethoxyphosphine were

synthesized according to the published procedures[19]. Column chromatography was carried out using Kieselgel 60 (40-63 μm, Merck). TLC was run on Kieselgel 60 F_{254} plates (Merck) with aluminium backing; the spots were visualized, if not specified otherwise, under shortwave (254 nm) UV light or (in the case of dimethoxytrityl-containing compounds) by exposing the plate to the trifluoroacetic acid vapour. The reactions and column chromatography were monitored by means of TLC.

^1H NMR spectra were obtained on a Bruker AC-500 spectrometer, ^{31}P NMR spectra were obtained on a Bruker AC-500 (in $CHCl_3$) and Varian XL-400 (in CD_3CN) spectrometers with 85% H_3PO_4 as an external standard. IR spectra (thin films or KBr pellets) were measured on a UR-20 spectrophotometer. Mass spectral data were obtained on mass spectrometers Varian MAT-44S (electron impact, EI), Kratos MS-50TC (fast atom bombardment, FAB), and ^{252}Cf plasma desorption time-of-flight (plasma desorption, PD) (Electron, Sumy, Ukraine). Elemental analyses were obtained on a Hewlett-Packard 185B C-H-N-Analyzer. Melting points were determined on a Boetius instrument and are uncorrected. Solid-phase oligonucleotide synthesis was carried out on an Applied Biosystems DNA Synthesizer 380B.

N,N'-Bis(4-hydroxybutyryl)-1,3-diaminopropanol-2 (2). A solution of 1,3-diaminopropanol-2 (0.90 g, 10 mmol), γ-butyrolactone (3.10 mL, 40 mmol) and DMAP (20 mg, 0.16 mmol) in methanol (15 mL) was kept under reflux for 5-6 h (visualization of spots in TLC with 2% CrO_3 in 10% aq. H_2SO_4). The solvent was removed on rotary evaporator, CH_2Cl_2 (20 mL) was added to the residue and the solution was allowed to crystallize (5°C, 20 h). After filtration and drying in vacuum, the pure diamidotriol **2** (2.54 g; 97%) was obtained as a white crystalline powder, mp 98°C, R_f 0.33 (acetone - 12.7% aq.NH_3 1:4 v/v). Found, %: C 50.31, H 8.28, N 10.50; calculated for $C_{11}H_{22}N_2O_5$, %: C 50.37, H 8.45, N 10.68. EI-MS $(m/z)^+$: 263 (M+H). IR, cm^{-1}: 1567 (ν_{N-H}), 1634, 1645 ($\nu_{C=O}$), 3305 ($\nu_{OH\cdots O}$). ^1H NMR (DMSO-d_6; δ, ppm): 7.73 (br. s, 2H, N*H*); 4.90 (d, 1H, *J*=5 Hz, *H*OCH); 4.44 (t, 2H, *J*=5 Hz, CH_2OH); 3.50 (m, 1H, OC*H*); 3.4-3.3 (m, 4H, CH_2O); 3.1-2.9 (m, 4H, CH_2N); 2.12 (t, 4H, *J*=7 Hz, $COCH_2$); 1.62 (quintet, 4H, *J*=7 Hz, CH_2CH_2O).

N,N′-Bis[4-(4,4′-dimethoxytrityloxy)butyryl]-1,3-diaminopropanol-2 (3). Diamidotriol **2** (1.31 g, 5.0 mmol) was coevaporated with pyridine (3x10 mL), dissolved in dry pyridine (15 mL), and, after cooling to 0°C, DMTCl (4.05 g, 13.0 mmol) in CH_2Cl_2 (15 mL) was added dropwise during 30 min with stirring. The reaction mixture was kept for 1-1.5 h at 20°C to complete, quenched with methanol (0.4 mL), diluted with $CHCl_3$ (300 mL), washed with 5% $NaHCO_3$ (250 mL) and brine (100 mL), dried over Na_2SO_4, and concentrated in vacuum. The residue was coevaporated with toluene (30 mL), and the crude product was purified by silica gel column chromatography in a gradient of methanol (0 to 5%) in $CHCl_3$ containing 0.5% Et_3N. The appropriate fractions were combined, evaporated, and dried in vacuum to give **3** (3.56 g; 82%) as a white foam, R_f 0.34 ($CHCl_3$-MeOH 9:1 v/v). FAB-MS $(m/z)^+$: 867 (M). IR, cm^{-1}: 1652 ($\nu_{C=O}$), 3425 ($\nu_{OH\cdots O}$). ^1H NMR (DMSO-d_6; δ, ppm): 7.77 (br. s, 2H, N*H*); 7.4-6.8 (m, 26H, Ar*H*); 4.94 (d, 1H, *J*=5 Hz, O*H*); 3.71 (s, 12H, OCH_3); 3.53 (m, 1H, OC*H*); 3.00 (m, 4H, CH_2N); 2.93 (t, 4H, *J*=6 Hz, CH_2O); 2.12 (t, 4H, *J*=7 Hz, COCH_2); 1.76 (m, 4H, *J*=7 Hz, CH_2CH_2O).

N,N′-Bis[4-(4,4′-dimethoxytrityloxy)butyryl]-O-(diisopropylamino-2-cyanoethoxyphosphinyl)-1,3-diaminopropanol-2 (4). Bis-DMT-derivative **3** (2.17 g, 2.5 mmol) was coevaporated with acetonitrile (2x20 mL), dissolved in dry acetonitrile (60 mL), and diisopropylammonium tetrazolide (0.17 g, 1.0 mmol) was added with stirring under argon followed by bis(diisopropylamino)-2-cyanoethoxyphosphine (0.90 mL, 2.8 mmol). The mixture was half evaporated (bath temperature below 30°C), allowed to react for 2-2.5 h with stirring, and evaporated. The residue was flash chromatographed on silica gel in $CHCl_3$-hexane 1:1 v/v (containing 0.5% Et_3N) to give 2.32 g of the TLC-pure product. It was dissolved in toluene (10 mL) and precipitated into pentane (500 mL), the solid was collected and dried in vacuum (0.04 mm, 24 h) to yield **4** (2.13 g, 80%) as a white powder, R_f 0.32 ($CHCl_3$-hexane-Et_3N 5:5:1 v/v/v). PD-MS $(m/z)^+$: 1090.0 (M+Na), 1105.7 (M+K), calculated for ($C_{62}H_{75}N_4O_{10}P$+Na) 1090.3, ($C_{62}H_{75}N_4O_{10}P$+K) 1106.4. ^{31}P NMR (δ, ppm): 148.70 ($CHCl_3$), 151.432 (CD_3CN). ^1H NMR ($CDCl_3$; δ, ppm):

7.5-6.7 (m, 26H, Ar*H*); 6.39 (br. s, 2H, N*H*); 3.9-3.6 (m), 3.79 (s) (19H, CH_2OP, C*H*OP, CH_2N (2H), C*H*N, OCH_3); 3.09 (t, 4H, *J*=6 Hz, CH_2ODMT); 3.00 (m, 1H, CH_2N); 2.86 (m, 1H, CH_2N); 2.62 (m, 2H, NCCH_2); 2.33 (m, 4H, COCH_2); 1.95 (m, 4H, COCH$_2$CH_2); 1.20 (d, 12H, *J*=6 Hz, CHCH_3).

Pentafluorophenyl 12-(4,4′-dimethoxytrityloxy)dodecanoate (6). 12-Hydroxydodecanoic acid **(5)** (2.16 g, 10.0 mmol) was dried by coevaporation with pyridine (3x15 mL), dissolved in dry pyridine (20 mL) and, upon cooling on ice (0°C) and stirring, DMTCl (3.38 g, 10.0 mmol) was added within 20 min. After 1 h, the ice bath was removed and the stirring continued for 24 h. A solution of pentafluorophenol (2.03 g, 11.0 mmol) in CH_2Cl_2 (10 mL) and pyridine (3 mL) was added, the reaction mixture was cooled (ice bath), and a solution of DCC (2.27 g, 11.0 mmol) in CH_2Cl_2 (15 mL) was added dropwise within 2 h. After 3 h the mixture was allowed to warm to ambient temperature and the stirring was continued overnight. The N,N′-dicyclohexylurea (DCU) precipitated was filtered off and washed with CH_2Cl_2 (150 mL), and the combined solutions were evaporated to dryness. After flash chromatography on silica gel (elution with 10% CH_2Cl_2 in hexane) and drying in vacuum, ester **6** (6.38 g, 93%) was obtained as a colorless oil, R_f 0.61 ($CHCl_3$). FAB-MS $(m/z)^+$: 684 (M). IR, cm^{-1}: 1790 ($\nu_{C=O}$). ^1H NMR ($CDCl_3$; δ, ppm): 7.5-6.8 (m, 13H, Ar*H*); 3.85 (s, 6H, OCH_3); 3.05 (t, 2H, *J*=6.5 Hz, CH_2O); 2.67 (t, 2H, *J*=7 Hz, COCH_2); 1.78 (m, 2H COCH$_2$CH_2); 1.62 (m, 2H, CH_2CH$_2$O); 1.5-1.2 (m, 14H, COCH$_2$CH$_2$(CH_2)$_7$).

N,N′-Bis[12-(4,4′-dimethoxytrityloxy)dodecanoyl]-1,3-diaminopropanol-2 (7). To a stirred solution of ester **6** (5.70 g, 8.3 mmol) in dry THF (30 mL) and pyridine (3 mL) was added amine **1** (364 mg, 4.0 mmol) in one portion, and the stirring was continued for 6 h. THF was removed in vacuum, the residue was dissolved in $CHCl_3$ (100 mL), washed with 5% $NaHCO_2$ (50 mL) and water (50 mL), dried (Na_2SO_4), concentrated, flash chromatographed on silica gel ($CHCl_3$, then 3% MeOH in $CHCl_3$) and dried in vacuum to yield derivative **7** (4.36 g, 100%) as a colorless oil, R_f 0.31 ($CHCl_3$-MeOH 20:1 v/v). FAB-MS (m/z)+: 1091 (M). IR, cm^{-1}:

1653 ($\nu_{C=O}$), 3310 ($\nu_{OH\cdots O}$). ^{1}H NMR (DMSO-d_6; δ, ppm): 7.71 (br. s, 2H, N*H*); 7.4-6.8 (m, 26H, Ar*H*); 4.92 (d, 1H, *J*=4.5 Hz, O*H*); 3.71 (s, 12H, OCH_3); 3.54 (m, 1H, OC*H*); 3.03 (m, 4H, CH_2N); 2.92 (t, 4H, *J*=6 Hz, CH_2O); 2.05 (t, 4H, *J*=7Hz, COCH_2); 1.6-1.4 (m, 8H, $COCH_2CH_2$, CH_2CH_2O); 1.3-1.1 (m, 28H, $COCH_2CH_2(CH_2)_7$).

N,N′-Bis[4-(4,4′-dimethoxytrityloxy)dodecanoyl]-O-(diisopropylamino-2-cyanoethoxyphosphinyl)-1,3-diaminopropanol-2 (8). Compound **7** (4.36 g, 4.0 mmol) was coevaporated with acetonitrile (2x20 mL), dissolved in dry acetonitrile (25 mL) and CH_2Cl_2 (25 mL), then diisopropylammonium tetrazolide (0.17 g, 1.0 mmol) and bis(diisopropylamino)-2-cyanoethoxyphosphine (1.35 mL, 4.25 mmol) were added under argon, and stirring was continued for 2.5-3 h. The mixture was concentrated in vacuum and chromatographed on silica gel ($CHCl_3$-hexane 1:1 v/v with 0.5% Et_3N) to give 3.52 g of the TLC-pure **8**. It was dissolved in toluene (15 mL) and precipitated into pentane (700 mL), then dissolved in dry acetonitrile (60 mL), evaporated and dried in vacuum to give amidite **8** (2.96 g, 57%) as a colorless oil. Reprecipitation of the product considerably (by 11%) decreases the yield, apparently because amidite **8** is lipophilic enough to be soluble in pentane. R_f 0.34 ($CHCl_3$-hexane-Et_3N 5:5:1 v/v/v). PD-MS $(m/z)^+$: 1314.7 (M+Na), calculated for ($C_{78}H_{107}N_4O_{10}P$+Na) 1314.7. ^{31}P NMR (δ, ppm): 148.70 ($CHCl_3$), 151.446 (CD_3CN). ^{1}H NMR ($CDCl_3$, δ, ppm): 7.4-6.7 (m, 26H, Ar*H*); 6.43 (br. s, 1H, N*H*); 6.33 (br. s, 1H, N*H*); 3.9-3.5 (m), 3.72 (s) (19H, CH_2OP, C*H*OP, CH_2N (2H), C*H*N, OCH_3); 3.03 (m, 1H, CH_2N); 2.99 (t, 4H, *J*=7 Hz, CH_2ODMT); 2.87 (m, 1H, CH_2N); 2.59 (m, 2H, NCCH_2); 2.17 (m, 4H, COCH_2); 1.57 (m, 8H, $COCH_2CH_2$, CH_2CH_2ODMT); 1.4-1.1 (m, 40H, $COCH_2CH_2$-$(CH_2)_7$, CHCH_3).

(+)-Biotin pentafluorophenyl ester (10). (+)-Biotin **(9)** (1.71 g, 7.0 mmol) was coevaporated with pyridine (35 mL), dissolved in dry pyridine (35 mL) at 90°C, cooled to room temperature, and then pentafluorophenol (1.31 g, 7.1 mmol) was added. The reaction mixture was cooled (0-5°C), and, with stirring, DCC (1.47 g, 7.1 mmol) in dry pyridine (10 mL) was added dropwise within 30 min. After 2 h the ice

bath was removed and stirring was continued for 16 h. The DCU formed was filtered off and washed with pyridine (10 mL), the combined solutions were half evaporated and stored at 20°C for 5 h. A small amount of DCU precipitated was filtered off, the solvent was removed, and the resulting syrup was triturated with a $CHCl_3$-hexane mixture (1:1 v/v; 50 mL). After filtration and drying, compound **10** (2.80 g, 98%) was obtained as a white crystalline powder, mp 188-189°C (pyridine-$CHCl_3$) (mp 186-189°C[16]), R_f 0.41 ($CHCl_3$-MeOH 5:1 v/v). EI-MS $(m/z)^+$: 410 (M).

Pentafluorophenyl N-(9-fluorenylmethoxycarbonyl)-6-aminohexanoate (13). The modified method[18] was used. 6-Aminohexanoic acid **(11)** (3.85 g, 29.3 mmol) and sodium carbonate (12.3 g, 116 mmol) were dissolved in water (80 mL) and dioxane (15 mL), cooled (0°C), and, with stirring, FmocCl (5.20 g, 20.1 mmol) in dioxane (35 mL) was added dropwise within 20 min. The reaction mixture was stirred for 12 h, diluted with water (300 mL), extracted with ether (5x125 mL), acidified with conc. HCl to pH 2, and kept for 3 h at 5°C. The precipitate was filtered off, dried in vacuum over phosphorus pentoxide, and recrystallized from nitromethane (45 mL) to give N-Fmoc-6-aminohexanoic acid **(12)** (5.90 g, 83%) as a white crystalline powder, mp 109-110°C.

A solution of DCC (3.57, 17.3 mmol) in THF (25 mL) was added dropwise to a cooled (0°C) solution of acid **12** (5.83 g, 16.5 mmol) and pentafluorophenol (3.19 g, 17.3mmol) in THF (100 mL) with stirring within 1 h. After 2 h the ice bath was removed, the reaction mixture was allowed to stir for 20 h, and the DCU formed was filtered off and washed with THF (20 mL). The combined filtrates were evaporated to dryness, and the residue was crystallized from $CHCl_3$-hexane 2:1 v/v to yield **13** (8.17 g, 95%) as a white crystalline powder, mp 119-120°C (mp 128-129°C[18]), R_f 0.42 ($CHCl_3$). EI-MS $(m/z)^+$: 519 (M), 335 ($FmocNH(CH_2)_5CO$). IR, cm^{-1}: 1775 ($\nu_{C=O}$, ester), 1680 ($\nu_{C=O}$, Fmoc). ^1H NMR ($CDCl_3$, δ, ppm): 7.8-7.3 (m, 8H, Ar*H*), 4.79 (br. s, 1H, N*H*), 4.43 (d, 2H, *J*=7 Hz, OCH_2), 4.24 (t, 1H, *J*=7Hz, OCH_2CH), 3.24 (m, 2H, NCH_2), 2.68 (t, 2H, *J*=7 Hz, CH_2CO), 1.82 (m, 2H, $COCH_2CH_2$), 1.60 (m, 2H, NCH_2CH_2), 1.48 (m, 2H, $NCH_2CH_2CH_2$).

O^1-(4,4′-Dimethoxytrityl)-3-(N-Fmoc-6-aminohexanoyl)-3-aza-1,5-pentanediol (15). Diethanolamine (0.48 mL, 5.0 mmol) in pyridine (15 mL) was added dropwise to a cooled (0°C) and stirred solution of ester **13** (2.60 g, 5.0 mmol) in dry pyridine (40 mL) within 1 h, and the mixture was left overnight at 20°C; TLC showed the quantitative conversion of **13** to **14**. The solution was half evaporated and cooled (0°C), and DMTCl (1.70 g, 5.0 mmol) was added in one portion. The mixture was stirred for 1 h, then allowed to warm to room temperature (2 h), evaporated to 15 mL, washed twice with 150 mL of brine + 20 mL of 5% $NaHCO_3$, dried over Na_2SO_4 and evaporated. The residue was chromatographed on silica gel in a gradient of hexane (50 to 0%) in chloroform followed by a gradient of methanol (0 to 15%) in chloroform. The appropriate fractions were combined, evaporated and dried in vacuum to give compound **15** and minor products **14** and **16**. Mono-DMT-derivative **15** was obtained as a white foam (1.75 g, 47%), R_f 0.38 ($CHCl_3$-MeOH 20:1 v/v). PD-MS $(m/z)^+$: 743.0 (M), 765.4 (M+Na), 782.3 (M+K); calculated for $C_{46}H_{50}N_2O_7$ 742.9 ($C_{46}H_{50}N_2O_7$+Na) 765.9, ($C_{46}H_{50}N_2O_7$+K) 782.0. ^1H NMR ($CDCl_3$; δ, ppm): 7.8-6.7 (m, 21H, Ar*H*); 4.96 (br. s, 1H, N*H*); 4.37 (d, 2H, *J*=7 Hz, CH_2OCO); 4.18 (t, 1H, *J*=7 Hz, $CHCH_2OCO$); 3.74 (s, 6H, OCH_3); 3.70-3.43 (m, 8H, CH_2CH_2OH, CH_2CH_2ODMT); 3.15 (m, 2H, CH_2NH); 2.37 (t, 2H, *J*=7 Hz, CH_2CO); 1.60 (m, 2H, CH_2CH_2CO); 1.47 (m, 2H, CH_2CH_2NH); 1.29 (m, 2H, $CH_2CH_2CH_2CO$).

O^1,O^5-Bis(4,4′-dimethoxytrityl)-3-[N-(9-fluorenylmethoxycarbonyl)-6-aminohexanoyl]-3-aza-1,5-pentanediol (16) eluted before **15** was obtained as a yellowish foam (0.67 g, 13%), R_f 0.35 ($CHCl_3$-MeOH 100:1 v/v). PD-MS $(m/z)^+$: 1044.8 (M), 1068.7 (M+Na); calculated for $C_{67}H_{68}N_2O_9$ 1045.3, ($C_{67}H_{68}N_2O_9$+Na) 1068.3. ^1H NMR ($CDCl_3$; δ, ppm): 7.9-6.9 (m, 34H, Ar*H*); 5.20 (br. s, 1H, N*H*); 4.58 (d, 2H, *J*=7 Hz, CH_2OCO); 4.39 (t, 1H, *J*=7 Hz, $CHCH_2OCO$); 3.95 (m, 12H, OCH_3); 3.82 (m, 2H), 3.70 (m, 2H) ($DMTOCH_2CH_2$); 3.43 (m, 4H, $DMTOCH_2$); 3.36 (m, 2H, CH_2 NH); 2.55 (t, 2H, *J*=7 Hz, CH_2CO); 1.80 (m, 2H, CH_2CH_2CO); 1.68 (m, 2H, CH_2CH_2NH); 1.50 (m, 2H, $CH_2CH_2CH_2CO$). Upon mild acid treatment (1 eq. of trifluoroacetic acid

in methylene chloride, 10 min at 20°C) **15** yielded a mixture of **16**, **17** and **15**, which can be separated as described above.

3-[N-(9-Fluorenylmethoxycarbonyl)-6-aminohexanoyl]-3-aza-1,5-pentanediol (14) eluted after **15** was obtained as a colorless oil (0.78 g, 35%) which solidified upon drying to give white crystals, mp 97-98°C (EtOAc), R_f 0.22 ($CHCl_3$-MeOH 9:1 v/v). PD-MS $(m/z)^+$: 440.8 (M), 463.4 (M+Na), 573.1 (M+Cs); calculated for $C_{25}H_{32}N_2O_5$ 440.5, ($C_{25}H_{32}N_2O_5$+Na) 463.5, ($C_{25}H_{32}N_2O_5$+Cs) 573.4. ^1H NMR ($CDCl_3$; δ, ppm): 7.8-7.2 (m, 8H, Ar*H*); 5.13 (br. s, 1H, N*H*); 4.34 (d, 2H, *J*=7 Hz, CH_2OCO); 4.16 (t, 1H, *J*=7 Hz, C*H*CH_2OCO); 3.95 (br. s, 1H, O*H*); 3.76 (t, 2H, *J*=5 Hz), 3.70 (t, 2H, *J*=5 Hz, 2CH_2OH); 3.48 (t, 2H, *J*=5 Hz), 3.42 (t, 2H, *J*=5 Hz, 2C$H_2$$CH_2$OH); 3.13 (m, 2H, C$H_2$NH); 2.36 (t, 2H, *J*=7 Hz, CH_2CO); 1.61 (m, 2H, C$H_2$$CH_2$CO); 1.48 (m, 2H, C$H_2$$CH_2$NH); 1.31 (m, 2H, C$H_2$$CH_2$$CH_2$CO).

O^1-(4,4′-Dimethoxytrityl)-3-(6-aminohexanoyl)-3-aza-1,5-pentanediol (17). Compound **15** (1.72 g, 2.31 mmol) and pyrrolidine (5.0 mL, 60 mmol) were mixed and stirred at ambient temperature, until deblocking of the amino function was complete (about 1 h). The reaction mixture was evaporated, and the residue was chromatographed on silica gel in a gradient of methanol (0 to 20%) in chloroform to give amine **17** (1.12 g, 93%) as a colorless oil, R_f 0.19 ($CHCl_3$-MeOH 1:1 v/v, visualization with 0.05% fluorescamine in acetone). ^1H NMR ($CDCl_3$; δ, ppm): 7.4-7.2, 6.9-6.7 (m, 13H, Ar*H*); 3.75 (s, 6H, OCH_3); 3.7-3.6, 3.55-3.4, 3.3-3.2 (m, 8H, CH_2CH_2OH, CH_2CH_2ODMT); 2.65 (m, 2H, C$H_2$$NH_2$); 2.58 (br. s, 2H, N$H_2$); 2.37 (t, 2H, *J*=7 Hz, CH_2CO); 1.59 (m, 2H, C$H_2$$CH_2$CO); 1.43 (m, 2H, C$H_2$$CH_2$$NH_2$); 1.30 (m, 2H, C$H_2$$CH_2$$CH_2$CO).

O^1-(4,4′-Dimethoxytrityl)-3-(N-biotinyl-6-aminohexanoyl)-3-aza-1,5-pentanediol (18). To a stirred solution of amine **17** (1.04 g, 2.0 mmol) in dry pyridine (40 mL) was added ester **10** (0.82 g, 2.0 mmmol) in one portion. After the reaction was completed (about 30 min), the solvent was evaporated and the residue flash chromatographed on silica gel with 5%

MeOH in $CHCl_3$ as an eluent and dried in vacuum (0.04 mm, 16 h) to yield **18** (1.49 g, 100%) as a white foam, R_f 0.25 ($CHCl_3$-MeOH 5:1 v/v). PD-MS $(m/z)^+$: 747.1 (M), 769.7 (M+Na), 786.4 (M+K), calculated for $C_{41}H_{54}N_4O_7S$ 747.0, ($C_{41}H_{54}N_4O_7S$+Na) 769.9, ($C_{41}H_{54}N_4O_7S$+K) 786.1. ^1H NMR ($CDCl_3$; δ, ppm): 7.4-7.2, 6.9-6.8 (m, 13H, Ar*H*); 6.45, 6.41 (2 br. s, 2H, N*H*CON*H*); 5.49 (br. s, 1H, CH_2NH); 4.46 (m, 1H), 4.28 (m, 1H, C*H*NHCONHC*H*); 3.83-3.42, 3.30-3.20 (m, 17H, $CH_2CH_2NCH_2CH_2$, OCH_3 (3.79), CH_2NH); 3.12 (m, 1H, C*H*S), 2.87 (m, 1H) ,2.70 (m, 1H, CH_2S); 2.42 (m, 2H), 2.18 (m, 2H, $2CH_2CO$); 1.8-1.2 (m, 12H, $CH_2CH_2CH_2CH_2$-NHCOCH$_2$CH_2CH_2CH_2).

O^1-(4,4′-Dimethoxytrityl)-O^5-(diisopropylamino-2-cyanoethoxyphosphinyl)-3-(N-biotinyl-6-aminohexanoyl)-3-aza-1,5-pentanediol (19). Compound **18** (1.36 g, 1.82 mmol) was coevaporated with acetonitrile (2x50 mL), dissolved in dry acetonitrile (120 mL), and diisopropyl ammonium tetrazolide (0.17 g, 1.0 mmol) was added with stirring under argon followed by bis(diisopropylamino)-2-cyanoethoxyphosphine (0.64 mL, 2.0 mmol). The reaction mixture was half evaporated (bath temperature 30°C) and stirred at ambient temperature for 1.5-2 h (disappearance of **18**). The mixture was concentrated in vacuum, diluted with EtOAc-$CHCl_3$ 1:1 v/v (300 mL), washed with brine (2x100 mL), dried over Na_2SO_4 and evaporated. The residue was flash chromatographed on silica gel ($CHCl_3$, then 5% MeOH and 0.5% Et_3N in $CHCl_3$) and dried in vacuum to give the TLC-pure **19** (1.22 g). It was dissolved in toluene (15 mL), precipitated into hexane (400 mL), filtered off and dried in vacuum (0.04 mm, 24 h) to yield **19** (1.12 g, 65%) as a white powder, R_f 0.26 (acetone-Et_3N 9:1 v/v). PD-MS $(m/z)^+$: 969.8 (M+Na), calculated for ($C_{50}H_{71}N_6O_8PS$+Na) 970.2. ^{31}P NMR (δ, ppm): 148.93, 148.46 ($CHCl_3$), 151.520, 151.239 (CD_3CN). ^1H NMR ($CDCl_3$; δ, ppm): 7.4-7.2, 6.9-6.8 (m, 13H, Ar*H*); 6.24, 6.33 (2 br.s, 2H, N*H*CON*H*); 5.49 (br. s, 1H, CH_2NH); 4.46 (m, 1H), 4.28 (m, 1H, C*H*NHCONHC*H*); 3.85-3.5, 3.3-3.2 (m, 20H, $CH_2OPOCH_2CH_2N$-CH_2CH_2, OCH_3 (3.79), C*H*CH_3, CH_2NH); 3.13 (m, 1H, C*H*S); 2.87 (m, 1H), 2.71 (m, 1H, CH_2S); 2.61 (m, 2H, CH_2CN); 2.38 (m, 2H), 2.18 (m, 2H, $2CH_2CO$); 1.8-1.2 (m, 12H, $CH_2CH_2CH_2CH_2$NH-COCH$_2$CH_2CH_2CH_2).

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