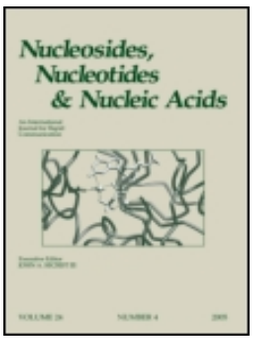

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ALDEHYDO-OLIGONUCLEOTIDES FOR BIOCONJUGATION

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Abstract: the synthesis of a new phosphoramidite building block with a masked aldehyde function is described. It was incorporated in an oligodeoxyribonucleotide. Coupling to amino derivatives by reductive amination was performed in high yield after unmasking the aldehyde function.

The immobilization of single stranded DNA to macromolecules is important for the design of diagnostic kits and biologically active substances. Immobilized oligonucleotides are indeed used to fish out a complementary sequence from a mixture of DNA or RNA[1,2]. The coupling of oligonucleotide effectors to peptides[3,4], basic polymers[5,6] and proteins[7,8] enhances their cellular penetration, and could allow their targeting to specific tissues or determine their intracellular routing. The link between the oligonucleotide and its support or vector may be reversible (typically a disulfide bridge) or irreversible (typically a thioether or an amide bond). The formation of an amine link by reductive amination was rarely performed. It is rather surprising, as reductive amination is largely used in protein chemistry and does not interfere with the functions present in DNA. The reason is probably because suitable aldehyde building blocks for automatic DNA synthesis are not currently available. A documented strategy to introduce aldehyde functions at the 3'-end of an oligonucleotide is the sodium periodate oxydation of a terminal ribonucleotide[5]. This procedure may be tricky however as β-elimination in the presence of an amine could be a major side-

reaction (Figure 1). Eliminations of this kind are indeed observed when apurinic DNA is treated by piperidine in the Maxam-Gilbert sequencing protocol[9].

Oligonucleotides bearing aldehyde functions could be conjugated to another molecule by either the hydrolyzable C=N imine, oxime, hydrazone or semicarbazone bond or the hydrolytically stable C-N bond obtained by reduction of the former. The hydrolytic stability of the C=N bond can be tuned according to the aim pursued (e.g. imines are far less stable than oximes[10]). Bioreversible bonds are of prime importance in the antisense research but the sole bioreversible bond available nowadays is the disulfide bridge. An enlargement of the palette of available bond types is thus convenient and in this connection, a reliable access to aldehydo-oligodeoxyribonucleotide is valuable.

We report here the synthesis of a nucleotide analog **8** (Figure 2) that allows the introduction of an aldehyde function in any position of an oligomer and in multiple copies, if necessary. The organocadmium **2**, obtained from 10-bromomagnesium-1-decene[11], was reacted with the readily accessible[12,13] protected deoxyribosyl chloride **1**. The coupling yield was good (85%), but the stereochemistry at the anomeric center was not controlled (R, 65%, S, 35%). The two epimers were separated by column chromatography and the synthesis was pursued with the S one[14]. Osmium tetroxyde oxidation[15], followed by silyl protection gave the fully protected nucleoside analog **5**. The *t*-butyldimethylsilyl group was used to protect the glycol because it is robust, compatible with the reagents used for automated DNA synthesis and easily removed at the oligonucleotide level. It is indeed the best 2'-O protecting group for RNA synthesis on solid support[16]. The selective *aminolysis* of the ester functions of **5** led to **6**, that was dimethoxytritylated on the primary alcohol function and then phosphitylated[17]. Compound **8** is a building block tailored for the automatic phosphoramidite-type DNA synthesis.

We then incorporated **8** in the sequence GTCGTGAC-(**8**)-GGGAAAAC. The trityl-on oligomer was cleaved from the glass beads by concentrated ammonia, detritylated, treated with tetrabutylammonium fluoride in THF to remove the silyl

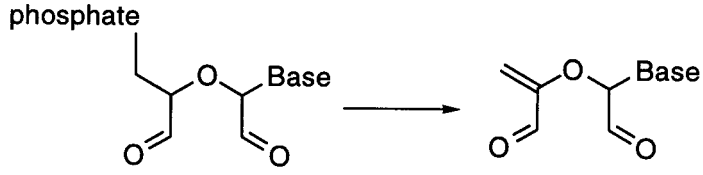


FIGURE 1

Possible β-elimination in oxidized 3' terminal ribonucleotide

protections and desalted. The resulting oligomer **9** was homogeneous in reversed phase C_{18} HPLC and PAGE. The oxidation of the glycol function of **9** by sodium periodate to give **10** (Figure 3) was conveniently followed by reversed phase HPLC. The scintillation counting after reduction of **10** by $NaBT_4$ proved that the oxidation was quantitative.

Oligonucleotide **10** was reductively coupled ($NaBH_3CN$) with amino derivatives of biotin, namely biotin hydrazide to give **11** and D-biotinyl-1,8-diamino-3,6-dioxaoctane, to give **12**. These couplings were also monitored by reversed phase HPLC, the conjugates being retarded by 1-1.5 minutes as compared to the aldehyde-oligomer **10**. The reactions were practically quantitative.

To get a further proof of the incorporation of one mole of biotin per mole of oligonucleotide, several mixtures of **12** + **9** (used as an internal standard) and avidin in different molar ratios were prepared. The mixtures were ultrafiltrated with a membrane cutoff that allowed the separation of the free oligonucleotides from avidin. They were quantified in the filtrate by reversed phase HPLC. The result of this titration of the conjugate **12** by avidin proved that indeed one molecule of biotin was immobilized per oligomer (see experimental part).

In conclusion, we described a practical synthesis of **8**. It is a convenient building block to introduce an aldehyde function in oligodeoxyribonucleotides and perform couplings with amino derivatives. Both 3' and 5' ends of glycol-oligonucleotides of type **9** remain free, so that attachment of other molecules at the extremities as well as 5' ^{32}P labeling remain feasible.

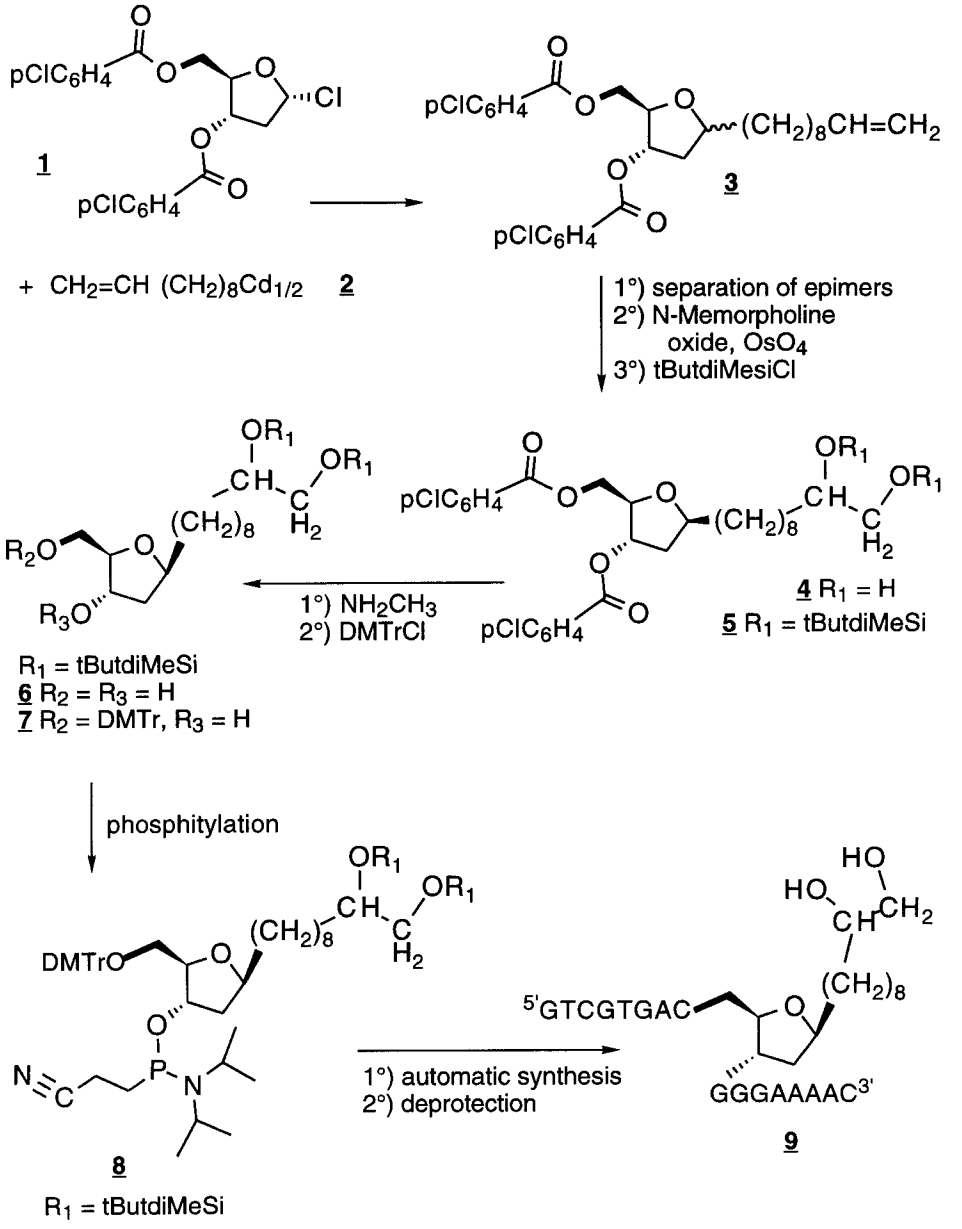
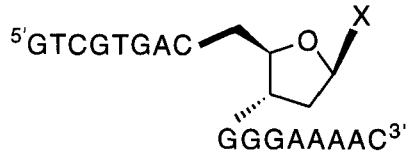
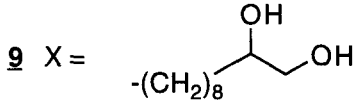


FIGURE 2





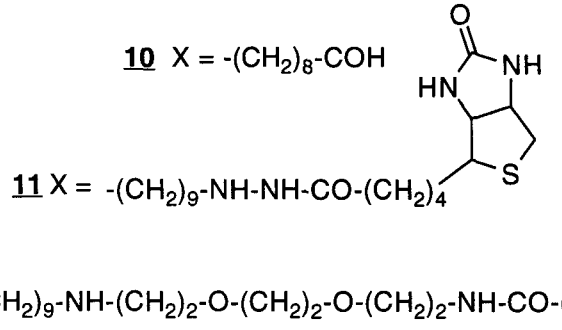


FIGURE 3

EXPERIMENTAL

The proportions in solvent and eluent mixtures are expressed as volumic fractions. The NMR spectra were recorded on a 500 MHz (^{1}H, 125 MHz ^{13}C, 81 MHz ^{31}P) instrument, except when otherwise stated. Chemical shifts are given in ppm relative to TMS (^{1}H, ^{13}C) or phosphoric acid (^{31}P). Biotin hydrazide was from Sigma and D-biotinyl-1,8-diamino-3,6-dioxaoctane from Boehringer Mannheim.

(2-R,3-S,5-S)-1-*O*,3-*O*-di(p-chlorobenzoyl)-1,2,3,5-tetrahydroxy-2,5-anhydropentadec-14-ene **3**

The reaction was performed under an argon atmosphere. A solution of 10-bromo-1-decene (40 mmoles) in dry THF (10 ml) was added portionwise to magnesium turnings (41 mmoles) magnetically stirred in dry THF (50 ml) containing 2-3 drops of

1,2-dibromoethane. The rate of addition was such that a constant reflux was maintained. Ten minutes after the end of addition, more THF (20 ml), and solid cadmium chloride (21 mmoles, dried at 100°C for 1 h, then kept under argon) was added. The mixture was kept for 1/2 h at room temperature. Dry hexane (30 ml) was added, followed by solid chloride **1** (10 mmoles, in 4-5 fractions). The mixture was warmed (67°C) for 1 h, then poured on cold water (100 ml, 0°C). The pH was adjusted to neutral with acetic acid. The water phase was extracted with toluene. The organic phase was washed (sat. $NaHCO_3$, H_2O), dried ($MgSO_4$), filtered and evaporated. The residue was chromatographed on silica (hexane/ethyl acetate, 95/5)(Yield: 85%; R, 65%, S, 35%). S Epimer: R_f = 0.15. 200 MHz ^1H NMR ($CDCl_3$): 7.96 and 7.41 (2 x m, ClBz), 5.78 (m, C**H**=CH_2), 5.45 (m, ClBzOC**H**), 4.95 (m, CH=C**H**$_2$), 4.48 (m, ClBzOC**H**$_2$CH), 4.32 (m, ClBzOCH$_2$C**H**), 4.20 [m, C**H**$(CH_2)_8$CH=CH_2], 2.23 [ddd, 1H, ring C**H**$_2$ (H exo)], 2.04 [m, CH$(CH_2)_7$C**H**$_2$CH=CH_2], 1.90 [m, ring C**H**$_2$ (H endo)], 1.65 [m, CHC**H**$_2(CH_2)_7$CH=CH_2], 1.30 [m, CHCH_2(C**H**$_2)_6$CH_2CH=CH_2]. ^{13}C NMR, 165.4, 139.8, 139.1, 131.1, 129.03 and 128.5 (ClBz and **C**H=CH_2), 114.2 (CH=**C**H$_2$), 82.1 and 79.5 (**C**HO**C**H), 77.6 (ClBzO**C**H), 65.1 (ClBzO**C**H$_2$), 38.5 (ring **C**H$_2$), 35.3, 33.9, 29.7, 29.6, 29.3, 29.2, 29.1 and 25.8 [(**C**H$_2)_8$]. C, H analysis, calc. C, 65.35, H, 6.43; found, C, 65.42, H, 6.53.

(2-R,3-S,5-S)-1-*O*,3-*O*-di(p-chlorobenzoyl)-1,2,3,5,14,15-hexahydroxy-2,5-anhydropentadecane **4**

Compound **3** (5 mmoles) was dissolved in an acetone/water mixture (9:1, 30 ml) kept at 0°C. *N*-methylmorpholin *N*-oxide (7.5 mmoles) was added, followed by osmium tetroxide (0.5 mmole, i.e. 0.5 ml of a 1M solution in t-butanol). The mixture was magnetically stirred and the progress of the reaction was followed by tlc (CH_2Cl_2/CH_3OH, 95:5; **3**, R_f = 0.8, **4**, R_f = 0.15). The mixture was evaporated after 6 h and partitioned between water and CH_2Cl_2. The organic phase was dried ($MgSO_4$), filtered and evaporated. The compound was purified by column chromatography (eluent, CH_2Cl_2). It is a colorless oil (Yield, 90%). ^1H NMR ($CDCl_3$): 7.97 and 7.42 (2 x m, ClBz), 5.46 (m, ClBzOC**H**), 4.50 (m, ClBzOC**H**$_2$CH), 4.34

(m, ClBzOCH$_2$CH), 4.25 [m, CH(CH$_2$)$_8$CH(OH)-CH$_2$(OH)], 3.67 [m, CH(CH$_2$)$_8$CH(OH)-CH$_2$(OH)], 3,40 [m, CH(CH$_2$)$_8$CH(OH)-CH$_2$(OH)], 3,05 [large signal, CH(CH$_2$)$_8$CH(OH)-CH$_2$(OH)], 2.22 [ddd, 1H, ring CH$_2$ (H exo)], 1.90 [m, ring CH$_2$ (H endo)], 1.75 and 1.40 [2 x m, CH(CH$_2$)$_7$CH$_2$CH(OH)-CH$_2$(OH) and CHCH$_2$(CH$_2$)$_7$CH(OH)-CH$_2$(OH)], 1.25-1.35 [m, CHCH$_2$(CH$_2$)$_6$CH$_2$CH(OH)-CH$_2$(OH)]. ^{13}C NMR, 165.2, 139.5, 130.8, and 128.0 (ClBz), 80.6 and 79.3 (CHOCH), 77.3 (ClBzOCH), 72,1 (CHOH), 66.6 (CH$_2$OH), 64.6 (ClBzOCH$_2$), 37.5 (ring CH$_2$), 35.7, 33.0, 29.5, 29.4, 29.3, 25.8 and 25.4 [(CH$_2$)$_8$]. C, H analysis, calc. C, 61.38, H, 6.39; found, C, 61.09, H, 6.38.

(2-R,3-S,5-S)-1-*O*,3-*O*-di(p-chlorobenzoyl)-14-*O*-15-*O*-di(*t*-butyldimethylsilyl)-1,2,3,5,14,15-hexahydroxy-2,5-anhydropentadecane **5**

The reaction was performed under argon with magnetic stirring. The diol **4** (2 mmoles) was dissolved in DMF (5 ml), dried imidazole (5 mmoles) was added, followed by *t*-butyldimethylsilylchloride (3 mmoles). The progress of the reaction was followed by tlc (eluent $CHCl_3$, R_f **4** = 0.1, R_f **5** = 0.7). After 48 h, the mixture was partitioned between water (20 ml) and CH_2Cl_2 (20 ml). The organic phase was washed (3 x) with water, dried ($MgSO_4$), filtered and evaporated. A filtration on silica (eluent $CHCl_3$) gave the product (Yield, 90%). ^{1}H NMR ($CDCl_3$): 7.97 and 7.42 (2 x m, ClBz), 5.46 (m, ClBzOCH), 4.52 (m, ClBzOCH$_2$CH), 4.34 (m, ClBzOCH$_2$CH), 4.25 [m, CH(CH$_2$)$_8$CH(OSiR$_3$)-CH$_2$(OSiR$_3$)], 3.61 [m, CH(CH$_2$)$_8$CH(OSiR$_3$)-CH$_2$(OSiR$_3$)], 3,41 [m, CH(CH$_2$)$_8$CH(OSiR$_3$)-CH$_2$(OSiR$_3$)], 2.18 [ddd, 1H, ring CH$_2$ (H exo)], 1.90 [m, ring CH$_2$ (H endo)], 1.70 and 1.40 [2 x m, CH(CH$_2$)$_7$CH$_2$CH(OSiR$_3$)-CH$_2$(OSiR$_3$) and CHCH$_2$(CH$_2$)$_7$CH(OSiR$_3$)-CH$_2$(OSiR$_3$)], 1.25-1.35 [m, CHCH$_2$(CH$_2$)$_6$CH$_2$CH(OSiR$_3$)-CH$_2$(OSiR$_3$)], 0.85 [s, C(CH$_3$)$_3$], 0.0 [s, Si(CH$_3$)$_2$]. ^{13}C NMR, 165.3, 139.6, 130.9, 128.7 and 128.3 (ClBz), 80.7 and 79.3 (CHOCH), 77.3 (ClBzOCH), 73,1 (CHOSiR$_3$), 67.4 (CH$_2$OSiR$_3$), 64.7 (ClBzOCH$_2$), 37.6 (ring CH$_2$), 35.7, 34.3, 29.6, 29.5, 29.4, 25.9, 25.7 and 25.0 [(CH$_2$)$_8$ and C(CH$_3$)$_3$], 18.2 [C(CH$_3$)$_3$], -4.7 [Si(CH$_3$)$_2$]. C, H, analysis, calc. C, 61.86, H, 8.10; found, C, 61.90, H, 8.13.

(2-R,3-S,5-S)-14-*O*-15-*O*-di(*t*-butyldimethylsilyl)-1,2,3,5,14,15-hexahydroxy-2,5-anhydropentadecane **6**

The diester **5** (2 mmoles) was dissolved in a mixture of THF (50 ml) and 24% aqueous trimethylamine (4 ml). After 3 h at 0°C, the solvent was evaporated. The residue was partitioned between water an ether. The ethereal phase was washed with water (5 x). The *N*-methyl-p-chlorobenzamide was precipitated by cooling the ethereal solution at -5°C. Yield of **6**, 90%.
^{1}H NMR ($CDCl_3$): 4.17 (m, HOC<u>H</u>), 4.05 [m, C<u>H</u>$(CH_2)_8$CH$(OSiR_3)$-$CH_2(OSiR_3)$], 3.73 (m, $HOCH_2$C<u>H</u>), 3.58 (m, HOC<u>H</u>$_2$CH), 3.55 [m, CH$(CH_2)_8$C<u>H</u>$(OSiR_3)$-$CH_2(OSiR_3)$], 3,40 [m, CH$(CH_2)_8$CH$(OSiR_3)$-C<u>H</u>$_2(OSiR_3)$], 2.9-3.2 (broad signal, 2 x O<u>H</u>), 1.88 [ddd, 1H, ring C<u>H</u>$_2$ (H exo)], 1.60 [m, ring C<u>H</u>$_2$ (H endo)], 1.50 and 1.35 [2 x m, CH$(CH_2)_7$C<u>H</u>$_2$CH$(OSiR_3)$-$CH_2(OSiR_3)$ and CHC<u>H</u>$_2(CH_2)_7$CH$(OSiR_3)$-$CH_2(OSiR_3)$], 1.18-1.28 [m, $CHCH_2$(C<u>H</u>$_2)_6CH_2$CH$(OSiR_3)$-$CH_2(OSiR_3)$], 0.85 [s, C(C<u>H</u>$_3)_3$], 0.0 [s, Si(C<u>H</u>$_3)_2$]. ^{13}C NMR, 86.6 and 78.7 (<u>C</u>HO<u>C</u>H), 73.2 (HO<u>C</u>H), 67.4 (<u>C</u>H$OSiR_3$), 63.2 (<u>C</u>H_2OSiR_3), 60.3 (HO<u>C</u>H_2), 41.1 (ring <u>C</u>H_2), 35.6, 34.3, 29.6, 29.5, 29.4, 26.0, 25.9 and 25.0 [(<u>C</u>$H_2)_8$ and <u>C</u>$(CH_3)_3$], 18.2 [C(<u>C</u>$H_3)_3$], -4.7 [Si(<u>C</u>$H_3)_2$]. C, H analysis, calc. C, 62.49, H, 11.27; found, C, 62.36, H, 11.37.

(2-R,3-S,5-S)-1-*O*-(4,4'-dimethoxytrityl)-14-*O*-15-*O*-di(t-butyldimethylsilyl)-1,2,3,5,14,15-hexahydroxy-2,5-anhydropentadecane **7**

Compound **6** (10 mmoles) was dissolved in dry pyridine (50 ml). Dimethoxytrityl chloride (12 mmoles) was added. The reaction was monitored by tlc (eluent $CH_2Cl_2/N(C_2H_5)_3$, 98:2). When the reaction was complete, methanol was added (10 ml). After 10 minutes, the mixture was diluted with CH_2Cl_2 and washed with aqueous Na_2CO_3 (5%). The organic phase was dried ($MgSO_4$), filtered and evaporated. The product was isolated by column chromatography (eluent $CH_2Cl_2/N(C_2H_5)_3$, 98:2, R_f = 0.4). Yield 75%.
^{1}H NMR ($CDCl_3$): 6.70-7.45 (trityl), 4.22 (m, HOC<u>H</u>), 4.12 [m, C<u>H</u>$(CH_2)_8$CH$(OSiR_3)$-$CH_2(OSiR_3)$], 3.88 (m, tritylOCH_2C<u>H</u>), 3.73 (s, CH_3O), 3.61 [m, CH$(CH_2)_8$C<u>H</u>$(OSiR_3)$-$CH_2(OSiR_3)$], 3.49 (m,

tritylOC$\underline{H}_2$CH), 3,15 [m, CH$(CH_2)_8$CH$(OSiR_3)$-C$\underline{H}_2(OSiR_3)$], 2.9-3.1 (broad signal, O$\underline{H}$), 1.89 [ddd, 1H, ring C$\underline{H}_2$ (H exo)], 1.65 [m, ring C$\underline{H}_2$ (H endo)], 1.48 and 1.40 [2 x m, CH$(CH_2)_7$C$\underline{H}_2$CH$(OSiR_3)$-$CH_2(OSiR_3)$ and CHC$\underline{H}_2(CH_2)_7$CH$(OSiR_3)$-$CH_2(OSiR_3)$], 1.18-1.32 [m, CHCH_2(C$\underline{H}_2)_6$CH_2CH$(OSiR_3)$-$CH_2(OSiR_3)$], 0.85 [s, C(C$\underline{H}_3)_3$], 0.0 [s, Si(C$\underline{H}_3)_2$]. ^{13}C NMR, 159.0, 145.3, 136.5, 130.5, 128.6, 128.3, 127.3, 113.7 and 86.5 (trityl), 84.0 and 78.8 (C$\underline{C}$HO$\underline{C}$H), 75.3 (HO$\underline{C}$H), 73.2 (tritylO-$\underline{C}H_2$), 67.8 ($\underline{C}$HOSiR_3), 65.3 ($\underline{C}H_2OSiR_3$), 55.7 ($\underline{C}H_3O$), 41.0 (ring $\underline{C}H_2$), 36.0, 34.1, 29.6, 29.5, 29.4, 26.2, 25.9 and 25.0 [($\underline{C}H_2)_8$ and $\underline{C}(CH_3)_3$], 18.2 [C(C$\underline{H}_3)_3$], -4.7 [Si(C$\underline{H}_3)_2$]. C, H analysis, calc. C, 70.26, H, 9.33; found, C, 70.46, H, 9.37.

{3-[(2-R,3-S,5-S)-1-*O*-(4,4'-dimethoxytrityl)-14-*O*-15-*O*-di(*t*-butyldimethylsilyl)-1,2,5,14,15-pentahydroxy-2,5-anhydro]pentadecoxy},(2-cyanoethoxy),(*N*,*N*-diisopropylamino)-phosphine **8**

The reaction was performed under argon. Compound **7** (0.1 mmoles) was coevaporated three times with acetonitrile, dried under high vacuum (3 h) and dissolved in dry CH_2Cl_2 (5 ml) containing diisopropylammonium tetrazolide (0.06 mmoles). Bis(diisopropylamino) (β-cyanoethoxy) phosphine (0.12 mmoles) was added. The mixture was magnetically stirred for 2 h [control by tlc, hexane/N$(CH_2CH_3)_3$, 6:1]. Sodium bicarbonate (5%, 5 ml) was added. The organic phase was washed with a phosphate buffer (pH 7, 5 ml), then with brine (sat., 5 ml), dried ($MgSO_4$), filtered and evaporated. The residue was chromatographed on silica [eluent: hexane/N$(CH_2CH_3)_3$, 6:1, R_f of **8** = 0.7]. Yield, 70 %. ^{31}P NMR, δ = 148.811 and 148.641 ppm. C, H analysis, calc. C, 67.02, H, 9.18; found, C, 67.35, H, 8.98.

Synthesis and purification of oligonucleotide **9**

The β-cyanoethyl phosphoramidite strategy was used[18]. At the end of the trityl-on synthesis, the glass beads were treated with conc. ammonia at 50°C for 24 h. The basic solution was passed

trough an Oligo-Pak^{TM} reversed phase cartridge (Pharmacia), that retained the tritylated oligomer. The column was washed with water. Treatment with TFA:H_2O (2%) cleaved the trityl protection and allowed to elute the desired product. The residue after lyophilization (0.02 µmoles of oligonucleotide) was dissolved in a solution of tetrabutylammonium fluoride in THF (0,1 M, 10 µl). After 2 h of shaking (the time necessary for full desilylation was checked by tlc on the monomeric compound **6**), the THF was evaporated and the residue was dissolved in water (1 ml). This solution was desalted on a Pharmacia NAP-10^{TM} microcolumn. The column was equilibrated with phosphate buffer (15 ml, 10 mM, pH 6.8). The sample was then loaded and eluted with the buffer. The first ml corresponded to the dead volume. The oligonucleotide was found in the following 1.5 ml of eluate.

Oxidation of **9** to **10**

A solution of $NaIO_4$ (120 mM) in phosphate buffer (20 mM, pH 7) was made. It was degazed by helium (to avoid overoxidation of the aldehyde to be obtained). This solution (1 ml) was added to the glycol-oligonucleotide (100 OD in 25 µl). The reaction was stopped after 30 min in the dark at room temperature by desalting on NAP-10^{TM}. The success of the reaction was checked by reversed phase HPLC [BIORAD Bio-Sil C_{18} HL 150x4.6 mm column. Linear gradient of eluent B (triethylammonium acetate 0.1 M in CH_3CN:H_2O, 60:40) in eluent A (triethylammonium acetate 0.1 M in CH_3CN:H_2O, 5:95), from 0 to 30% in 25 min. Flow, 1 ml/min.]. The retention times were 15.4 and 16.3 min. for **9** and **10** respectively. The solution of the aldehyde-oligomer was not kept, but immediately used for the next step.

Titration of **10** by $NaBT_4$

9 (5 nmoles) was dissolved in water in an eppendorf tube. A 5 mM solution of $NaIO_4$ in 20 mM acetate buffer (pH 4.75) was added (2.5 µl). The mixture was stirred in the dark for 5 h. A 10 mM solution of $NaBT_4$ in 40 mM borate buffer (pH 9) was added

(2.5 µl). The mixture was stirred at 4°C for 5 h, then diluted with water (300 µl). The yield of tritium incorporation was determined by passing the sample trough NAP-5™ cartridges, isolating the high molecular weight fraction and determining the immobilized tritium content by scintillation counting.

Synthesis of **11**

A solution of biotin hydrazide (10 µl, 15 mM) and acetate buffer (5 µl, 500 mM, pH 5) and a solution of sodium cyanoborohydride (5 µl, 100 mM in acetate buffer) were added to the aldehyde-oligonucleotide **10** dissolved in water (1.5 nmoles in 500 µl). The mixture was desalted on NAP-10 ™ after 2 h at room temperature. The conjugate was isolated by semi-preparative HPLC, in the same conditions as indicated above.

Synthesis of **12**

A solution of D-biotinoyl-1,8-diamino-3,6-dioxaoctane (10 µl, 15 mM) and phosphate buffer (100 µl, 20 mM, pH 6.8) and a solution of sodium cyanoborohydride (10 µl, 5 mM in water) were added to the aldehyde-oligonucleotide **10** dissolved in water (1.5 nmoles in 200 µl). After 4 h at room temperature, the mixture was diluted by water to 500 µl and desalted on NAP-5 ™. The conjugate was isolated by semi-preparative HPLC, in the same conditions as indicated above.

Titration of **12** by avidin (Figure 4)

Aliquots of a solution of avidin (Sigma, 5.2 nanomoles/ml of sites of complexation) were added to solutions of the biotin-oligonucleotide conjugate **12** (66 picomoles of oligonucleotide in 200 µl). Each solution was ultrafiltrated (ULTRAFREE-MC™ from Millipore, membrane UFC3-LTK, cutoff 30.000), and the filtrate checked by HPLC. Oligomer **9** was used as a not retained internal

relative areas under the HPLC peaks. Upper line, **9**, lower line, **12**.

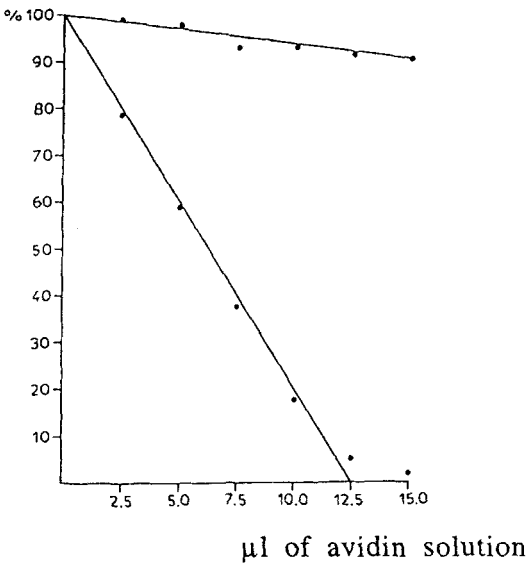


FIGURE 4

standard. The addition of 12.5 µl of the solution of avidine (65 picomoles of sites of complexation) was necessary to completely retain the biotinylated oligomer on the filter.

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