C-Glycoside phosphoramidite building block for versatile functionalization of oligodeoxyribonucleotides

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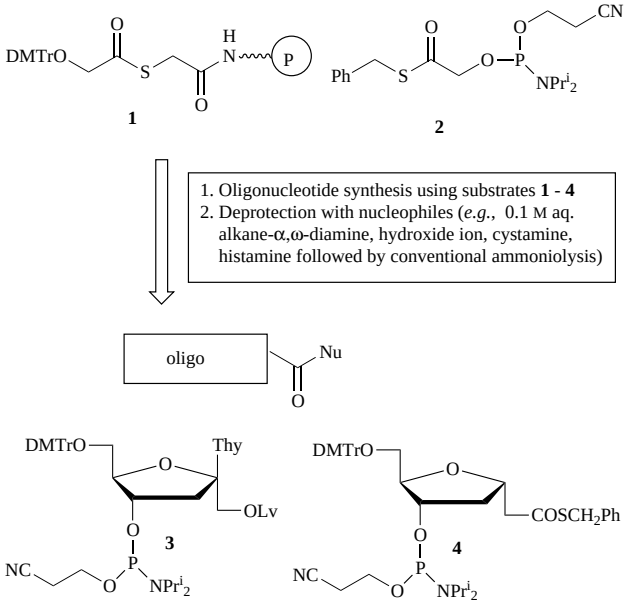
A chiral non-nucleosidic phosphoramidite building block containing a thioester function in its structure, 4, is synthesized in six steps starting from '2-deoxy-D-ribose'. It is used in the machine-assisted oligonucleotide synthesis in a conventional manner. Upon completion of the oligonucleotide chain assembly the thioester bond is cleaved with various nucleophiles (hydroxide ion, propane-1,3-diamine, cystamine, histamine), resulting in oligonucleotide conjugates with a tether group in the middle of the oligonucleotide chain.

Introduction

Synthetic oligonucleotides covalently linked to various ligands have been increasingly used as research tools in molecular biology.[1] They have been applied to genetic analysis, and to elucidate the mechanisms of gene function.[2–6] Oligonucleotides carrying reporter groups have had widespread use in automated sequencing,[7–9] hybridization affinity chromatography[10,11] and fluorescence microscopy.[12,13] Oligonucleotide–biotin conjugates are widely used as hybridization probes.[6] Antisense oligonucleotides covalently linked to intercalators, chain-cleaving or alkylating agents have been shown to be efficient as gene-expression regulators. The sequence-specific artificial nucleases, when targeted against messenger RNA (mRNA), may find applications even as chemotherapeutics.

We have recently developed a versatile strategy for oligonucleotide tethering: an ester[14–17] or thioester[18] function is attached to the oligonucleotide during chain assembly, and upon completion of the oligonucleotide synthesis the desired functional group is introduced by treating the oligomer, when still anchored to the solid support, with an appropriate nucleophile (Scheme 1). Finally, the oligonucleotide is allowed to react



Scheme 1

in solution with electrophiles, such as succinimido, isothiocyanato, maleimido or chlorosulfonyl derivatives of conjugate groups. For the introduction of a functional group in the 3′- or 5′-end of an oligonucleotide the method is straightforward. However, for the introduction of reporter groups in the middle of the oligonucleotide chain a multistep synthesis of a phosphoramidite building block derived from 3′-deoxypsicothymidine **3** is needed.[19,20] After completion of the oligonucleotide-chain assembly the laevulinyl group (Lv) is removed selectively with hydrazinium acetate, and the phosphoramidite **2** is coupled to the free hydroxy group.[18] Here is presented a simple synthesis of a non-nucleosidic phosphoramidite building block, **4**, starting from '2-deoxy-D-ribose' (2-deoxy-D-*erythro*-pentose). This *C*-glycoside derivative enables attachment of various functional groups in the middle of the oligonucleotide chain by use of our tethering strategy. The use of compound **4** results in oligonucleotide conjugates where the structure of the entire oligomer is minimally modified. The introduction of an abasic site in the middle of the oligonucleotide chain destabilizes the duplex formed upon hybridization with a complementary oligonucleotide to the same extent as a spot mutation.[21]

Results and discussion

Synthesis of the phosphoramidite

Preparation of the phosphoramidite building block, **4**, is outlined in Scheme 2. Treatment of '2-deoxy-D-ribose'[22] with an equimolar amount of 4,4′-dimethoxytrityl chloride (DMTrCl) yielded 2-deoxy-5-*O*-(4,4′-dimethoxytrityl)-D-ribose **5** in moderate yield (51%). When compound **5** was allowed to react with (ethoxycarbonylmethylene)triphenylphosphophorane in dry tetrahydrofuran (THF) at reflux, compound **6** was formed. It was cyclized without isolation to the epimeric furanoses **7a**,**b** by treatment with ethanol containing traces of sodium ethoxide. The epimers were separated on a silica gel column and were characterized based on the following observations: ^{1}H, ^{1}H 2D nuclear Overhauser enhancement (NOESY) spectrum of the faster migrating isomer had distinct cross-peaks between H-2b and H-3 (the latter proton is undoubtedly on the β-face of the *C*-glycoside ring; for numbering, see Scheme 2) as well as between H-2b and H-3. By contrast, there was no cross-peak between H-1 and H-2a. Thus, H-2b and the anomeric proton are on the same face of the ring, and the compound is the α-anomer, **7a**. The opposite effect was observed in the case of the slower migrating epimer: there was a cross-peak between H-3 and H-2b, H-2a and H-2, but not between H-2b and H-1. Hence this compound can be judged as the β-anomer, **7b**. The α:β ratio according to ^{1}H NMR analysis‡ of the unpurified

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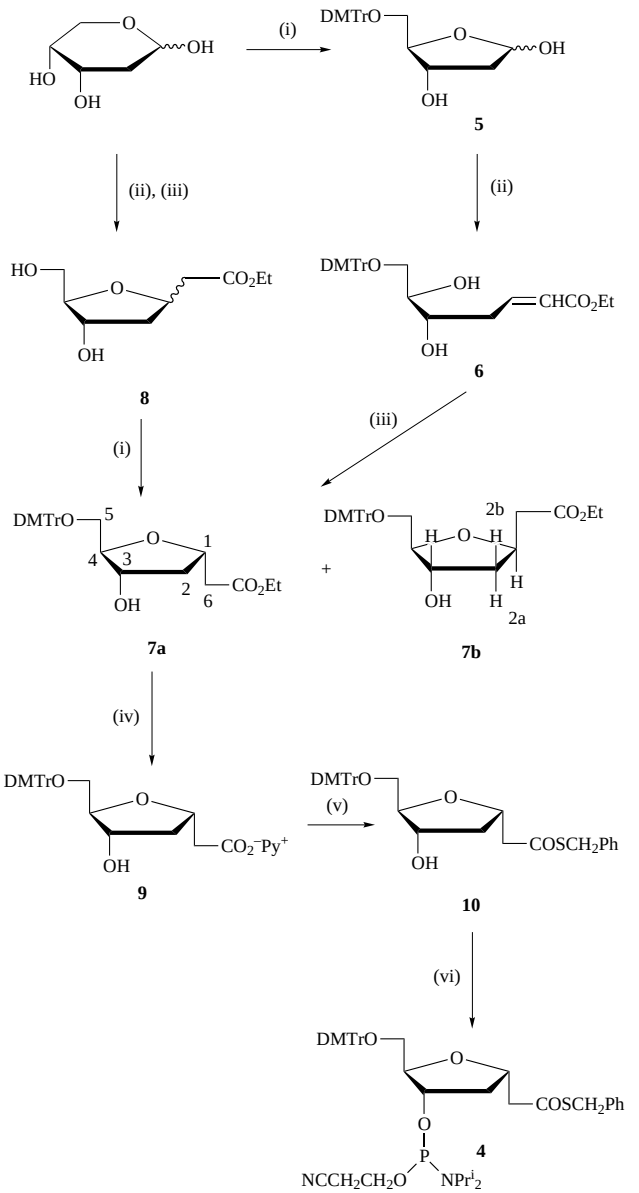
‡ Determination of the α:β ratio is based on the integral ratio of the well separated 3-protons of isomers **7a**,**b**.



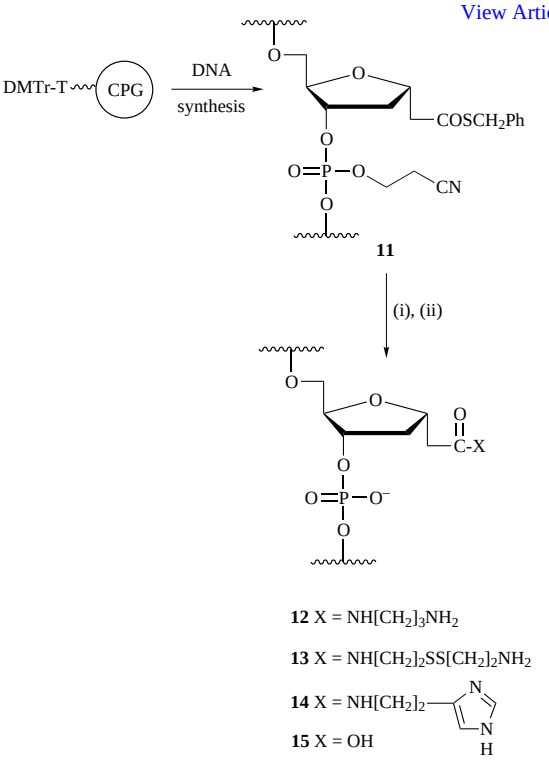
Scheme 2 *Reagents and work-up conditions:* (i) DMTrCl in pyridine; (ii) $Ph_3P{=}CHCO_2Et$ in THF; (iii) NaOEt (trace) in EtOH; (iv) 1 mol dm^{-3} NaOH–1,4-dioxane (1 : 4 v/v); then Dowex-50 (pyridinium form); (v) DCC and toluene-α-thiol in dichloromethane; (vi) 2-cyanoethyl *N*,*N*,*N*′,*N*′-tetraisopropylphosphorodiamidite and 1*H*-tetrazole in acetonitrile

reaction mixture was 1 : 2.4. This is quite surprising, since it is known that Wittig-mercurio-[23] and -iodocyclizations[24] of 2-deoxy-5-*O*-trityl-D-ribofuranoses afford a 1 : 1 mixture of epimers. Furthermore, when '2-deoxy-D-ribose' was converted into compound **8** as described by Rokach *et al.*[25] followed by dimethoxytritylation, products **7a** and **7b** were obtained in an equimolar ratio.

The α-epimer **7a** was converted into the phosphoramidite building block, **4**, as follows: saponification of the ester function gave the corresponding acid, **9**, dicyclohexylcarbodiimide (DCC)-assisted thioesterification of which with toluene-α-thiol gave rise to thioester **10** (75% overall yield). An excess of the thiol was used in order to suppress the reaction of the unprotected 3-hydroxy group. Compound **10** was finally phosphitylated using 2-cyanoethyl *N*,*N*,*N*′,*N*′-tetraisopropylphosphorodiamidite in the presence of 1*H*-tetrazole to give, after silica gel column chromatography, thioester **4** as the final product.

Synthesis of the oligonucleotide conjugates

In order to demonstrate the suitability of thioester **4** for oligonucleotide tethering a model sequence dT_6XT_7 (X = abasic site) was prepared (Scheme 3). The phosphoramidite **4** was used in the 7th coupling step in conventional manner (0.1 mol dm^{-3} **4** in acetonitrile, coupling time 25 s). No difference between the coupling efficiency of compound **4** and commercial nucleosidic phosphoramidite building blocks was detected. When the oligonucleotide-chain assembly was completed the solid-support-bound material was treated with various nucleophiles as shown in Table 1. We have already shown that thioesters react smoothly with nitrogen nucleophiles under mild conditions.[16] Indeed, also in the present case, treatment of the protected oligonucleotide **11** with dil. aq. amines gave the desired oligonucleotide conjugates **12**–**14**, according to HPLC analysis, in over 90% yield (peaks of capped sequences are not included). When 0.1 mol dm^{-3} NaOH was used as the cleavage agent, the corresponding acid, **15**, was obtained. The deprotection procedure was completed by conventional ammonolysis to verify that all of the oligonucleotide was released from the solid support.



Scheme 3 *Reagents:* (i) aq. amine or NaOH; (ii) aq. ammonia

Characterization of the oligonucleotides prepared

The oligonucleotides prepared were isolated on ion-exchange high-performance liquid chromatography (HPLC), further purified on a reversed-phase (RP) column and desalted by gel filtration.[14] An ion-exchange HPLC profile of the oligonucleotide tethered to cystamine, compound **13** (crude reaction mixture), is shown in Fig. 1 as an illustrative example. The structures of the oligonucleotide conjugates were verified on matrix-isolated laser-desorption time-of-flight (MALDI-TOF) mass spectrometry.[26] In all the cases the observed relative molecular masses were in agreement with the expected values (Table 1).

Experimental

General

Reagents for oligonucleotide synthesis were purchased from Cruachem. 2-Cyanoethyl *N*,*N*,*N*′,*N*′-tetraisopropylphosphorodiamidite was prepared according to a published procedure.[27] Cystamine dihydrochloride was converted into the free-base form[28] which was used without purification. NMR spectra were recorded on a JEOL LA-400 spectrometer operating at 399.8

Table 1 Deprotection procedures for compound **11** to obtain tethered oligonucleotides **12**–**15**, their ion-exchange HPLC retention times[a] and relative molecular masses obtained by MALDI-TOF mass spectrometry[b]

Product	Deprotection procedure	t_R/min[c]	M(Found)	*M*(Calc.)
12	(i) 0.1 M aq. propane-1,3-diamine[d] for 6 h, (ii) ammonolysis[e]	13.3	4184.3	4186.8
14	(i) 0.2 M aq. histamine for 6 h,[d] (ii) ammonolysis[e]	13.5	4221.2	4223.9
13	(i) 0.1 M aq. cystamine for 6 h,[d] (ii) ammonolysis[e]	13.2	4267.3	4265.0
15	(i) 0.1 M aq. NaOH for 4 h,[d] (ii) ammonolysis[e]	17.3	4128.7	4130.7

[a] For conditions, see Experimental section. [b] The molecular ion of dT_{14} (4196.8 Da) was used as an external standard. [c] t_R of dT_{14} was 15.0 min. [d] At room temperature. [e] Conc. aq. ammonia, 7 h, at 55 °C.

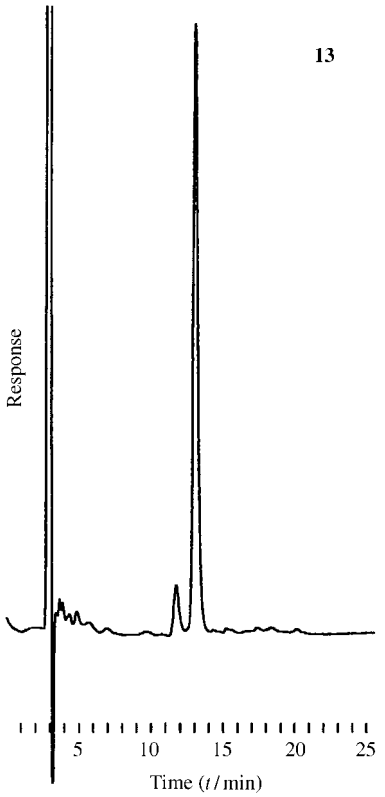


Fig. 1 Ion-exchange HPLC profile of **13** (crude reaction mixture) at 260 nm. The large peak at $t_R \sim 3$ min is the result of non-nucleosidic material. For gradient limits, see the Experimental section.

and 161.9 MHz for ^1H and ^{31}P, respectively. $SiMe_4$ was used as an internal (^1H) and H_3PO_4 as an external (^{31}P) reference. *J*-Values are given in Hz. When reported, characterization of NMR signals is based on ^1H, ^1H 2D homonuclear chemical-shift correlation spectroscopy (COSY) and NOESY experiments. MALDI-TOF mass spectra of oligonucleotides were recorded on a Finnigan Lasermat spectrometer (negative detection mode) using the procedure described by Pieles *et al.*[26] Elemental analyses were performed on a Perkin-Elmer 2400 Series II instrument.

HPLC Techniques

HPLC analyses were carried out as described previously in detail.[14] Chromatographic conditions in the present work were: Ion exchange (SynChropak AX-300, 4.6 × 250 mm, 6 μm), flow rate 1 $cm^3\ min^{-1}$, buffer A = 0.05 mol dm^{-3} KH_2PO_4 in 50% (v/v) aq. formamide, pH 5.6; Buffer B = A + 0.6 mol dm^{-3} $(NH_4)_2SO_4$, from 10 to 60% B in 30 min. Reversed phase (Nucleosil 300-5C18, 4.0 × 250 mm, 5 μm), flow rate 1 $cm^3\ min^{-1}$, Buffer A = 0.05 mol dm^{-3} NH_4OAc, Buffer B = A + 50% (v/v) MeCN, from 0 to 40% B in 40 min. Gel filtration (TSKgel G2000SW, 7.5 × 300 mm), flow rate 1 $cm^3\ min^{-1}$, water.

2-Deoxy-5-*O*-(4,4′-dimethoxytrityl)-D-*erythro*-pentofuranose 5

'2-Deoxy-D-ribose' (5.0 g, 37.3 mmol) was dried by coevaporations with dry pyridine and was dissolved in the same solvent (500 cm^3). DMTrCl (12.6 g, 37.3 mmol) was added and the mixture was stirred overnight at room temperature. The reaction was quenched with ice–water, and all volatile material was removed *in vacuo*. The residue was dissolved in dichloromethane and washed with saturated aq. $NaHCO_3$. The organic phase was dried over $MgSO_4$ and concentrated. Purification on silica gel (eluent CH_2Cl_2–MeOH–Et_3N 97:3:0.1; v/v/v) yielded the title compound as a foam (8.3 g, 51%); $\delta_H([^2H_6]DMSO)$ 7.44–7.20 (9 H, m), 6.88 (4 H, d, *J* 6.9), 6.21 and 6.17 (tot. 1 H, *J* 2 × 4.9, exch. with D_2O), 5.37 (1 H, m), 5.00 and 4.96 (tot. 1 H, 2 d, *J* 5.0 and 5.4, exch. with D_2O), 4.08 and 3.96 (tot. 1 H, 2 m), 3.89 and 3.77 (tot. 1 H, m), 3.76 (6 H, s), 3.03 (2 H, m), 2.27 (1 H, m) and 1.83 (1 H, m).

Ethyl [2-deoxy-5-*O*-(4,4′-dimethoxytrityl)-α- and -β-D-*erythro*-pentofuranosyl]acetate 7a,b

Method A. Compound **5** (10 g, 22.9 mmol) was dissolved in dry THF (100 cm^3). (Ethoxycarbonylmethylene)triphenylphosphorane (Fluka; 8.88 g, 25.2 mmol) was added, and the mixture was heated at reflux for 6 h before being allowed to cool, and all volatile material was removed *in vacuo*. The residue was dissolved in ethanol (100 cm^3) containing traces of sodium ethoxide and kept 1 h at ambient temperature. The solvent was removed, the residue was dissolved in dichloromethane, and the solution was washed with brine and dried ($MgSO_4$). The residue was applied onto a silica gel column and eluted with a mixture of hexane and ethyl acetate (3:2, v/v) in order to remove triphenylphosphine oxide. Fractions containing a mixture of the epimers **7a**,**b** were pooled and the rechromatographed (eluent CH_2Cl_2–MeOH–Et_3N, 98:2:0.1, v/v/v) to give the pure anomers. Compound **7a** (2.32 g, 20%) (Found: C, 71.4; H, 6.8. $C_{30}H_{34}O_7$ requires C, 71.1; H, 6.8%); $\delta_H(CDCl_3)$ 7.42–7.19 (9 H, m, ArH), 6.82 (4 H, d, *J* 6.8, ArH), 4.50 (1 H, m, H-1), 4.31 (1 H, m, H-3), 4.15 (2 H, q, *J* 7.2, $OCOCH_2$), 4.05 (1 H, m, H-5), 3.79 (6 H, s, 2 × OCH_3), 3.24 (1 H, dd, *J* 4.6 and 9.6, H^a-5), 3.07 (1 H, dd, *J* 6.2 and 9.6, H^b-5), 2.73 (1 H, dd, *J* 6.4 and 15.9, H^a-6), 2.65 (1 H, dd, *J* 5.9 and 15.9, H^b-6), 2.48 (2 H, m, H^a-2 and 3-OH), 1.79 (1 H, m, H^b-2) and 1.26 (3 H, t, *J* 7.2, CH_3). Isomer **7b** (5.67 g, 49%) (Found: C, 71.3; H, 6.5%); $\delta_H(CDCl_3)$ 7.69–7.19 (9 H, m, ArH), 6.82 (4 H, d, *J* 6.8, ArH), 4.55 (1 H, m, H-1), 4.33 (1 H, m, H-3), 4.15 (2 H, q, *J* 7.2, $OCOCH_2$), 3.93 (1 H, m, H-4), 3.79 (6 H, s, 2 × OCH_3), 3.23 (1 H, dd, *J* 4.4 and 9.6, H^a-5), 3.07 (1 H, dd, *J* 6.0 and 9.6, H^b-5), 2.66 (1 H, dd, *J* 7.2 and 15.4, H^a-6), 2.51 (1 H, dd, *J* 6.0 and 15.4, H^b-6), 2.06 (1 H, ddd, *J* 2.4, 5.7 and 13.1, H^a-2), 1.84 (2 H, m, H^b-2 and 3-OH) and 1.25 (3 H, t, *J* 7.2, CH_3).

Method B. '2′-Deoxy-D-ribose' (5.0 g, 37.3 mmol) was treated with (ethoxycarbonylmethyl)triphenylphosphorane (1.5 mol equiv.) as described above for compound **5**. After base-catalyzed cyclization, all volatile material was removed *in vacuo*. The residue was coevaporated with dry pyridine and dissolved in the same solvent (50 cm^3). DMTr-Cl (12.6 g, 37.3 mmol) and a catalytic amount of 4-(dimethylamino)pyridine (DMAP) were added and the mixture was stirred overnight at ambient temperature. Work-up and purification was performed as described above in **Method A**. The isolated yields of isomers **7a** and **7b** were both 35%.

S-Benzyl [2-deoxy-5-*O*-(4,4′-dimethoxytrityl)-α-D-*erythro*-pentofuranosyl]thioacetate 10

Compound **7a** (2.0 g, 3.95 mmol) was dissolved in a mixture of 1,4-dioxane and 0.1 mol dm^3 NaOH (20 cm^3; 1:1 v/v) and the solution was stirred for 1 h at room temperature. Solvent was evaporated off, the residue was dissolved in a mixture of pyridine–water–methanol (2:1:1, v/v/v) and the solution was passed through a Dowex-50 resin (pyridinium form) using the same solvent system as the eluent. All volatile material was removed *in vacuo*. After coevaporation with pyridine the residue was dissolved in dichloromethane (20 cm^3). Toluene-α-thiol (0.93 cm^3, 7.90 mmol) and DCC (0.82 g, 3.95 mmol; predissolved in 5 cm^3 of CH_2Cl_2) were added and the mixture was stirred for 4 h at ambient temperature. The dicyclohexylurea formed was filtered off, and the filtrate was washed with brine and dried ($MgSO_4$). Purification on a silica gel column (eluent CH_2Cl_2–MeOH 97:3, v/v) yielded the title compound as an oil (1.73 g, 75%) (Found: C, 72.1; H, 6.43; S, 5.75. $C_{35}H_{36}O_6S$ requires C, 71.9; H, 6.21; S, 5.48%); $\delta_H(CDCl_3)$ 7.42–7.19 (14 H, m, ArH), 6.82 (4 H, d, *J* 6.8, ArH), 4.58 (1 H, m, H-1), 4.30 (1 H, m, H-3), 4.12 (2 H, AB, *J* 13.9, SCH_2Ph), 3.83 (1 H, m, H-4), 3.76 (6 H, s, 2 × OCH_3), 3.18 (1 H, dd, *J* 4.2 and 9.8, H^a-5), 3.10 (1 H, dd, *J* 5.4 and 9.8, H^b-5), 2.93 (1 H, dd, *J* 7.1 and 14.9, H^a-6), 2.76 (1 H, dd, *J* 5.6 and 14.9, H^b-6), 2.02 (1 H, ddd, *J* 2.4, 5.6 and 7.8, H^a-2), 1.54 (1 H, m, H^b-2) and 1.74 (1 H, br, 3-OH).

Synthesis of the phosphoramidite 4

Predried alcohol **10** (1.75 g, 3.0 mmol) and 2-cyanoethyl *N*,*N*,*N*′,*N*′-tetraisopropylphosphorodiamidite (1.39 g, 4.60 mmol) were dissolved in dry acetonitrile (1.5 cm^3). 1*H*-Tetrazole (3.0 mmol, 6.70 cm^3; 0.45 mol dm^{-3} in acetonitrile) was added, and the mixture was stirred for 30 min at room temperature before being poured into 5% aq. $NaHCO_3$ (150 cm^3) and extracted with dichloromethane (2 × 75 cm^3). The extracts were combined, dried ($MgSO_4$), and concentrated. Purification on a silica gel column (eluent hexane–ethyl acetate–triethylamine, 5:4:1, v/v/v) yielded compound **4** as an oil (1.8 g, 80%), which could be stored at −20 °C; $\delta_H(CDCl_3)$ 7.45–7.20 (14 H, m, ArH), 6.82 (4 H, 2 × d, *J* 6.8, ArH), 4.69 (1 H, m, H-1), 4.46 (1 H, m, H-3), 4.20 (1 H, m, H-4), 3.78 and 3.77 (tot. 6 H, 2 × s, OCH_3), 3.71 (3 H, m, OC$H_2$$CH_2$CN and C$H$$Me_2$), 3.31 (3 H, m, H^a-6a and H_2-5), 2.85 (1 H, m, H^b-6), 2.53 and 2.40 (tot. 2 H, CH_2CN), 2.40 (1 H, m, H^a-2), 1.92 and 1.82 (tot. 1 H, 2 × m, H^b-2) and 1.15 (12 H, Pr^i); $\delta_P(CDCl_3)$ 148.31 (0.5 P) and 148.22 (0.5 P).

Preparation of the oligonucleotides

The oligonucleotides were assembled on an Applied Biosystems 392 DNA Synthesizer in 0.2 μmol scale using phosphoramidite chemistry and recommended protocols (DMTr-Off synthesis). The phosphoramidite **4** was used in the 7th coupling step in a conventional manner (0.1 mol dm^3 solution in dry acetonitrile, coupling time 25 s). When the oligonucleotide-chain assembly was completed the fully protected oligonucleotide was treated with aqueous nucleophiles and ammoniolysed (for reaction times and concentrations, consult Table 1).

Acknowledgements

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