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An expeditious route to methylphosphonate analogues of DNA

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Abstract. Protection of the exocyclic amino groups of both nucleobases adenine and guanine with the *N,N*-dimethylformamidine and cytosine with the isobutyryl group presents a convenient and economical N-protective-group-strategy for the solid-phase synthesis of DNA fragments containing methylphosphonate linkages at predetermined locations. Thus, the post-synthesis removal of the N-protective groups with hydrazine hydrate (16 h at 20°C) afforded immobilized fragments, which were then cleaved from the solid support and deprotected at the phosphorus by treatment with ammonia in methanol (2 h at 20°C) to yield high-quality products.

Introduction [a]

Methylphosphonate oligodeoxynucleotides (MPO's) present an important member of backbone modified nucleic acids in which the negatively charged phosphodiester oxygen has been replaced by sterically undemanding and neutral methyl groups. The attractiveness of MPO's as therapeuting agents[1–10] (*i.e.* potential inhibitors of gene expression in several systems) is mainly due to the fact that they not only survive enzymatic degradation by endo- and exonucleases, but also exhibit cell membrane permeability. However, the accessibility of these promising antisense tools is severely hampered by the intrinsic base lability of the neutral (3′-5′)-internucleotidic methylphosphonate linkage.

In order to overcome this limitation two distinct approaches have been pursued. In the first one, the final and crucial deblocking of the commonly applied N-acyl protective groups X, Y and Z (see Table I) in the respective methylphosphonamidite building units **1a–c** (R = CH_3) could be effected using a mild two-step deprotection procedure. Thus, deacylation of the N-benzoyl groups X and Y by hydrazine hydrate and subsequent removal of the isobutyryl (iBu) group Z by 1,2-ethanediamine (EDA), was reported by *Miller*[11] et al. (see procedure A in Table I). On the other hand, deprotection of the same set of protective groups could be accomplished, as reported by *Agrawal*[12] et al. with aqueous ammonia followed by EDA treatment (see procedure B). Recently, *Hogrefe*[13] et al. revealed that deprotection of the above-mentioned combination of N-acyl protective groups, in which the N-benzoyl group X in the deoxycytidine unit **1a** was replaced by the more base labile iBu group, could be realized via the one-pot procedure C in Table 1. In the second approach, hydrolysis of the methylphosphonate linkage was suppressed by mild ammonolysis (procedures D and E in Table I) of the rather base labile *tert*-butylphenoxyacetyl[14] [tBu(PhO)Ac] or 2-(acetoxymethyl)benzoyl[15] (AMB) groups, each of which may serve as a suitable protective group for the exocyclic amino function of the three nucleobases A, G and C.

In a preliminary paper, *Vu*[16] et al. showed that the readily accessible (cyanoethyl)phosphoramidite derivatives **1a,d,e** (R = 2-cyanoethoxy), in which the pyrimidine and purine bases are protected with iBu and *N,N*-dimethylformamidine (dmf) groups, respectively, fulfill all criteria in terms of application and performance (*i.e.* enhanced resistance to depurination and fast deprotection in ammonium hydroxide[17]) for the rapid production of high-quality unmodified oligodeoxynucleotides.

As part of a programme to synthesize DNA fragments containing methylphosphonate linkages at predetermined positions[18,19], we report here that the use of the similarly N-protected methylphosphonamidites **1a,d,e** (R = CH_3) and their deprotection with hydrazine hydrate, under the same conditions as those previously applied by *Miller* et al. (see first step of procedure A in Table I), presents an economical and efficient route to methylphosphonate analogues of DNA.

[a] **Abbreviations and synonyms**

AMB = 2-(acetoxymethyl)benzoyl
Bz = benzoyl
t-Bu(PhO)Ac = *tert*-butylphenoxyacetyl
CGE = Capillary Gel Electrophoresis
DMAP = 4-(dimethylamino)pyridine
dmf = *N,N*-dimethylformamidine
DMT = 4,4′-dimethoxytrityl
EDA = 1,2-ethanediamine
FPLC = Fast Protein Liquid Chromatography
iBu = isobutyryl
MPO = methylphosphonate oligodeoxynucleotide
OCEt = 2-cyanoethoxy
TCA = trichloroacetic acid
TEAA = triethylammonium acetate
Tris = 2-amino-2-(hydroxymethyl)-1,3-propanediol

Results and discussion

It may be concluded from the prolonged hydrazine treatment (*i.e.* first step of procedure A in Table I) that methylphosphonate functions are compatible with hydrazinolysis. It has also been reported[20] that the same conditions are equally well suitable for the rapid removal of the dmf group from the purine base adenine. Furthermore, it was to be expected[13,21] that replacement of the N^4-benzoyl group in cytosine by the relatively less base-stable isobutyryl would have a beneficial effect on the rate of hydrazinolysis. Indeed, hydrazinolysis of 5′-*O*-(4,4′-dimethoxytrityl)-N^4-isobutyryl-2′-deoxycytidine went to completion in 6 h at 20°C (*cf.* removal of the N^4-benzoyl which required[22] 24 h) to give 5′-*O*-(4,4′-dimethoxytrityl)-2′-deoxycytidine, which was in every respect (*i.e.* NMR spectroscopy as well as mass spectrometry) identical with the same deoxycytidine derivative obtained after ammonolysis of the starting compound.

In order to evaluate the viability of the iBu-dmf protective-group strategy, we prepared the three pentamers **4** (*i.e.* dTTB$_P$(Me)TT: B = C, A or G) using an automated Gene Assembler and controlled-pore glass as the solid support. The requisite methylphosphonamidites **1a,d,e** (R = CH_3) were readily accessible by *sym*-collidine-HCl-mediated condensation of the 5′-*O*-DMT protected derivatives of A^{dmf}, G^{dmf} and C^{iBu} with bis(*N*,*N*-diisopropylamino)methylphosphine. Thus, work-up and purification of the crude products by silica-gel chromatography led to the isolation of the phosphonamidites **1a,d,e** (R = CH_3), the homogeneity and identity of which was firmly established by NMR spectroscopy and mass spectrometry. In the first place, the assembly of the fully protected and immobilized pentamer **2** (B = C^{iBu}) was executed by stepwise elongation of immobilized (3′-*O*-succinyl linkage) thymidine with the phosphoramidite **1** (B = T; R = 2-cyanoethoxy) and phosphoramidite **1a** (R = Me) according to the protocol summarized in Table II. The efficacy of each elongation step was greater than 97%, as gauged spectrophotometrically by the released DMT cation. Hydrazinolysis of immobilized pentamer **2** (B = C^{iBu}) gave, after washing of the polymer with acetonitrile, the N-deprotected pentamer **3** (B = C) still attached to the solid support. Cleavage of the 3′-*O*-succinyl bond and elimination of the cyanoethyl groups in **3** with ammonia in methanol afforded crude pentamer **4** (B = C). Analysis (see Figure 1) of **4** (B = C) by fast protein liquid chromatography (FPLC) and capillary gel electrophoresis (CGE) indicated that both elongation and deblocking steps proceeded with a high degree of efficiency. Purification of the crude pentamer by reversed phase liquid chromatography yielded homogeneous **4** (B = C), as evidenced by NMR spectroscopy (see Figures 2 and 3) and mass spectrometry.

Table I Procedures thus far reported for the removal of exocyclic amino protective groups X, Y and Z from immobilized DNA fragments containing ethylphosphonate linkages.

Combination of protective groups			Deblocking procedure [a]
X	Y	Z	
Bz	Bz	iBu	A[11] or B[12]
iBu	Bz	iBu	C[13]
tBu(phO)Ac	tBu(PhO)Ac	tBu(PhO)Ac	D[14]
AMB	AMB	AMB	E[15]

[a] A: NH_2NH_2 (0.5M) in pyridine/AcOH (4:1) 24 h at 20°C, then EDA/EtOH (1:1) 6 h at 20°C; B: NH_4OH 2 h at 20°C, then EDA/EtOH (1:1) 6 h 20°C; C: EDA/CH_3CN/NH_4OH (45:45:10) 6 h at 20°C; D: EtOH/NH_3 2 h at 20°C; E: MeOH/NH_3 16 h at 20°C.

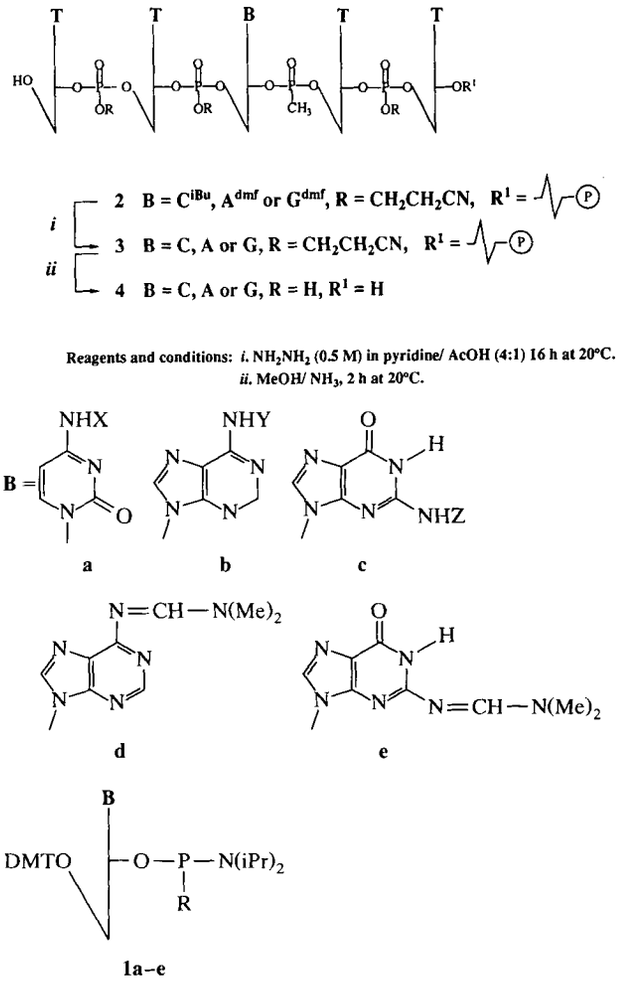

Table II Chemical steps involved in each elongation cycle [a].

Step	Manipulation	Solvents and reagents	Time (min)
1	Detritylation	2% TCA in DCE	2.5
2	Coupling	**1** [b] (B = T, R = OCEt), 1*H*-tetrazole [c], CH_3CN	3.0
		or **1** [b]**a,d,e** (R = CH_3), 1*H*-tetrazole [c], CH_3CN	6.0
3	Oxidation	0.02M I_2/CH_3CN/*sym*-collidine/H_2O, 11/1/5 (v/v/v)	1.0
4	Capping	0.25M DMAP in Ac_2O/*sym*-collidine/CH_3CN, 1/1/8 (v/v/v)	1.2

[a] Reactions were performed on 10 mg (0.2 μmol) of resin (loading 20 μmol/g). [b] 0.1M **1** (0.05 ml) in CH_3CN. [c] 0.5M 1*H*-tetrazole (0.2 ml) in CH_3CN. [a] When reactions were performed on 100 mg (5 μmol) of resin (loading 50 μmol/g), then 0.1M of **1** (0.25 ml) in CH_3CN and 0.5M 1*H*-tetrazole (0.4 ml) in CH_3CN were used in the coupling step.

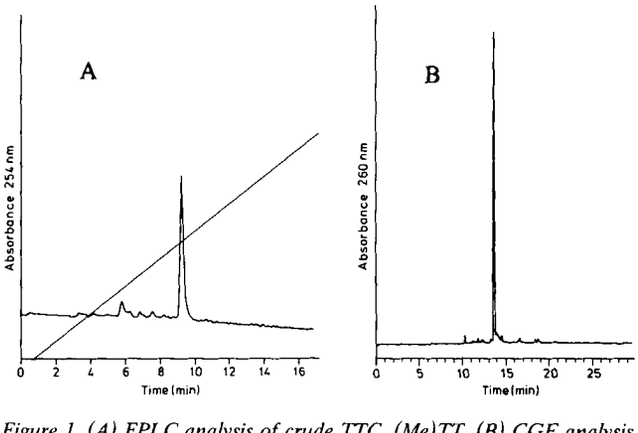

Figure 1. (A) FPLC analysis of crude TTC$_P$(Me)TT. (B) CGE analysis of crude TTC$_P$(Me)TT.

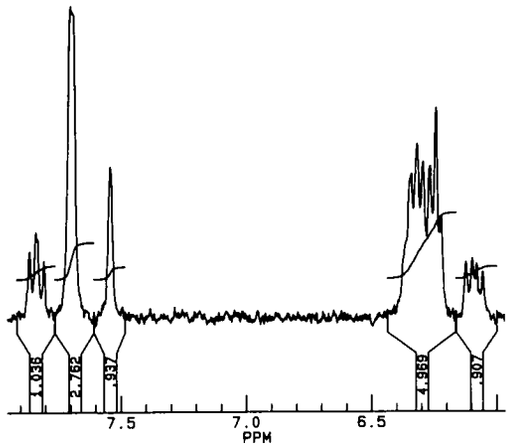

Figure 2. Part of the ^1H NMR (D_2O, pD 6.5–7.0) spectrum of purified $TTC_p(Me)TT$.

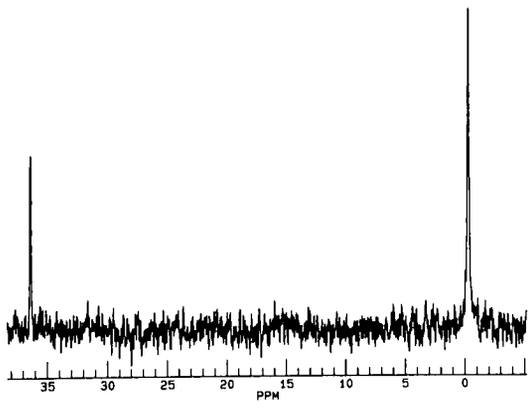

Figure 3. ^{31}NMR spectrum of purified $TTC_p(Me)TT$.

The potency of the approach was further illustrated by the successful solid-phase preparation of the heptamer $TC_p(Me)TC_p(Me)TC_p(Me)T$ containing three methylphosphonate linkages. Thus, FPLC analysis of the crude heptamer resulting after hydrazinolysis followed by short ammonolysis revealed the presence of mainly one product, the purification of which led to the isolation of the target heptamer. In this respect it is also of interest to note that the removal of the N^4-isobutyryl and 2-cyanoethyl groups as well as the cleavage of the succinyl linkage can be realized, in analogy with the removal of the AMB groups[15], in one step with ammonia in methanol. The latter is indeed substantiated by the FPLC pattern (see Figure 4) of the resulting crude product, and the fact that the purified compound comigrated with the modified

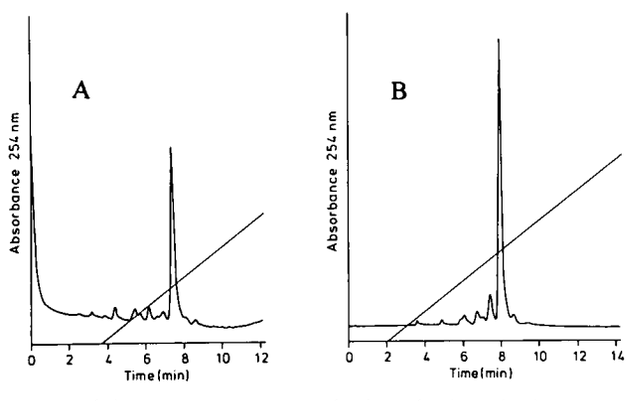

Figure 4. (A) FPLC analysis of $TC_p(Me)TC_p(Me)TC_p(Me)T$ obtained after deprotection with hydrazine and (B) after deprotection with NH_3 / MeOH.

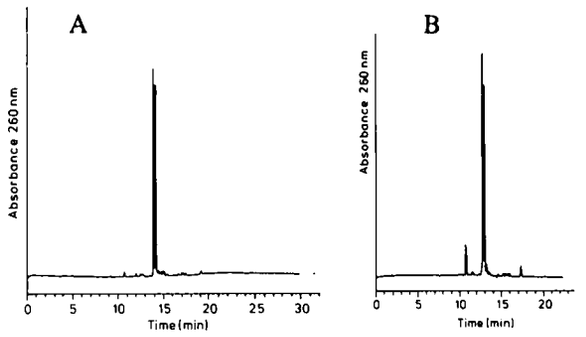

Figure 5. (A) CGE analysis of crude $TTA_p(Me)TT$. (B) CGE analysis of crude $TTG_p(Me)TT$.

heptamer obtained via the two-step procedure mentioned above.

At this stage, the applicability of the *N,N*-dimethylformamidine-protected methylphosphonamidites **1d,e** was assessed in the preparation of the two pentamers **4** (B = A or G) following the same protocol as used for the synthesis of pentamer **4** (B = C). FPLC and CGE analysis of the individual pentamers, resulting from the two-step deprotection of **3** ($B = A^{dmf}$ or G^{dmf}), clearly showed that the assembly and deprotection proceeded as expected. Thus, CGE analysis showed in each case (see Figure 5) the presence of the expected two diastereomers of the methylphosphonate function in the pentamers **4** (B = A or G). Furthermore, it was established by FPLC and CGE analysis that the pentamers **4** (B = A or G), prepared in a similar fashion starting from the N-AMB-protected methylphosphonamidites **1b,c** (Y = Z = AMB), were indistinguishable from the same two pentamers mentioned above. In conclusion, the easily accessible methylphosphonamidites **1a,d,e** show great promise for the future synthesis of well-defined DNA fragments having methylphosphonate linkages at predetermined locations.

Experimental

Materials and methods

Hydrazine monohydrate 98% was purchased from Aldrich. 4,4′-Dimethoxytrityl chloride and *N,N*-dimethylformamide dimethyl acetal were obtained from Janssen. 5′-*O*-(4,4′-Dimethoxytrityl)-N^2-dimethylformamidine-2′-deoxyguanosine, 5′-*O*-(4,4′-dimethoxytrityl)-N^6-dimethylformamidine-2′-deoxyadenosine, 5′-*O*(4,4′-dimethoxytrityl)-N^4-isobutyryl-2′-deoxy-cytidine and their 3′-(2-cyanoethyl-*N,N*-diisopropyl)phosphoramidites were prepared according to published procedures[16,17]. Fully protected 3′-polymer-supported 2′-deoxynucleosides were prepared[24] by succinylation (50°C) of the suitably protected nucleosides followed by condensation with aminopropyl-CPG (30-45 mesh, pore size 375A). Schleicher and Schull DC Fertigfolien F1500 LS254 were used for TLC.

Synthesis of the oligonucleotides on the solid-support was performed using the Gene Assembler (Pharmacia, Uppsala Sweden). FPLC analysis was carried out on a Pharmacia LCC-500 liquid chromatograph equipped with a gradient mixing system, UV-absorption detector (254 nm) and a photometer output recorder. Pre-packed strong anion-exchange resin Mono-Q HR 5/5 (Pharmacia) was used. Gradient elution was performed at 20°C by building up a gradient starting with buffer A (0.01M NaOH, pH 12.0) and applying buffer B (0.01M NaOH, 1.2M NaCl, pH 12.0) with a flow rate of 2.0 ml/min and a pressure of 3.0 MPa. The gradient used was a linear gradient (system 1) starting from pure buffer A (1.0 min) and applying 4% buffer B each min.

Capillary gel electrophoresis (CGE) was carried out on an Applied Biosystems 270A Capillary electrophoresis system using a column of Micro-Gel_{100}, gel filled capillary, diameter 50 μm, length 50 cm. Elution was performed with Micro-Gel_{100} buffer (75 mM Tris-phosphate, 10% methanol, pH 7.6) at 30°C and a voltage of 300 V/cm. For detection a variable-wavelength detector was used operating at 260 nM.

Pharmacia FPLC-system equipment with a preparative reversed phase C-18 column was used for purification of $TTC_P(Me)TT$. Gradient elution was performed by building up a linear gradient starting from buffer A [0.1M triethylammonium acetate (TEAA) in water] and applying 2% per min of buffer B (0.1M TEAA in water and 90% acetonitrile).
^{31}P-, ^{13}C- and ^{1}H-NMR spectra were measured at 80.7, 50.1 and 200 MHz, respectively, using a JEOL JNM-FX 200 spectrometer equipped with a PG 200 computer and operating in the Fourier-transform mode. ^{31}P- and ^{1}H-NMR were also measured using the Bruker WM-300 (300 MHz) spectrometer, equipped with an ASPECT-2000 computer operating in the Fourier-transform mode. Chemical shifts are given in ppm (δ) relative to tetramethylsilane (TMS) for ^{13}C and ^{1}H-NMR spectra and 85% H_3PO_4 as an external reference for ^{31}P-NMR spectra.
Mass spectra (LCMS) were recorded on a Finnigan MAT SSQ 710 mass spectrometer equipped with an electrospray interface operating in a positive ion-mode.

Synthesis of bis(N,N-diisopropylamino)methylphosphine

To a stirred and cooled (ethanol/liquid-N_2 bath) solution of chloro-bis(*N*,*N*-diisopropylamino)phosphine[23] (13.33 g, 50.0 mmol) in dry diethyl ether (75 ml) under a nitrogen atmosphere was added dropwise a solution of CH_3MgBr (16.7 ml, 3.0M in diethyl ether). After stirring for 30 min, the reaction mixture was slowly warmed up and stirred for 1 h at 20°C. The salts were removed by filtration under anhydrous conditions and the filtrate was concentrated *in vacuo*. The residual crude oil was distilled under reduced pressure. Yield 68%, based on chloro-bis(*N*,*N*-diisopropylamino)phosphine. B.p. 77–78°C at 0.8 mmHg. ^{31}P NMR (CH_2Cl_2): 39.7 ppm.

*5'-O-(4,4'-dimethoxytrityl)-N^2-dimethylformamidine-2'-deoxyguanosine-3'-(N,N-diisopropyl)methylphosphonamidite (**1e**)*

5'-*O*-(4,4'-Dimethoxytrityl)-N^2-dimethylformamidine-2'-deoxyguanosine (2.0 mmol, 1.25 g) was dissolved in dry dioxane (20 ml), evaporated to dryness and then redissolved in CH_2Cl_2 (20 ml). To this solution was added bis(diisopropylamino)methylphosphine (4.0 mmol, 0.98 g) and *sym*-collidine-HCl (0.4 mmol, 64 mg). The reaction mixture was stirred overnight at 20°C. The mixture was diluted with CH_2Cl_2 (100 ml), washed with sodium bicarbonate (1M, 75 ml) containing triethylamine (1.0 ml), NaCl solution (1M, 75 ml) containing triethylamine (1.0 ml), dried over Na_2SO_4 and concentrated to dryness. The crude amidite was purified by flash column chromatography using ethyl acetate triethylamine (98/2, v/v) and applying a methanol gradient (0–5%) as the eluent. Yield 80% (1.6 mmol, 1.23 g). R_f 0.18 (EtOAc/MeOH/Et_3N 96/2/2, v/v/v) ^{31}P NMR (CH_2Cl_2): 122.5 and 120.1 ppm.
^{1}H NMR ($CDCl_3$): 8.60 and 8.58 (d, 1H, N = CHN, dmf); 7.72 and 7.70 (d, 1H, H-8, dG); 7.44-6.78 (13H, DMT); 6.32 (q, 1H, H-1'; dG); 4.70-4.45 (2m, 1H, H-3', dG); 4.19 (d, 1H, H-4', dG); 3.78 and 3.71 (2s, 6H, OMe, DMT); 3.51-3.25 (2m, 4H, H-5', H-5" and 2CH of isopropyl); 3.15-3.08 (2d, 6H, N-Me, dmf); 2.61-2.40 (2m, 2H, H-2' and H-2", dG); 1.76-1.72 (dd, 3H, PMe); 1.25-1.04 (12H, 4Me of isopropyl).
^{13}C NMR ($CDCl_3$): 86.43 (C-DMT); 85.99 and 85.91 (C-1'); 83.25 and 83.04 (C-4'); 75.92 and 75.69 (C-3'); 64.04 and 63.80 (C-5'); 55.22 (OMe, DMT); 44.36, 44.27, 44.18 and 44.09 (CH, isopropyl); 41.23 (Me, dmf); 40.09 (C-2'); 35.13 (Me, dmf); 24.70, 24.62, 24.15, 24.00 and 18.22, 17.99 (Me of isopropyl and PMe). Mass spectrum of **1e** recorded in a positive ion-mode, confirmed a molecular weight of 769 as expected for $C_{41}H_{53}N_7O_6P$.

*5'-O-(4,4'-dimethoxytrityl)-N^6-(dimethylformamidine)-2'-deoxyadenosine-3'-(N,N-diisopropyl)methylphosphonamidite (**1d**)*

Compound **1d** was prepared as described above for the deoxyguanosine derivative **1e**. Yield 83% (1.66 mmol, 1.25 g). R_f 0.23 (EtOAc/MeOH/Et_3N 96/2/2, v/v/v). ^{31}P NMR (CH_2Cl_2): 121.5 and 119.8 ppm. ^{1}H NMR ($CDCl_3$): 8.93 (s, 1H, N = CHN, dmf); 8.51 (d, 1H, H-8, dA); 8.09 (d, 1H, H-2, dA); 7.44-6.76 (13H, DMT); 6.51-6.45 (dd, 1H, H-1', dA); 4.60 (m, 1H, H-3', dA); 4.25-4.23 (m, 1H, H-4', dA); 3.77 and 3.67 (2s, 6H, OMe, DMT); 3.56-3.31 (4H, H-5', H-5" and 2CH isopropyl); 3.25 and 3.18 (2s, 6H, NMe, dmf); 2.83-2.47 (1m and 2ddd, 2H, H-2' and H-2", dA); 1.27-1.05 (15H, PMe and 4Me isopropyl).
^{13}C NMR ($CDCl_3$): 86.20 (C-DMT); 85.88 (broad, C-1'); 84.12 and 83.89 (C-4'); 75.94 and 75.72 (C-3'); 63.68 (broad, C-5'); 54.93 (OMe, DMT); 44.03 and 43.83 (CH, isopropyl); 40.97 (Me, dmf); 39.74 (C-2'); 34.87 (Me, dmf); 24.41, 23.91 and 18.25 and (Me of isopropyl and PMe). Mass spectrum of **1d** recorded in a positive-ion mode, confirmed a molecular weight of 753 as expected for $C_{41}H_{53}N_7O_5P$.

*5'-O-(4,4'-dimethoxytrityl)-N^4-isobutyryl-2'-deoxycytidine-3'-(N,N-diisopropyl)methylphosphonamidite (**1a**)*

Compound **1a** was prepared as described above for the deoxyguanosine derivative **1e**. Yield 78% (1.56 mmol, 1.16 g). R_f 0.66 (EtOAc/Et_3N 98/2, v/v). ^{31}P NMR ($CDCl_3$): 118.9 and 117.4 ppm. ^{13}C NMR ($CDCl_3$): 86.43 (C-1'); 86.08 (C-DMT); 85.55 and 85.47 (C-4'); 74.66, 74.43, 73.82 and 73.47 (C-3'); 62.40 and 61.61 (C-5'); 55.01 (OMe, DMT); 44.50, 44.39, 43.57 and 43.39 (CH, isopropyl); 40.79 (broad, C-2'); 35.45 (CH, iBu); 23.86, 23.42, 22.25, 21.99, 18.46, 18.19, 17.44 and 17.20 (Me of iBu, isopropyl and PMe).
Mass spectrum of **1a** recorded in a positive-ion mode, confirmed a molecular weight of 744 as expected for $C_{41}H_{53}N_4O_7P$.

Synthesis of deoxyoligonucleotides on a polymer support

The polymer-supported synthesis was performed on a fully automated synthesizer (Pharmacia, Gene Assembler) using 2'-deoxythymidine 3'-(2-cyanoethyl-*N*,*N*-diisopropylphosphoroamidites) **1** (B = T, R = OCEt), and the 2'-deoxynucleosides 3'-(*N*,*N*-diisopropylmethylphosphonoamidites) **1a,d,e**, (R = CH_3) synthesized above. Controlled-pore glass (CPG-AP, 100 mg), covalently linked to the appropriate nucleoside (loading 50 μmol/g or 20 μmol/g), was used as solid support. The individual steps of one complete elongation cycle are depicted in Table II and, after each step, the column was washed to remove impurities and excess reagents. In the last step the 5'-DMT group was removed (see step 1 in Table II) and the solid support was washed with CH_3CN (12.5 ml) for 5 min.

Deprotection and purification

The resin-bound modified oligodeoxynucleotide (0.2 μmol) was added to hydrazine hydrate (0.5M in pyridine/acetic acid, 4/1, v/v, 1ml) and the mixture was kept 16 h at 20°C. After washing with dry acetonitrile, 1 ml saturated solution of NH_3 in methanol was added, and the mixture was left for 2 h at 20°C. The support was removed by centrifugation and the resulting solution was lyophilized.
The crude unprotected DNA fragment was purified by applying it on a preparative reversed phase C-18 column. Gradient elution was performed by building up a linear gradient starting from buffer A (0.1M TEAA in water) and applying 2% per min buffer B (0.1M TEAA in water and 90% acetonitrile).
The appropriate fractions were pooled, concentrated to small volume, analyzed by FPLC analysis and lyophilized. The pure product was converted into the Na^+ form by passing it through a column of Dowex 50W X4 cation-exchange resin (100–200 mesh, Na^+ form). The resulting UV-positive fractions were pooled, concentrated and relyophilized.
Methylphosphonate containing DNA fragments can also be purified on a Q-Sepharose Hiload™ 16/10 (Pharmacia) pre-packed, strong anion-exchange resin. The column was eluted with buffer A (0.01M NaOH, pH 12) and a gradient was build by addition of buffer B (0.01M NaOH containing 1.2M NaCl). Elution was performed as follows: 16 min and 40 sec pure buffer A (0% B); in 66 min and 40 sec 0–20%; 83 min and 20 sec 20–70%; 1 min, 70–100%. Flow rate was 3 ml per min. After purification, each fraction was neutralized with acetic acid and the combined fractions were lyophilized. The purified DNA fragment was then freed from salts by passing it through a Superdex-75 Hiload™ 16/120 preparative-grade (Pharmacia) gel filtration column which was suspended and eluted with 0.15M TEAB in 10% methanol in water. The flow rate was 1.5 ml per min. The conversion into the sodium form was performed as described above for $TTC_P(Me)TT$.

Pentamer $TTC_P(Me)TT$. ^{31}P NMR (D_2O): 36.38, 36.30 and −0.34 ppm.
^{1}H NMR 300 MHz (D_2O): 7.83 (dd, 1H, C-6, dC); 7.69 (d, 3H, 3H-6, dT); 7.54 (d, 1H, H-6, dT); 6.30 (m, 5H, 5H-1'); 6.09 (dd, 1H, H-5, dC); 5.13-3.76 (20H, 5H-3', 5H-4', 5H-5' and 5H-5"); 2.68-2.32 (2m, 10H, 5H-2' and 5H-2"); 1.89-1.82 (m, 12H, 4Me, dT); 1.73-1.66 (dd, 3H, PMe). Mass spectrum recorded in a positive-ion mode, confirmed a molecular weight of 1507 as expected for $C_{50}H_{64}Na_3N_{11}O_{31}P_4$. FPLC Rt 9.2 min (mono Q, system 1). CE Rt 13.6 min.

Pentamer $TTA_P(Me)TT$: FPLC Rt 9.1 min (mono Q, system 1). CE Rt 14.0 min.

Pentamer $TTG_P(Me)TT$: FPLC Rt 10.7 min (mono Q, system 1). CE RT 12.8 min.

Heptamer $TC_P(Me)TC_P(Me)TC_P(Me)T$: FPLC Rt 7.9 min. (mono Q, system 1).

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