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Solid-phase synthesis of oligodeoxynucleotides containing 4-alkoxythymidine residues[1]

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Abstract. Immobilized and fully protected oligodeoxynucleotides containing a 4-(1,2,4-triazolyl)-thymidine residue at a predetermined position were prepared according to a well-established phosphite triester methodology using 2-cyanoethyl phosphoramidites of a 4-(1,2,4-triazolyl)-substituted thymidine and standard protected nucleosides. Treatment of the immobilized oligomer with methanol, ethanol or *n*-propanol in the presence of DBU at 50 °C gave the corresponding oligonucleotides containing 4-methoxy, 4-ethoxy- or 4-*n*-propoxythymidine residue.

Abbreviations

A = adenin-9-yl
Ac = acetyl
alkoxyT = 4-alkoxythymine
Bz = benzoyl
C = cytosin-1-yl
CE = 2-cyanoethyl
sym-collidine = 2,4,6-trimethylpyridine
CPG = controlled-pore glass
CPG-AP = 3-aminopropyl-protected CPG
DBU = 1,8-diaza[5.4.0]undec-7-ene
DCC = dicyclohexylcarbodiimide
DCE = 1,2-dichloroethane
DIPEA = diisopropylethylamine
DMAP = 4-(dimethylamino)pyridine
DMT = 4,4′-dimethoxytrityl
= bis(4-methoxyphenyl)phenylmethyl
FPLC = Fast Protein Liquid Chromatography
G = guanin-9-yl
HOBt = 1-hydroxy-1*H*-benzotriazole
HPLC = High-Performance Liquid Chromatography
iBu = isobutyryl
= 2-methylpropanoyl
NMI = *N*-methyl-1*H*-imidazole
T = thymin-1-yl
T^{alk} = 4-alkoxythymidine
T^{Tr} = 4-(1,2,4-triazol-1-yl)thymidine
TCA = trichloroacetic acid
TEAB = triethylammonium bicarbonate
TLC = Thin-Layer Chromatography
TMS = tetramethylsilane
Tr = 1,2,4-triazol-1-yl

Introduction

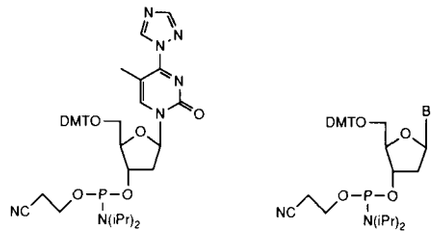
4-Alkoxythymine (alkoxyT) adducts are one of the many possible lesions that are generated by the reaction of alkylating reagents with various nucleophilic sites present in DNA[2,3]. *In-vitro* experiments[4,5] showed that alkoxyT residues can be considered as strong miscoding bases[2-4] predominantly resulting in T → C transitions. However, the effect of these adducts on *in-vivo* mutagenicity has been studied less extensively[6,7]. Furthermore, there is increasing evidence that their persistency and probably also their mutagenicity in mammalian cells varies considerably with the size of the alkyl group[8,9]. The latter aspect was recently explored using the synthetic dT^{Et}-containing DNA fragment AATTCAT^{Et}CGATATCTA for incorporation into a shuttle vector system in *Hela* cells. It was demonstrated[10] that, after replication of the vector, an average percentage of 23% of the progeny molecules possessed a mutation in the ethylT region.

We report here in detail the preparation of the modified DNA fragments, *i.e.*, hexamer CGT*ACG and hexadecamer AATTCAT*CGATATCTA (T* stands for a T^{Me}-, T^{Et}- or T^{Pr} residue) via a solid-phase approach using 4-(1,2,4-triazolyl)thymidine 2-cyanoethyl phosphoramidite **1** and the standard protected nucleoside 2-cyanoethyl phosphoramidites **2**.

Results and discussion

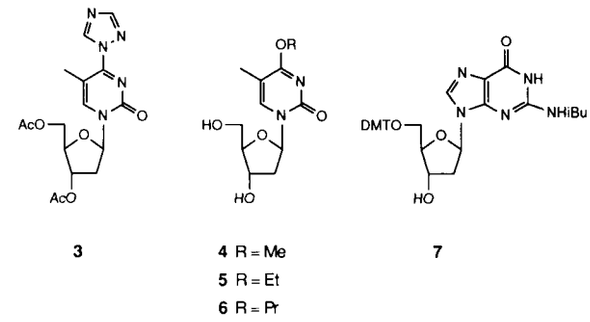
In 1987, *Li* et al.[11] showed that replacement of the 4-(3-nitro-1,2,4-triazolyl) function in a properly protected thymidine derivative by a methoxy group could be realized (10 min at −5 °C) with the methoxide ion generated *in situ* from methanol and DBU. In addition, it was also established that removal of *N*-acyl protecting groups (*i.e.*, N^4- or

N^6-benzoyl and N^2-acetyl from the deoxynucleobases cytosine or adeine and guanine) could be affected (60 h, 20°C) under the same basic conditions (DBU/MeOH). Shortly before this, *Webb* et al.[12,13] reported that the 4-(1,2,4-triazolyl)thymidine amidite **1** could be applied as a synthon in a solid-phase synthesis of modified DNA fragments. On the basis of this information, we were anxious to find out whether solid-support synthesis of DNA fragments containing a alkoxythymidine residue could be concluded successfully via the following two-stage process. Thus it was to be expected that the first stage, which implies a solid-support assembly of an immobilized DNA fragment bearing a 4-(1,2,4-triazolyl)thymidine (T^{Tr}) unit (*e.g.*, immobilized fragment **9** in Scheme 1), could be realized without any difficulties using the T^{Tr} amidite **1** and the commercially available d-nucleoside amidites **2**[14]. However, the expected outcome of the second stage, which entails treatment of the immobilized T^{Tr}-containing fragment with the respective alcohol in the presence of DBU to give the corresponding

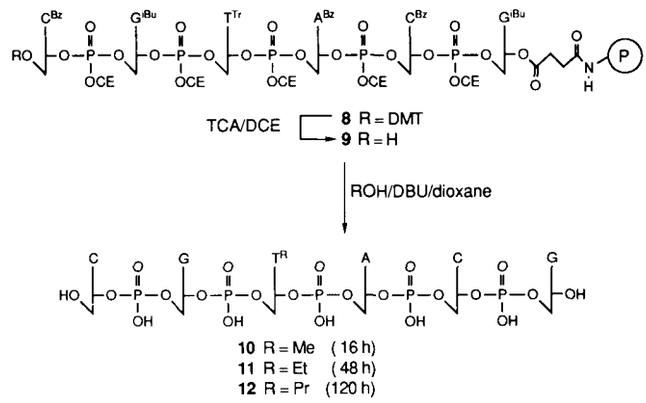


4-alkoxythymidine-containing oligonucleotides (*e.g.*, fragments **10–12** in Scheme 1), is far less clear. Thus, it is not *a priori* evident whether the crucial substitution of the 4-(1,2,4-triazolyl) function by methoxide, ethoxide or *n*-propoxide ions will proceed quantitatively and with an acceptable rate. The same holds for the removal of the *N*-acyl protecting groups (especially the rather base-stable N^2-isobutyryl group[15]) and the release (cleavage of the succinyl ester bond) of the modified fragments from the solid support. In order to evaluate these factors in more detail, the model compound **3**, prepared by triazolation of 3′,5′-di-*O*--acetyl-2′-deoxythymidine[16] according to the procedure of *Reese* et al.[17], was first subjected to the action of methanol/DBU/dioxane (9/2/9, v/v/v) at 50°C. Monitoring of the process by TLC revealed that the substitution and deacetylation reactions were complete within 5 and 20 min, respectively. Similarly, substitution of **3** with ethoxide or *n*-propoxide ions was completed in 5 and 15 min, respectively. On the other hand, fully deacetylation of the intermediate 4-OEt(OPr)-thymidine derivative proceeded rather slowly (*i.e.*, 2 and 5 h, respectively). Work-up and purification of the products resulting from individual alcohol/DBU treatment gave the 4-alkoxythymidines **4–6** in 78–85% yield. Furthermore, TLC analysis showed that complete *N*-deacylation of the isobutyryl group from the d-guanosine derivative **7** could be effected by treating it with methoxide, ethoxide and *n*-propoxide ions for 5, 8 and 30 h, respectively, to give exlusively 5′-*O*-(4,4′-dimethoxytrityl)-2′--deoxyguanosine in an excellent yield.

The above results clearly indicate that the reliable and fast conversion of 4-(1,2,4-triazolyl)thymidine into 4-alkoxythymidines (*i.e.*, **4–6**) by the action of the appropriate alkoxides can be adopted for the two-stage solid-phase approach. However, it remains to be seen whether the same holds for the observed alkoxide-dependent decrease in the rate of *O*- and *N*-deacylation.



In order to substantiate the viability of the solid-support concept, it was decided first to prepare the hexamer $CGT^{Me}ACG$ (*i.e.*, **10** in Scheme 1) using an automated Gene Assembler (Pharmacia) and the amidites **1** and **2** as



Scheme 1

incoming d-nucleoside synthons. Thus, stepwise extension of a 2′-deoxyguanosine unit, linked by a 3′-*O*-succinyl bond to controlled pore glass (CPG), was carried out according to the standard protocol summarized in Table I. The individual coupling steps proceeded, as gauged by spectrophotometric determination of the released DMT cation, with a coupling efficiency of 98% throughout the whole elongation process.

After the last elongation cycle, the fully protected and immobilized hexamer **8** was detritylated with trichloroacetic acid in 1,2-dichloroethane (see step 1 in Table I) to **9**, which was washed with acetonitrile for 5 min and treated with MeOH/DBU/dioxane (9/2/9, v/v/v) at 50°C under strictly anhydrous conditions. Monitoring of the process by anion-exchange FPLC analysis indicated that the reaction was complete after 16 h and that the reaction mixture contained mainly one product (hexamer **10** as shown in Figure 1, pattern a). In addition, FPLC analysis of the mixture obtained by subjecting the controlled pore glass, isolated after the MeOH/DBU treatment, to aqueous ammonia (25%) did not reveal residual deoxynucleic acid components, thus confirming complete release (*O*-desuccinylation) of **10** from the solid support.

In a similar fashion, crude hexamers $CGT^{Et}ACG$ (**11**) and $CGT^{Pr}ACG$ (**12**) were obtained (see Figure 1 patterns b and c) by treating **9** with ethanol or *n*-propanol/DBU at 50°C for 48 and 120 h, respectively. Purification of the crude oligomers by gel filtration and anion-exchange chromatography gave homogeneous **10–12**, the identity of which was confirmed by ^1H NMR spectroscopy.

The scope of the solid-phase approach was further illustrated by the preparation of the three hexadecamers 5′-d(AATTCAT*CGATATCTA)-3′ (**13**, T* = T^{Me}; **14**,

Table I Chemical steps involved in each elongation cycle.

Step	Manipulation	Solvents and reagents[a]	Time (min)
1	detritylation	2% TCA in DCE	2.5
2	coupling	**1**[b] or **2**[b], 1*H*-tetrazole[c], MeCN	3.0
3	oxidation	0.02M I_2 in MeCN/*sym*-collidine/H_2O, 11:1:5 (v/v/v)	1.0
4	capping	0.25M DMAP in Ac_2O/*sym*-collidine/MeCN, 1:1:8 (v/v/v)	1.2

[a] Reactions were performed on 200 mg (10 μmol) of resin. [b] 0.1M amidite (0.4 ml) in MeCN. [c] 0.5M 1*H*-tetrazole (0.6 ml) in MeCN.

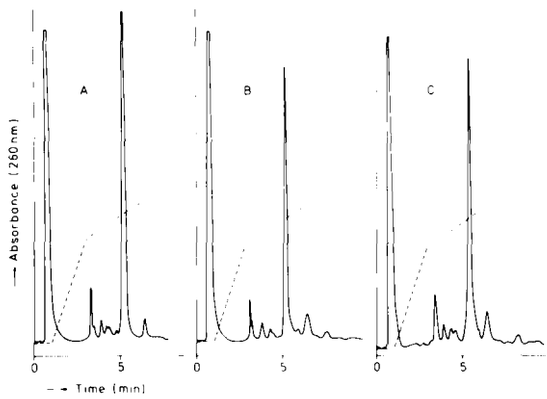


Figure 1. Chromatography profiles (FPLC) of crude hexamers: (A) **10**, *(B)* **11** *and (C)* **12**.

T* = T^{Et} and **15**, T* = T^{Pr}). Thus, treatment of the immobilized T^{Tr}-containing hexadecamer with the respective alkoxides under the same conditions as mentioned above for the preparation of the modified hexamers **10–12** gave, after purification, the required oligomers **13–15** (see further, Table II). The identity and homogenity of the hexadecamers was ascertained by quantitative analysis of the d-nucleosides released after enzymatic hydrolysis (see Figure 2). Quantitation of the separate digestion products showed the presence of the modified and natural d-nucleosides in the expected molar ratios (see Table II).

Thus, it has been shown that the amidites **1** and **2** can be applied in a solid-phase phosphite triester methodology to give, in contrast with an earlier report[18], high-quality DNA fragments bearing either 4-methoxy, 4-ethoxy or 4-*n*-propoxythymine moieties. It was also illustrated that the rate of N^2-deisobutyrylation, in the final stage of the synthesis, with an appropriate linear alcohol in the presence of DBU, decreased rapidly in the order $CH_3OH < C_2H_5OH < C_3H_7OH$. The latter feature may prohibit future synthesis of DNA fragments containing 4-*O*-$[CH_3(CH_2)_{n>2}]$-thymidine units. However, it is expected that this drawback may be overcome by using the recently proposed[19] more base-labile phenoxyacetyl group for protection of the exocyclic N^2-amino function of the guanosine base.

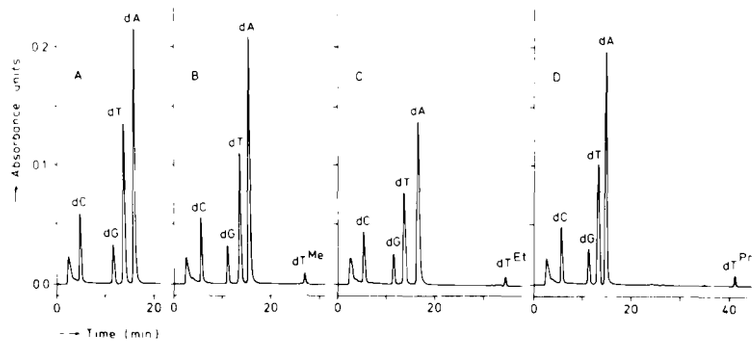


Figure 2. Chromatography profiles (HPLC) of the d-nucleosides and modified nucleosides resulting after enzymatic digestion of natural (i.e., **16**) *and modified* (i.e., **13–15**) *oligonucleotides . Hydrolysate of: (A)* **16**, *(B)* **13**, *(C)* **14** *and (D)* **15**.

Table II Relevant data on synthesis and enzymatic digestions of oligodeoxynucleotides **13–16**.

Oligomer	Yield[a] in mg (%)	Retention[b] time (min)	Base composition[c]						
			A	C	G	T	T^{Me}	T^{Et}	T^{Pr}
13 AATTCAT^{Me}CGATATCTA	8.0 (15)	9.6	6.10	3.10	1.00	5.11	1.04		
14 AATTCAT^{Et}CGATATCTA	9.1 (18)	9.8	5.93	3.04	1.00	4.88		1.06	
15 AATTCAT^{Pr}CGATATCTA	7.9 (15)	10.2	5.85	2.96	1.00	4.83			1.01
16 AATTCATCGATATCTA	19.2 (37)	9.5	6.12	2.89	1.00	6.09			

[a] Yields of the purified oligonucleotides are based on 10 μmol resin. [b] FPLC conditions are described in materials and methods. [c] HPLC conditions are described in materials and methods.

Experimental procedures

Materials and methods

Pyridine, dioxane and triethylamine were dried by refluxing with calcium hydride (CaH_2, 5 g/l) for 16 h and then distilled. Dioxane was redistilled from $LiAlH_4$ (5 g/l). Toluene and dichloromethane (CH_2Cl_2) were dried by heating under reflux with P_2O_5 (30 g/l) for 2 h and then distilled. Pyridine, dioxane and CH_2Cl_2 were stored over molecular sieves 4 Å, 5 Å and 4 Å, respectively. Toluene was stored over sodium wire. Acetonitrile (MeCN, Rathburn HPLC-grade) was stored over molecular sieves 3 Å. For use on the Gene Assembler, special MeCN (Rathburn, HPLC-grade) was used and stored on molecular sieve rods (Fluka, Carbide Type 3 Å, 1/8″ rods). 1,2-Dichloroethane (DCE, Rathburn, HPLC-grade) was used and stored on molecular sieves 3 Å. Methanol (MeOH) was dried over methoxide, prepared *in situ* by iodine (catalytic) activated magnesium curls (5 g/l) in methanol (200 ml, p.a. Baker), refluxed for 1 h and distilled. In an analogous way, ethanol (EtOH, Baker) and *n*-propanol (PrOH, Baker) were dried from ethoxide and *n*-propoxide and distilled after reflux for 1 h. *N*-Methylimidazole (NMI, Aldrich) was dried by heating under reflux with CaH_2 (5 g/l) and distilled under reduced pressure. *N,N*-Diisopropylethylamine (DIPEA) was distilled from chlorosulfonic acid (5 g/l) and then redistilled from KOH pellets (20 g/l). 4,4′-Dimethoxytrityl chloride (DMT-Cl, Janssen), acetic acid anhydride (Ac_2O, Baker), dicyclohexylcarbodiimide (DCC, Merck), trichloroacetic acid (TCA, Baker), 4-(dimethylamino)pyridine (DMAP, Merck), iodine (I_2, Merck), *sym*-collidine (Fluka) and 1*H*-tetrazole (Janssen) were of pure analysis and used without further purification. 1-Hydroxybenzotriazole (HOBt) was purchased from Aldrich and dried *in vacuo* (P_2O_5) for 72 h at 50°C before use. Triethylammonium bicarbonate buffer (TEAB, 2M) was prepared by passing a stream of carbon dioxide (CO_2) gas through a cooled (ice–water bath) mixture of triethylamine (825 ml) and deionized water (2175 ml) until pH 7.0–7.5.

5′-*O*-(4,4′-Dimethoxytrityl)-2′-deoxynucleoside 3′-(2-cyanoethyl *N,N*-diisopropylphosphoramidites) [N^4-benzoylcytosin-1-yl (C^{Bz}); N^6-benzoyladenin-9-yl (A^{Bz}); N^2-isobutyrylguanin-9-yl (G^{iBu}) and thymin-1-yl (T)] (**2**) were obtained from Synorchem and used as 0.1M solutions in special dry MeCN. Compound **7** was prepared according to a published procedure[20].

Aminopropyl-derivatized controlled-pore glass (CPG-AP) was obtained as described before[21] using CPG-Silica carrier (30–45 mesh, pore size 375 Å), purchased from Fluka.

Schleicher and Schüll DC Fertigfolien F1500 LS254 were used for TLC analysis in solvent system A: CH_2Cl_2/CH_3OH (92/8, v/v), B: CH_2Cl_2/CH_3OH (90/10, v/v) or C: EtOAc/Et_3N (95/5, v/v), unless otherwise stated. Short-column chromatography was performed on Kieselgel 60 (230–400 mesh ASTM) suspended in CH_2Cl_2. Sephadex LH20 was purchased from Pharmacia (Uppsala, Sweden).

UV-absorption spectra were measured using a Cary C14 recording spectrophotometer.

^{31}P NMR spectra were measured at 80.7 MHz using a JEOL JNM-FX 200 spectrophotometer equipped with a PG 200 computer and operating in the Fourier-transform mode. Chemical shifts are given in ppm (δ) relative to 85% H_3PO_4 as external standard. ^1H NMR spectra were measured at 200 MHz as described above for ^{31}P NMR spectra or at 300 MHz using a Bruker WM-300 spectrometer, equipped with an ASPECT-2000 computer and operating in the Fourier-transform mode. ^{13}C NMR spectra were measured at 50.1 MHz as described above for ^{31}P NMR spectra. Chemical shifts for ^1H- and ^{13}C-NMR are given in ppm (δ) relative to TMS.

NMR samples of the unprotected oligonucleotides were made by lyophilizing the purified oligonucleotide (5–10 mg, sodium salt), after adjusting the pH to 6.8–7.0 (meter reading), twice from D_2O (99.75%) and then redissolved in D_2O (99.95%). Chemical shifts are given in ppm (δ) relative to sodium 3-(trimethylsilyl)propanesulfonate.

Gel filtration was performed on a Hiload 16/120 Superdex grade column using a 0.15M TEAB buffer as eluent with a flow rate of 1.0 ml/min.

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. Prepacked strong anion-exchange resin Hiload 16/10 Q Sepharose High Performance (Pharmacia) was used. Gradient elution was performed at 20°C 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 3.0 ml/min and a pressure of 3.0 MP. The gradient was as follows: 1 min at 0%, 2 min from 0 to 30%, 3 min from 30 to 40%, 4 min from 40 to 48%, 9 min from 48 to 57%, 5 min from 57 to 70% and 7.5 min from 70 to 100%.

Cation-exchange resin (sodium form) was prepared by passing 2M NaOH in water (100 ml) through a column (1.5 × 10 cm) packed with resin (Dowex 50W X4, 100–200 mesh, Fluka, H^+ form) followed by eluting the column with water until pH 7.0. Cation-exchange resin (pyridinium form) was prepared by treatment of resin (Dowex 50W X4, 100–200 mesh, Fluka, H^+ form) with pyridine/water (250 ml, 1/4, v/v) followed by washing the resin with water until pH 7.0.

(HPLC) analysis was carried out on a Waters 990 Photodiode Array Detector equipped with a NEC APC IV Powermate 2 Computer and a Waters 990 Plotter or NEC Pinwriter PC6.

4-(1,2,4-Triazol-1-yl)-5′-O-(4,4′-dimethoxytrityl)-2′-deoxythymidine 3′-(2-cyanoethyl N,N-*diisopropylphosphoramidite)* (**1**)[13]

A solution of **2** (B = T; 2.23 g, 3.00 mmol), dried by evaporation with dioxane (2 × 25 ml), in anhydrous MeCN (30 ml) was added to a freshly prepared solution of phosphoryl tristriazolide reagent[15] [$OP(C_2H_2N_3)_3$] (15.0 mmol) in MeCN (30 ml). After stirring for 3 h, the mixture was diluted with CH_2Cl_2 (100 ml) and poured into aqueous $NaHCO_3$ solution (50 ml, 5% w/v). The organic layer was separated, extracted with brine (50 ml), dried over $MgSO_4$ and concentrated to an oil. Crude **1** was dissolved in CH_2Cl_2 (5 ml) and purified by flash column chromatography (50 g). Elution with EtOAc/Et_3N (95/5, v/v) gave, after concentration *in vacuo*, pure **1** as a mixture of diastereomers in a yield of 2.24 g (2.81 mmol, 94%); R_f 0.53 and 0.48 (B). ^{31}P NMR (CH_2Cl_2, external D_2O lock): δ 150.3. ^1H NMR ($CDCl_3$): δ 9.28 (s, 1H, H-5, Tr), 8.37 and 8.34 (2 s, 1H, H-6), 8.08 (s, 1H, H-3, Tr), 7.42–6.81 (m, 13H, arom. H's, DMT), 6.33 (m, 1H, H-1′), 4.68 (m, 1H, H-3′), 4.26 (m, 1H, H-4′), 3.79 and 3.78 (2 s, 6H, OCH_3, DMT), 3.67–3.52 (m, 4H, H-5′, H-5″ and OCH_2, CE), 3.47–3.33 (m, 1H, H-2′ or H-2″), 2.91–2.76 (m, 1H, H-2′ or H-2″), 2.42 (m, 2H, CH_2CN, CE), 1.95 and 1.94 (2 s, 3H, CH_3, T), 1.29–1.05 (m, 14H, 2 CH and 4 CH_3, iPr).

*4-(1,2,4-Triazol-1-yl)-3′,5′-di-*O*-acetyl-2′-deoxythymidine* (**3**)

Ac_2O (2.84 ml, 30.0 mmol) was added to a solution of 2′-deoxythymidine (2.42 g, 10.0 mmol), dried by evaporation with pyridine, in pyridine (25 ml). After stirring for 2 h, MeOH (2.0 ml) was added to quench the reaction and the mixture was concentrated to dryness. The residue was dissolved in CH_2Cl_2 (100 ml) and washed with aqueous $NaHCO_3$ solution (50 ml, 10% w/v) and water (50 ml). The organic layer was separated, dried with $MgSO_4$, filtered, concentrated *in vacuo* to a small volume and triturated with light petroleum (40–60°C, 100 ml). The precipitate was dissolved in CH_2Cl_2 (5 ml) and purified by short-column chromatography (50 g). Elution of he column was effected by a 0 to 3% gradient of MeOH in CH_2Cl_2 and gave **3** as a white foam in a quantitative yield (3.26 g, 10.0 mmol); R_f 0.50 (B). ^1H NMR ($CDCl_3$): δ 9.07 (bs, 1H, NH), 7.28 [d, J(6,CH_3) 1.0 Hz, 1H, H-6], 6.43 [dd, J(1′,2′) 3.1 Hz, J(1′,2″) 5.5 Hz, 1H, H-1′], 5.22 (m, 1H, H-3′), 4.38–4.35 (m, 2H, H-5′ and H-5″), 4.26 (m, 1H, H-4′), 2.54–2.43 (m, 1H, H-2′ or H-2″), 2.24–2.09 (m, 1H, H-2′ or H-2″) coinciding with 2.14 and 2.12 (2 s, 6H, 2 CH_3, Ac), 1.95 [d, J(6,CH_3) 1.0 Hz, 3H, CH_3, T]. ^{13}C NMR ($CDCl_3$): δ 170.2 and 170.0 (2 s, 2 C=O, Ac), 163.8 (C-4), 150.5 (C-2), 134.3 (C-6), 111.4 (C-5), 84.6 (C-1′), 81.9 (C-4′), 74.0 (C-3′), 63.7 (C-5′), 37.9 (C-2′), 20.7 and 20.6 (2 s, 2 CH_3, Ac), 12.5 (CH_3, T).

3′,5′-Di-*O*-acetyl-2′-deoxythymidine (0.98 g, 3.00 mmol), dried by evaporation with dioxane, was dissolved in anhydrous MeCN (30 ml). A freshly prepared solution of phosphoryl tristriazolide reagent[15] (15.0 mmol) in MeCN (30 ml) was then added. After stirring for 3 h, the mixture was diluted with CH_2Cl_2 (100 ml) and poured into aqueous $NaHCO_3$ solution (50 ml, 5% w/v). The organic layer was separated, extracted with brine (50 ml), dried over $MgSO_4$, filtered and concentrated *in vacuo* to give a solid. Purification by short-column chromatography (20 g) using a

0-to-6% gradient of CH_3OH in CH_2Cl_2 as eluent gave **3** as a white foam in a yield of 1.02 g (2.70 mmol, 90%); R_f 0.49 (B). ^1H NMR ($CDCl_3$): δ 9.22 (bs, 1H, H-5, Tr), 8.06 (bs, 1H, H-3, Tr), 8.00 [d, $J(6,CH_3)$ 0.9 Hz, 1H, H-6], 6.21 [dd, $J(1',2')$ 2.2 Hz, $J(1',2'')$ 5.7 Hz, 1H, H-1'], 5.18 (m, 1H, H-3'), 4.35 (bs, 3H, H-4', H-5' and H-5''), 2.90–2.79 (m, 1H, H-2' or H-2''), 2.41 [d, $J(6,CH_3)$ 0.9 Hz, 3H, CH_3, T], 2.14–2.00 (m, 1H, H-2' or H-2'') coinciding with 2.09 and 2.00 (2 s, 6H, 2 CH_3, Ac). ^{13}C NMR ($CDCl_3$): δ 169.9 and 169.8 (2 s, 2 C=O, Ac), 157.7 (C-4), 153.3 (C-2), 152.7 (C-6), 145.6 (CH, Tr), 105.5 (C-5), 87.3 (C-1'), 82.8 (C-4'), 73.5 (C-3'), 63.0 (C-5'), 38.2 (C-2'), 20.2 (s, 2 CH_3, Ac), 16.5 (CH_3, T).

General procedure for the synthesis of 4-alkoxy-2'-deoxythymidines (**4–6**)

A freshly prepared solution of alcohol/DBU/dioxane (9/2/9, v/v/v, 25 ml) was added to an anhydrous solution of compound **3** (473 mg, 1.25 mmol) in dioxane (1 ml). The mixture was heated in a sealed flask at 50°C. After 24 h (MeOH), 24 h (EtOH) and 48 h (PrOH), respectively, the mixture was neutralized with Dowex 50W X4 (pyridinium form). The resin was removed by filtration and the filtrate concentrated *in vacuo*. The crude oil was dissolved in CH_2Cl_2/MeOH (2/1, v/v, 4 ml) and purified over a Sephadex LH20 column, equilibrated and eluted with the same solvent mixture. The fractions containing alkylated product were collected and concentrated to a solid, which was crystallized from EtOAc/MeOH.

4: yield 240 mg (0.94 mmol, 75%); m.p. 171–173°C; R_f 0.20 (**B**); λ_{max} (pH 3.5) 280 nm (ε $6.6 \cdot 10^3$). ^1H NMR (DMSO): δ 8.01 (s, 1H, H-6), 6.14 [t, $J(1',2')$, $J(1',2'')$ 6.4 Hz, 1H, H-1'], 5.24 [d, $J(3',OH)$ 4.1 Hz, 1H, 3'-OH, exchangeable], 5.07 [t, $J(5',OH)$, $J(5'',OH)$ 5.3 Hz, 1H, 5'–OH, exchangeable], 4.25 (m, 1H, H-3'), 3.84 (s, 3H, OCH_3, Me), 3.81 (m, 1H, H-4'), 3.59 (m, 2H, H-5' and H-5''), 2.24–2.15 (m, 1H, H-2' or H-2''), 2.06–1.96 (m, 1H, H-2' or H-2''), 1.88 (s, 3H, CH_3, T). ^{13}C NMR (DMSO): δ 169.9 (C-4), 154.7 (C-2), 140.9 (C-6), 103.0 (C-5), 87.6 (C-1), 85.5 (C-4'), 70.0 (C-3'), 61.0 (C-5'), 54.0 (OCH_3, Me), 40.6 (C-2'), 11.9 (CH_3, T).

5: yield 241 mg (0.89 mmol, 71%); m.p. 183–185°C; R_f 0.23 (B); λ_{max} (pH 3.5) 280 nm (ε $6.5 \cdot 10^3$). ^1H NMR (DMSO): δ 8.02 (s, 1H, H-6), 6.15 [t, $J(1',2')$, $J(1',2'')$ 6.4 Hz, 1H, H-1'], 5.25 [d, $J(3',OH)$ 4.1 Hz, 1H, 3'-OH, exchangeable], 5.08 [t, $J(5',OH)$, $J(5'',OH)$ 5.3 Hz, 1H, 5'-OH, exchangeable], 4.31 (q, J 7.1 Hz, 2H, OCH_2, Et), 4.28 (m, 1H, H-3'), 3.82 (m, 1H, H-4'), 3.61 (m, 2H, H-5' and H-5''), 2.27–2.15 (m, 1H, H-2' or H-2''), 2.07–1.94 (m, 1H, H-2' or H-2''), 1.89 (s, 3H, CH_3, T), 1.30 (t, J 7.1 Hz, 3H, CH_3, Et). ^{13}C NMR (DMSO): δ 169.6 (C-4), 154.7 (C-2), 140.9 (C-6), 103.0 (C-5), 87.6 (C-1'), 85.5 (C-4'), 70.0 (C-3'), 62.5 (OCH_2, Et), 61.0 (C-5'), 40.6 (C-2'), 14.1 (CH_3, Et), 11.9 (CH_3, T).

6: yield 248 mg (0.88 mmol, 70%); m.p. 145–146°C; R_f 0.27 (B); λ_{max} (pH 3.5) 280 nm (ε $6.3 \cdot 10^3$). ^1H NMR (DMSO): δ 8.01 (s, 1H, H-6), 6.13 [t, $J(1',2')$, $J(1',2'')$ 6.7 Hz, 1H, H-1'], 5.24 [d, $J(3',OH)$ 4.4 Hz, 1H, 3'-OH, exchangeable], 5.07 [t, $J(5',OH)$, $J(5'',OH)$ 5.4 Hz, 1H, 5'-OH, exchangeable], 4.21 (m, 3H, H-3' and OCH_2, Pr), 3.80 (m, 1H, H-4'), 3.59 (m, 2H, H-5' and H-5''), 2.24–2.11 (m, H, H-2' or H-2''), 2.08–1.92 (m, (H, H-2' or H-2''), 1.88 (s, 3H, CH_3, T), 1.70 (m, 2H, CH_2, Pr), 0.94 (t, J 7.0 Hz, 3H, CH_3, Pr). ^{13}C NMR (DMSO): δ 169.4 (C-4), 154.4 (C-2), 140.6 (C-6), 102.7 (C-5), 87.3 (C-1'), 85.2 (C-4'), 69.8 (C-3'), 67.6 (OCH_2, Pr), 60.8 (C-5'), 40.4 (C-2'), 21.2 (CH_2, Pr), 11.6 (CH_3, T), 10.0 (CH_3, Pr).

Deprotection of **7**

Compound **7**[20] (100 mg, 0.16 mmol) was dried by evaporation with dioxane (5 ml) and dissolved in alcohol/DBU/dioxane (2 ml, 9/2/9, v/v/v). The mixture was kept in a sealed flask at 50°C and the reaction was monitored by TLC analysis. After complete removal of the iBu group, the mixture was neutralized with HOAc and concentrated *in vacuo* to dryness. Crude product was dissolved in CH_2Cl_2 (5 ml) and purified by short-column chromatography. Elution with a gradient of 0 to 10% MeOH in CH_2Cl_2 followed by concentration of the appropriate fractions gave pure 5'-*O*-(4,4'-dimethoxytrityl)-2'-deoxyguanosine as a white solid. Yield 86 mg (94%) in thecase of MeOH, 85 mg (93%) in the case of EtOH and 82 mg (90%) in the case of PrOH; R_f 0.15 (B). ^1H NMR ($CDCl_3/CD_3OD$): δ 7.41–6.94 (m, 9H, arom. H's, DMT), 6.93–6.71 (m, 4H, arom. H's, DMT), 5.69 [dd, $J(1',2')$ 5.1 Hz, $J(1',2'')$ 5.7 Hz, 1H, H-1'], 4.27 (m, 1H, H-3'), 3.70 (m, 1H, H-4'), 3.64 (s, 3H, OCH_3, DMT), 3.38–3.12 (m, 2H, H-5' and H-5''), 2.62–2.37 (m, 1H, H-2' or H-2''), 2.33–1.89 (m, 1H, H-2' or H-2''). ^{13}C NMR ($CDCl_3/CD_3OD$): δ 158.4, 144.5, 136.7, 135.6 (C-arom., DMT), 151.1 (C-6), 149.6 (C-2), 139.8 (C-8), 129.9, 127.6, 126.8, 113.0 (CH-arom., DMT), 117.4 (C-5), 86.3 (Cq., DMT), 85.0 (C-1'), 83.6 (C-4'), 71.6 (C-3'), 64.1 (C-5'), 55.1 (OCH_3, DMT), 40.2 (C-2).

Functionalisation of the solid support (CPG)

HOBt (202 mg, 1.5 mmol) and DCC (309 mg, 1.5 mmol) was added to a solution of 5'-*O*-(4,4'-dimethoxytrityl)-2'-deoxynucleoside 3'-*O*-succinate[22] (1.0 mmol) in dioxane (10 ml). The mixture was stirred for 5 h at 20°C. After removing the precipitate by filtration, the filtered solution was added to the amino support (2 g, CPG-AP) and the mixture was shaken overnight at 20°C in the presence of NMI (0.4 ml, 5.0 mmol). The support was isolated by filtration and washed successively with dioxane (5 ml), MeCN (5 ml), methanol (2 × 5 ml) and CH_2Cl_2 (2 × 5 ml). Unreacted NH_2 groups were capped by treatment with 5% NMI in Ac_2O/*sym*-collidine/dioxane (3/3/13, v/v/v) for 30 min at 20°C followed by extensive washing of the support with dioxane, methanol and finally with CH_2Cl_2. The extent of loading of the first nucleoside on the solid support was established according to a described procedure[23].

Synthesis of oligodeoxynucleotides on polymer support CPG

The polymer-supported synthesis of oligodeoxynucleotides was performed on a fully automated synthesizer (Pharmacia, Gene Assembler) using both the above synthesized phosphoramidite **1** and the commercial available phosphoramidites **2**. Controlled-pore glass (CPG-AP, 200 mg), covalently linked to the appropriate nucleoside (loading 50 μmol/g), was used as solid phase. The individual steps of one complete elongation cycle are depicted in Table I and, after each step, the column was washed with MeCN to remove impurities and excess reagents. In the last step, the 5'-DMT group was removed (see step 1 in Table I) and the solid support washed with MeCN (12.5 ml) for 5 min.

Deprotection and purification of **10–16**

The solid support, containing immobilized oligodeoxynucleotides, was treated with a mixture of alcohol/DBU/dioxane (20 ml, 9/2/9, v/v/v) and kept in a sealed flask for 24 h (for MeOH), 48 h (for EtOH) and 120 h (for PrOH) at 50°C. After neutralization of the individual mixtures with acetic acid, the support was removed by filtration and the filtrate was evaporated under reduced pressure. The crude unprotected DNA fragments were purified over a Sepharose 75 column, suspended in and eluted with TEAB buffer (0.15M). The appropriate fractions, checked by FPLC analysis, were pooled, concentrated *in vacuo* to a small volume, evaporated several times with water and lyophilized. The obtained oligonucleotides were further purified on a Q Sepharose high-performance column, neutralized with acetic acid and desalted on a Hiload Sephadex 75 column as described above. Finally, the pure oligonucleotides were converted into the Na^+ form by passing them through a column of Dowex 50W X4 cation-exchange resin (100–200 mesh, Na^+ form). The resulting UV-positive fractions were pooled, concentrated to a small volume, checked by FPLC analysis and lyophilized.

Hexamer **10**: d-CpGpT^{Me}pApCpG; isolated yield 7.9 mg (52%, Na^+ salt); retention time: 5.2 min using anion-exchange FPLC; 26.8 min using reversed-phase HPLC. ^1H NMR (D_2O): δ 8.27 (s, 1H, H-8, A), 8.04 (s, 1H, H-2, A), 7.96 (s, 1H, H-8, G), 7.95 (s, 1H, H-8, G), 7.58 [d, $J(5,6)$ 7.5 Hz, 1H, H-6, C], 7.50 [d, $J(5,6)$ 7.6 Hz, 1H, H-6, C], 7.45 (s, 1H, H-6, T), 6.27 [dd, $J(1',2'')$ 7.8 Hz, $J(1',2')$ 6.3 Hz, 1H, H-1'], 6.13–5.96 (m, 4H, 4 H-1'), 5.95 (dd, $J(1',2'')$ 8.6 Hz, $J(1',2')$ 6.2 Hz, 1H, H-1'), 5.88 [d, $J(5,6)$ 7.5 Hz, 1H, H-5, C], 5.81 [d, $J(5,6)$ 7.6 Hz, 1H, H-5, C), 3.82 (s, 3H, OCH_3, Me), 1.70 (s, 3H, CH_3, T).

Hexamer **11**: d-CpGpT^{Et}pApCpG; isolated yield 8.9 mg (46%, Na^+-salt); retention time: 5.4 min using anion-exchange FPLC; 34.2 min using reversed-phase HPLC. ^1H NMR (D_2O): δ 8.26 (s, 1H, H-8, A), 8.03 (s, 1H, H-2, A), 7.96 (s, 1H, H-8, G), 7.95 (s, 1H, H-8, G), 7.57 [d, $J(5,6)$ 7.6 Hz, 1H, H-6, C], 7.50 [d, $J(5,6)$ 7.6 Hz, 1H, H-6, C], 7.46 (s, 1H, H-6, T), 6.27 [dd, $J(1',2'')$ 7.4 Hz, $J(1',2')$ 6.4 Hz, 1H, H-1'], 6.13–6.00 (m. 4H, 4 H-1'), 5.95 [dd, $J(1',2'')$

8.6 Hz, $J(1',2')$ 6.5 Hz, 1H, H-1'], 5.88 [d, $J(5,6)$ 7.5 Hz, 1H, H-5, C], 5.81 [d, $J(5,6)$ 7.5 Hz, 1H, H-5, C], 4.15 (m, OCH_2, Et) coinciding with other sugar protons as determined by saturation of 1.31 (t, J 7.21 Hz, 3H, CH_3, Et), 1.68 (s, 3H, CH_3, T).
Hexamer **12**: d-CpGpT^{Pr}pApCpG; isolated yield: 7.4 mg (38%, Na^+-salt); retention time: 5.5 min using anion-exchange FPLC; 41.4 min using reversed-phase HPLC. ^{1}H NMR (D_2O): δ 8.26 (s, 1H, H-8, A), 8.02 (s, 1H, H-2, A), 7.97 (s, 1H, H-8, G), 7.96 (s, 1H, H-8, G), 7.58 [d, $J(5,6)$ 7.5 Hz, 1H, H-6, C], 7.50 [d, $J(5,6)$ 7.6 Hz, 1H, H-6, C], 7.49 (s, 1H, H-6, T), 6.27 [dd, $J(1',2'')$ 7.2 Hz, $J(1',2')$ 6.6 Hz, 1H, H-1'], 6.14–5.99 (m, 4H, 4 H-1'), 5.92 [dd, $J(1',2'')$ 8.3 Hz, $J(1',2')$ 5.6 Hz, 1H, H-1'], 5.89 [d, $J(5,6)$ 7.4 Hz, 1H, H-5, C], 5.81 [d, $J(5,6)$ 7.5 Hz, 1H, H-5, C], 4.23–4.21 (m, OCH_2, Pr) coinciding with some sugar protons as determined by saturation of 1.72–1.68 (m, CH_2, Pr) coinciding with 1.69 (s, 3H, CH_3, T), 0.91 (t, J 7.4 Hz, 3H, CH_3, Pr).
Yields and other relevant data of hexadecamers **13–16** are presented in Table II.

Analysis of the nucleotide composition of oligonucleotides **13–16**

The composition of the oligonucleotides was verified by hydrolyzing 10 μg oligonucleotide to nucleosides using a modification of a known procedure[24]. In addition to DNAse I, alkaline phosphatase and nuclease P1 12 mU Venom phosphodiesterase were added for each reaction. Nucleosides were separated on a Supelco LE-18C reversed-phase HPLC column. Elution was carried out with a buffer of 40mM triethylammonium formate (pH 3.5) with a linear gradient of 0–50% MeCN (see Figure 2). The alkoxyT adducts were identified both by their UV-absorbance spectrum and by comigration with the synthetic prepared reference alkoxyT nucleosides. The molar ratios of the nucleosides from the digestions are presented in Table II.

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