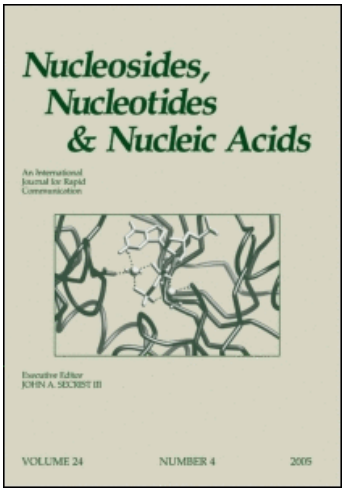

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7-Deaza-2'-Deoxy-O⁶-Methylguanosine: Selective N²-Formylation Via a Formamidine, Phosphoramidite Synthesis and Properties of Oligonucleotides

F. Seela[a]; H. Driller[a]
[a] Laboratorium für Organische und Bioorganische Chemie, Fachbereich Biologie/Chemie, Universität Osnabrück, Osnabrück, West-Germany

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7-DEAZA-2′-DEOXY-O^6-METHYLGUANOSINE: SELECTIVE N^2-FORMYLATION VIA A FORMAMIDINE, PHOSPHORAMIDITE SYNTHESIS AND PROPERTIES OF OLIGONUCLEOTIDES

F. Seela * and H. Driller

Laboratorium für Organische und Bioorganische Chemie, Fachbereich Biologie/Chemie, Universität Osnabrück, D-4500 Osnabrück, West-Germany

ABSTRACT Hexa- and dodecanucleotides containing 7-deaza-2′-deoxy-O^6-methylguanosine ($m^6c^7G_d$, 2) have been prepared by solid-phase synthesis employing methyl- as well as cyanoethyl phosphoramidites of 2. As the N^2-isobuturyl group of protected 2 was difficult to remove after oligonucleotide synthesis the more labile formyl group was introduced. Regioselective N^2-formylation was carried out without sugar protection by in-situ hydrolysis of a formamidine intermediate. Replacement of dG within the oligomer d(CGCGAATTCGCG) next to dA increased hairpin formation, whereas duplexes were formed if one of the outer dG residues was replaced by $m^6c^7G_d$. Tertiary structure changes of the oligomers were detected by comparing the melting curves at 260 and 280 nm or duplex specific cleavage by the endodeoxyribonuclease Eco RI.

INTRODUCTION

Recent work on 2′-deoxy-O^6-methylguanosine (1, m^6G_d) containing oligonucleotides has shown that side reactions occur during alkaline deprotection [1]. These reactions are caused by the new nucleophilic centre introduced at C-6 by O^6-methylation. This methylation also changes the reactivity of the 2-amino group which results in difficulties during the deprotection procedure. In consequence oligonucleotides containing (i) 2,6-diaminopurine deoxyribofuranoside-, (ii) 2′-deoxyguanosine-, and (iii) amino-protected m^6G_d-residues apart from the regular reaction products were formed [1-3].

If an oligonucleotide contains more than one modified residue it becomes very heterogeneous and difficult to purify.

Recently we have synthesized 7-deaza-2'-deoxy-O^6-methylguanosine (2, $m^6c^7G_d$) a pyrrolo[2,3-d]pyrimidine isostere of m^6G_d (1), containing an O^6-methoxy group which is difficult to displace by nucleophiles [4]. This property is caused by replacement of nitrogen-7 by a methine group. Additionally, structural modification results in a very stable N-glycosylic bond. Due to these characteristics compound 2 is attractive for site-directed mutagenesis or as regioselective probe within DNA.

In the following the synthesis of phosphoramidites of 2 and their application in solid-phase oligonucleotide synthesis are described. Moreover, it will be shown that replacement of dG by $m^6c^7G_d$ within the oligomer d(CGCGAATTCGCG) 11 changes its tertiary structure depending on the position of incorporation.

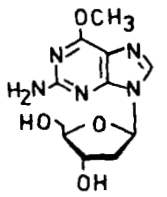
RESULTS AND DISCUSSION

Synthesis of the Phosphoramidites 5a,b and 6a,b and Their Application during Synthesis of the Oligomers 7 - 14

The application of the phosphoramidites 5a and 6a in oligonucleotide synthesis was studied first on the hexamers 8 and 10 containing one $m^6c^7G_d$ instead of dG. The parent oligomers 7 and 9 were also synthesized as reference. Syntheses were carried out on an automated DNA-synthesizer employing solid-phase chemistry and phosphoramidite methodology. As starting material the nucleoside 2 was used [4]. Its toluoylated precursor 3d was prepared by solid-liquid phase-transfer glycosylation of 2-amino-4-methoxy-7H-pyrrolo[2,3-d]pyrimidine with 2-deoxy-3,5-di-O-(p-toluoyl)-α-D-erythro-pentofuranosyl chloride in 61% yield [5-8]. Detoluoylation as described earlier gave 2. ^{13}C NMR chemical shifts of 2 were assigned by a gated-decoupled NMR spectrum;

two dimensional [^{1}H,^{13}C]-correlation spectrum (XHCORR) confirmed the assignment of ^{1}H NMR data as reported earlier [4].

Protection of the 2-amino group of 2 was accomplished by triisobutyrylation to give 3a as an intermediate followed by selective deprotection of the sugar protecting groups according to the protocol of Khorana (3b) [9]. Transient protection failed, instead unreacted starting material was isolated [10].



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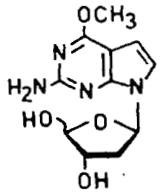

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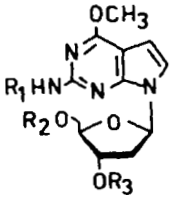
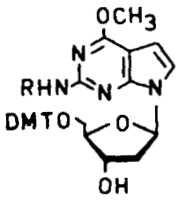
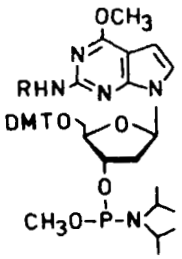


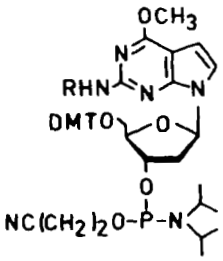
	R_1	R_2	R_3
3a	iBu	iBu	iBu
3b	iBu	H	H
3c	CHO	H	H
3d	H	Tol	Tol



4a R = iBu
4b R = CHO



5a R = iBu
5b R = CHO



6a R = iBu
6b R = CHO

Reaction of 3b with 4,4'-dimethoxytrityl chloride yielded 4a, which was isolated pure after flash chromatography. Compounds 3a - 4b were characterized by elemental analysis and ^{1}H NMR spectroscopy. ^{13}C NMR data are shown in TABLE 1. Phosphitylation of 4a with either β-cyanoethoxy [11] or methoxy diisopropylaminophosphine [12] gave the phosphoramidites 5a and 6a, respectively. They were purified by flash chromatography and characterized by ^{31}P NMR spectroscopy.

TABLE 1: ^{13}C NMR Chemical Shifts of Compound 2 and Related Nucleosides in [D_6]DMSO. [a] Tentative assignement.

Compound	C-2	C-4	C-4a	C-5	C-6	C-7a	OCH_3
2	159.4	163.0	97.3	98.9	119.5	154.2	52.5
3a	151.6[a]	162.5	101.4	99.2	122.9	152.4[a]	53.3
3b	151.5[a]	162.3	101.1	98.8	122.8	152.4[a]	53.2
4a	151.6[a]	162.4	101.2	98.9	122.6	152.1[a]	53.2
3c	152.0[a]	162.7	101.5	99.5	122.8	152.4[a]	53.7
4b	152.1[a]	162.8	101.7	99.5	122.9	152.4[a]	53.7

Compound	C-1′	C-2′	C-3′	C-4′	C-5′	C=O
2	82.4	39.3	70.9	86.9	62.0	-
3a	81.1	DMSO	74.2	83.4	63.4	174.9
3b	82.6	DMSO	70.8	87.1	61.8	174.9
4a	82.7	40.6	70.8	85.4	64.2	174.9
3c	82.7	DMSO	71.1	87.3	62.0	163.6
4b	82.9	39.4	70.9	85.5	64.4	163.6

Both phosphoramidites were employed together with those of dG and dC in solid-phase synthesis of the oligomers 8 and 10 on an automated DNA synthesizer. As references compounds 7 and 9 were also prepared. The synthesis followed a reaction cycle of detritylation, condensation, oxidation and capping already described earlier [13]. The oligomers were split off

7	d(CGCGCG)	11	d(CGCGAATTCGCG)
8	d(CGCm^{6}c^{7}GCG)	12	d(Cm^{6}c^{7}GCGAATTCGCG)
9	d(GCGCGC)	13	d(CGCm^{6}c^{7}GAATTCGCG)
10	d(GCm^{6}c^{7}GCGC)	14	d(CGCGAATTCm^{6}c^{7}GCG)

from the polymer support by ammonia. In case of the $POCH_3$ protected oligomers demethylation was carried out with thiophenol. From $POCH_2CH_2CN$ protected oligomers the cyanoethyl group was removed together with base protecting groups by

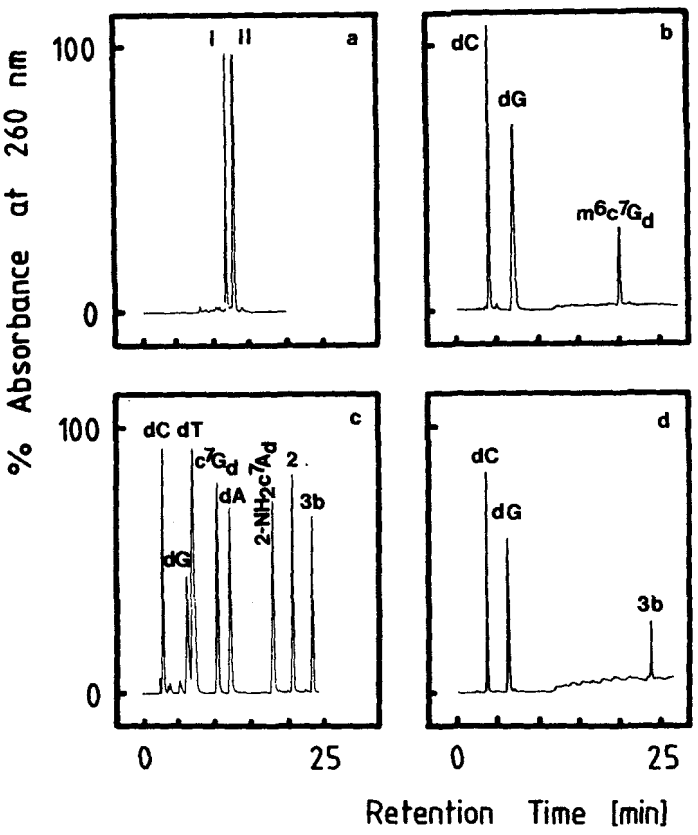


FIGURE 1: HPLC profile of d(CGCm^6c^7GCG) (8) employing the phosphoramidite 5a during synthesis (a). Nucleoside content of peak I (b) and of peak II (d) after tandem hydrolysis with snake venom phosphodiesterase followed by alkaline phosphatase. HPLC separation of a mixture of the four regular nucleosides, and c^7G_d [4], $2\text{-}NH_2\text{-}c^7A_d$ [14] , $m^6c^7G_d$ (2), and compound 3b (solvent system III) (c).

treatment with ammonia. The 5'-dimethoxytritylated oligomers were purified by reverse-phase HPLC. Then DMT-residues were removed by the action of 80% acetic acid yielding the oligomers 7 - 10, which were purified again by reverse-phase HPLC, desalted and isolated as colorless solids.

Figure 1 shows the HPLC profile obtained from compound 8 synthesis employing the isobutyrylated phosphoramidite 5a. From the HPLC pattern it is apparent that two types of

oligomers were formed. The content of both peaks was isolated and then hydrolysed with snake venom phosphodiesterase followed by alkaline phosphatase. Only the hydrolysis products of the slower migrating material (I) yielded the nucleoside content ($m^6c^7G_d$:dG:dC; 1:2:3) as expected from structure 8 (FIG. 1a). The other oligomer did not contain 2. Instead isobutyrylated 3b was present (FIG. 1d) which was confirmed (i) by an experiment which showed complete resolution of the regular nucleosides together with c^7G_d, 2-NH_2-c^7A_d, $m^6c^7G_d$, and 3b (FIG. 1b) and (ii) by cochromatography of authentic 3b with the products of hydrolysis of peak II. Complete removal of the isobutyryl residue afforded exhaustive treatment with ammonia for at least 4 days at 60°C. In that case the isobutyrylated oligomer was no more detectable and 8 was the main reaction product.

As the deprotection protocol of 3b was time-consuming we looked for another protecting group more labile than the isobutyryl residue. As amidines have been used as substitutes for acyl protecting groups in oligonucleotide synthesis we tried to introduce the formamidine function in 2 by reaction with N,N-dimethylformamide diethylacetal [15,16]. If the procedure was carried out at room temperature in methanolic solution the expected amidine was detectable on TLC. However, trace amounts of water present in the reaction mixture decomposed the protecting group leading to another uniform reaction product of faster mobility. This product was the main one if the reaction was carried out at 60°C. In contrast to the amidine, which could not be isolated pure, this product was obtained crystalline directly from the reaction mixture in 90% yield. Elemental analysis as well as ^1H and ^{13}C NMR data (TABLE 1) confirmed the structure of the formyl compound 3c. The formation of 3c was fairly unexpected and in contrast to 7-deaza-2′-deoxyguanosine (c^7G_d), which formed the amidine under the same reaction conditions [17].

As compound 3c could be prepared by in situ hydrolysis of the corresponding formamidine without protection of the

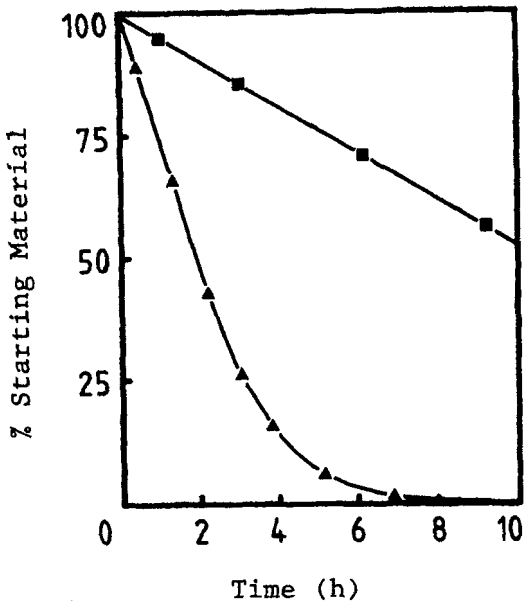


FIGURE 2: Deprotection kinetics of compound 3b (■) and 3c (▲) in 25% aq. ammonia at 25°C (30 µM). The kinetics was followed UV-spectrophotometrically in sealed cuvettes at 280 nm (compound 3b), and 285 nm (compound 3c).

3'- and 5'-hydroxyl groups, it is recommended for further experiments. In order to show that the formyl group was also suitable for oligonucleotide synthesis the kinetics of deprotection were studied under controlled conditions (25% aq. ammonia, 25°C) and compared with the hydrolysis rate of 3b. The reaction was followed photometrically at 285 nm (compound 3c) and 280 nm (compound 3b). As it can been seen from figure 2 the half-life of deformylation was 2 h (3c), compared to 12 h for deisobutyrylation of 3b. Next, compound 3c was reacted with DMT-Cl under identical conditions as described for 3b. The reaction product 4b was isolated after chromatographical work up and was characterized by ^{1}H and ^{13}C NMR spectroscopy (TABLE 1). From 4b the methyl amidite 5b as well as the cyanoethyl amidite 6b

were prepared similar as described before. They were then employed during synthesis of the oligomers 11 - 14. The oligomers 11 - 14 were selected as it has been shown that compound 11 forms hairpin as well as duplex structures in dilute aqueous solution [18].

DNA-synthesis followed the same scheme as reported above. HPLC analysis showed, that brief ammonia treatment was neccessary to obtain the deprotected oligonucleotides 12, 13, and 14. The nucleoside content of each of the oligomers was confirmed by complete enzymatic hydrolysis as described before. The oligomers 11 - 14 were then compared with respect to tertiary structure and regioselective cleavage by the Eco RI endonuclease, inhibited or induced by $m^6c^7G_d$ modification.

Physical Properties of d(CGCGAATTCGCG) Containing $m^6c^7G_d$ Instead of dG at Various Positions.

Earlier we have shown that replacement of three dG residues by c^7G_d within the hexamer $d(G\text{-}C)_3$ (9), reduces the T_m value from 45°C (9) to 35°C in case of $d(c^7G\text{-}C)_3$ [19]. In contrast, replacement of one dG by $m^6c^7G_d$ (oligomers 8 and 10) reduces the duplex T_m so strongly that no complete melting profile could be measured. Similar findings were already reported for the parent purine compound $d(CGCm^6GCG)$ [2].

It is worth noting that melting of these oligomers can be only observed at 280 nm whereas at 260 nm only a minor absorbance change is detectable. This is a known phenomenon which is due to very different hypochromicity changes of dA-dT vs. dG-dC base pairs depending on the wavelength [20]. In contrast to that the dodecamers 11 - 14 exhibited cooperative melting profiles at 260 and 280 nm, as they contain dG-dC and dA-dT base pairs which are opened during melting.

Recent observations in our laboratory have shown that compound 11 exhibits different T_m values at 260 and 280 nm (ΔT_m 5°C). This is the result of hairpin formation in

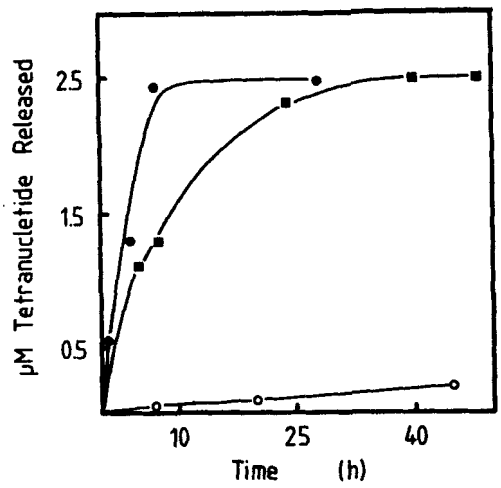


FIGURE 3: Hydrolysis curves of phosphodiester cleavage of the oligomers 11, 12, and 14 by the endodeoxyribonuclease Eco RI. The concentration of each oligomer was 2.5 μM. 1 mL samples (water) containing 10 mM TRIS/HCl, pH 7.5, 20 mM $MgCl_2$, and 80 mM NaCl were incubated at 30°C. Aliquots were analysed by reverse phase HPLC (solvent system III). The amount of tetranucleotide released was calculated as described in the Experimental section. (o) d(CGCGAATTCGCG (11), (■) d(Cm^6c^7GCGAATTCGCG (12), and (●) d(CGCGAATTCm^6c^7GCG) (14).

dilute aqueous solution and takes place as the d(AATT) fragment of 11 (two hydrogen bonds/base pair) is flanked by a number of stable dG-dC base pairs. Intramolecular Watson-Crick base pairing is then more favourable than the intermolecular one [18]. However, in more concentrated solution or in the crystal the duplex is still the preferred species which makes the phenomenon difficult to study by ^{1}H NMR spectroscopy or by single crystal X-ray analysis.

As the endodeoxyribonuclease Eco RI is a sensitive probe for DNA duplex structures, regioselective cleavage of the oligomer 11 was used to differentiate between duplex and hairpin structures. In agreement with the high d(GC) content in the flanking regions and the reduced number of duplexes compound 11 was cleaved very slowly by the endodeoxyribonuclease (FIGURE 3). The stronger difference between 260 and

TABLE 2. T_m Values of the Oligomers 7 - 14 at 260 and 280 nm

oligomer		260 nm[a)]	280 nm[a)]
d(CGCGCG)	(7)	- [b)]	44
d(CGCm^{6}c^{7}GCG)	(8)	- [b)]	-
d(GCGCGC)	(9)	- [b)]	44
d(GCm^{6}c^{7}GCGC)	(10)	- [b)]	-
d(CGCGAATTCGCG)	(11)	60	65
d(Cm^{6}c^{7}GCGAATTCGCG)	(12)	47	47
d(CGCm^{6}c^{7}GAATTCGCG)	(13)	25	41
d(CGCGAATTCm^{6}c^{7}GCG)	(14)	37	37

a) T_m values (°C) were determined in 10 mM Tris/HCl buffer, pH 7.5, containing 20 mM $MgCl_2$, 80 mM NaCl and an oligomer concentration of 2.5 µM. b) not detectable.

280 nm T_m values (Δ T_m 16°C) for the oligomer 13 (TABLE 2) pointed to an even more pronounced tendency of hairpin formation. This is a consequence of further reduction of hydrogen bonds due to the replacement of an inner dG by $m^6c^7G_d$. Unfortunately, $m^6c^7G_d$ (2) is located at a dG position, which contributes two hydrogen bonds to Eco RI endonuclease-DNA binding [21]. Therefore the resistance of 13 against endonuclease Eco RI cleavage can be due to base modification and/or hairpin formation.

The situation becomes different if $m^6c^7G_d$-residues are incorporated into the d(GC) motives flanking either the 5′-or the 3′-end of the recognition site. In that case T_m values are identical at 260 and 280 nm (TABLE 2) indicating that only duplexes are present in solution. As a consequence rapid cleavage of the oligomers 12 and 14 occured (FIGURE 3-5).

In conclusion, it was shown that the formyl residue is the appropriate protecting group for the phosphoramidites of 2 employed in automated oligonucleotide synthesis.

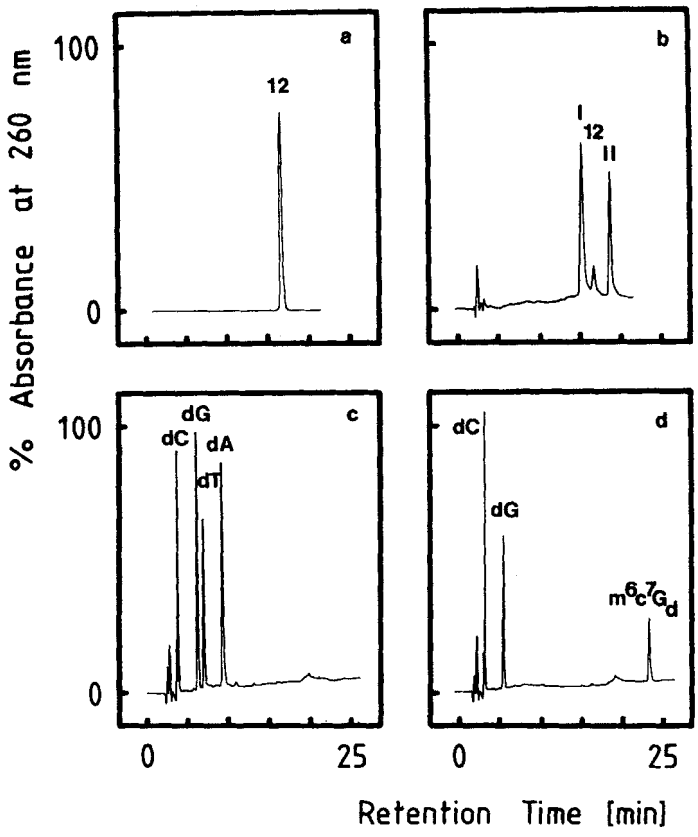


FIGURE 4. HPLC profiles of phosphodiester hydrolysis of d(Cm^{6}c^{7}GCGAATTCGCG) (12) by the endodeoxyribonuclease Eco RI under conditions described in the Experimental Section. HPLC profile of the reaction mixture at the beginning (a) and the reaction after 6 h (b). HPLC pattern of the cleavage products of I (c) and of II (d) were taken after tandem hydrolysis with snake venom phosphodiesterase followed by alkaline phosphatase. Solvent system III was used as eluent.

Replacement of dG by $m^6c^7G_d$ residues at different positions of the oligomer 11 showed that d(GC) clusters flanking the 5′ and 3′ site of a short oligomer induce tertiary structure changes (e.g. duplex-hairpin transitions). For high-molecular-weight DNA the situation is somewhat different but hairpin or cruciform structures should be also favoured if a d(AT) rich sequence is flanked by d(GC) fragments.These d(GC) induced changes of teriary structure can be altered if the

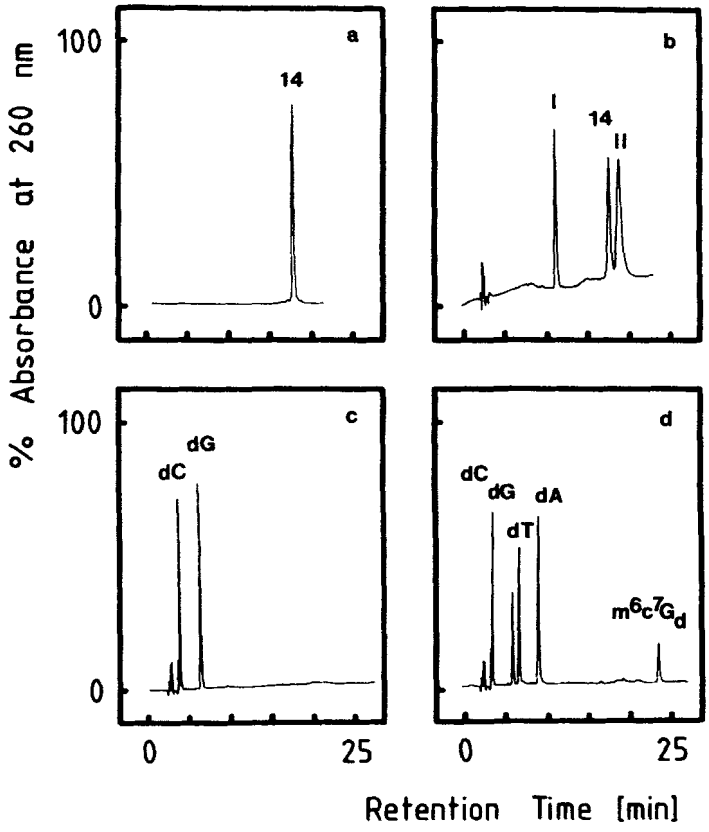


FIGURE 5. HPLC profiles before (a) and after hydrolysis (b) of 14 with the endodeoxyribonuclease Eco RI; (c) and (d) show the nucleoside contents of the hydrolysis reaction carried out with I and II. Same conditions as Figure 4.

number of hydrogen bonds or the stacking interactions within alternating d(GC) regions are reduced. As base-modified deoxynucleosides as compound 2 or its congener c^7G_d show such properties they can be used to induce such changes [22].

EXPERIMENTAL SECTION

Melting points were determined on a Linström apparatus (Wagner & Munz, FRG) and were not corrected. Elemental analyses were performed by Mikroanalytisches Labor Beller

(Göttingen, FRG). NMR spectra were measured on an AC 250 spectrometer equipped with an Aspect 3000 data system and an array processor (Bruker, FRG). Operational frequencies: ^{1}H: 250.133 MHz; ^{31}P: 101.256; ^{13}C: 62.898 MHz. δ values are in ppm relativ to tetramethylsilane as internal standard (^{1}H and ^{13}C) or to external 85% phosphoric acid (^{31}P). Chemical shifts are positiv when downfield from the appropriate standard. Digital resolutions: ^{1}H: 0.275 Hz/pt; ^{31}P: 1.907 Hz/pt; ^{13}C: 0.526 Hz/pt. UV spectra were recorded on a U 3200 spectrophotometer (Hitachi, Japan). Thin-layer chromatography (TLC) was performed on silica gel SIL G-25 UV_{254} plates (Macherey & Nagel, FRG). Silica gel 60 (70-230 mesh, Merck, FRG) was used for column chromatography, flash chromatography was performed with silica gel 60 H (Merck, FRG) at 0.5 bar (N_2). Solvent systems: (A) CH_2Cl_2, (B) $CHCl_3$-MeOH (95:5), (C) CH_2Cl_2-acetone (8:2), (D) CH_2Cl_2-ethyl acetate-triethyl amine (45:45:10), (E) CH_2Cl_2-acetone (9:1), (F) $CHCl_3$-MeOH (9:1), (G) CH_2Cl_2-ethyl acetate (9:1). 2'-Deoxyadenosine, 2'-deoxycytidine, 2'-deoxyguanosine and thymidine were purchased from Pharma-Waldhof (FRG). The protected phosphoramidites were synthesized according to the procedure of McBride and Caruthers [12] and Sinha et al. [11] in the case of the cyanoethylphosphoramidites. CPG (70 µmol of immobilized protected 2'-deoxynucleoside/g of solid support was purchased from Biosyntech (Hamburg, FRG). Snake venom phosphodiesterase (EC 3.1.16.1, from crotallus durissus), alkaline phosphatase (EC 3.1.3.1, from E. Coli), and endodeoxyribonuclease Eco RI (EC 3.1.23.13, from E. Coli BS 5) were products of Boehringer Mannheim (FRG).

7-[2-Deoxy-3,5-di-O-(2-methylpropionyl)-β-D-erythro-pentofuranosyl]-4-methoxy-2-[(2-methylpropionyl)amino]-7H-pyrrolo[2,3-d]pyrimidine (3a):

Compound 2 [4-8] (0.5 g, 1.78 mmol) was dissolved in DMF (2 mL), treated with isobutyric anhydride (35 mL), and heated under reflux for 3.5 h. The solution was evaporated in vacuo and the residue chromatographed on silica gel (column:3x35

cm, solvent A). The main zone was separated and evaporation of the solvent resulted in a colorless amorphous foam (530 mg, 61%). TLC (silica gel, solvent B) R_f 0.8; UV ($CHCl_3$) λ_{max} 279 (ε 8700); ^1H NMR ([D_6]DMSO) δ 1.05 (d, CH_3, J = 7 Hz), 1.07 (d, CH_3, J = 4.7 Hz), 1.09 (d, CH_3, J = 4.7 Hz), 1.14 (d, CH_3, J = 7 Hz), 2.40, 2.80 (m, CH and 2′-$H_{a,b}$), 4.03 (s, OCH_3), 4.15 (m, 5′-H), 4.26 (m, 4′-H), 5.36 (m, 3′-H), 6.48 (m, 1′-H), 6.52 (d, 5-H, J = 3.6 Hz), 7.43 (d, 6-H, J = 3.6 Hz), 10.22 (s, NH). Anal. calcd. for $C_{24}H_{34}N_4O_7$ (490.5): C, 58.76; H, 6.99; N, 11.42. Found: C, 58.76; H, 6.94; N, 11.38.

7-(2-Deoxy-β-D-erythro-pentofuranosyl)-4-methoxy-2-[(2-methylpropionyl)amino]-7H-pyrrolo[2,3-d]pyrimidine (3b):

Compound 3a (500 mg, 1.02 mmol) was dissolved in a mixture of MeOH-25% aq. NH_3 (40 mL, 1:1) and stirred for 3 h at room temperature. The solution was evaporated, the oily residue redissolved in $CHCl_3$ and chromatographed on silica gel (column: 20 x 2 cm, solvent B). Compound 3b (294 mg, 82%) was isolated from the main zone as colorless amorphous foam. TLC (silica gel, solvent B) R_f 0.4. UV (MeOH) λ_{max} 278, 232 (ε 12000, 17500). ^1H NMR ([D_6]DMSO) δ 1.07, 1.10 (2s, 2 CH_3), 2.32, 2.80 (2m, 2′-$H_{a,b}$ and CH), 3.52 (m, 5′-H), 3.81 (m, 4′-H), 4.02 (s, OCH_3), 4.35 (m, 3′-H), 4.88 (s, 5′-OH), 5.28 (s, 3′-OH), 6.48 (d, 5-H, J = 3.7 Hz). 6.54 (dd, 1′-H, J = 6.1 Hz), 7.47 (d, 6-H, J = 3.7 Hz), 10.17 (s, NH). Anal. calcd. for $C_{16}H_{22}N_4O_5$ (350.3): C, 54.85; H, 6.33; N, 15.99. Found: C, 54.84; H, 6.38; N, 16.00.

7-[2-Deoxy-5-O-[bis(4-methoxyphenyl)phenylmethyl]-β-D-erythro-pentofuranosyl]-4-methoxy-2-[(2-methylpropionyl)-amino]-7H-pyrrolo[2,3-d]pyrimidine (4a):

To a solution of 3b (785 mg, 2.2 mmol) in anhydrous pyridine, 4,4′-dimethoxytrityl chloride (1.0 g, 3.0 mmol) dissolved in p-dioxane (20 mL) and N-ethyl diisopropylamine (400 µL, 2.3 mmol) were added. It was stirred for 3 h at room temperature, then poored into 5% aq. $NaHCO_3$ (50 mL) and

extracted with CH_2Cl_2 (2 x 50 mL). The combined organic layers were dried over sodium sulfate, filtered, and evaporated. The residue was redissolved in solvent C and applied to silica gel (column 20x5 cm, solvent C). The main zone was evaporated yielding colorless amorphous 4a (1.17 g, 80%). TLC (silica gel, solvent B) R_f 0.4. UV (MeOH) λ_{max} 276 (ε 15200). ^{1}H NMR ($CDCl_3$) δ 1.21, 1.24 (2s, 2 CH_3), 2.45, 2.59 (m, CH and 2'-$H_{a,b}$), 3.15 (m, 4'-H), 3.34 (m, 5'-H), 3.75 (s, 2 OCH_3), 4.04 (s, OCH_3), 4.06 (m, 3'-H), 4.60 (d, 3'-OH, J = 2.4 Hz), 6.40 (d, 5-H, J = 3.7 Hz), 6.64 (pt, 1'-H, J = 6.7 Hz), 6.76, 6.79 (2s, trityl), 7.05 (d, 6-H, J = 3.7 Hz), 7.30 (m, trityl), 7.77 (s, NH). Anal. calcd. for $C_{37}H_{40}N_4O_7$ (652.7): C, 68.08; H, 6.18; N, 8.58. Found: C, 67.93; H, 6.19; N, 8.40.

7-[5-O-[Bis(4-methoxyphenyl)phenylmethyl]-[3-O-(diisopropylamino)methoxyphosphino]-2-deoxy-β-D-erythro-pentofuranosyl]-4-methoxy-2-[(2-methylpropionyl)amino]-7H-pyrrolo[2,3-d]pyrimidine (5a):

Compound 4a (200 mg, 0.31 mmol) dissolved in anhydrous CH_2Cl_2 (1 mL) was stirred in the presence of N-ethyldiisopropylamine (138 mg, 1.07 mmol) at room temperature. Chlorodiisopropylamino methoxyphosphine [12] (85 mg, 0.43 mmol) was added by a syringe (argon). After 1 h the solution was diluted with ethyl acetate, extracted with saturated aq. NaCl dried over $NaSO_4$ and filtered. After evaporation the resulting foam was purified by flash chromatography on silica gel (column 15 x 3 cm, solvent D). From the main zone colorless amorphous 5a (188 mg, 75%) was isolated. TLC (silica gel, solvent E) R_f 0.9. ^{31}P NMR ($CDCl_3$) δ 149.62 ppm.

7-[5-O-[Bis(4-methoxyphenyl)phenylmethyl]-[3-O-β-cyanoethoxy(diisopropylamino)phosphino]-2-deoxy-β-D-erythro-pentofuranosyl]-4-methoxy-2-[(2-methylpropionyl)amino]-7H-pyrrolo[2,3-d]pyrimidine (6a):

To a solution of compound 4a (250 mg, 0.38 mmol) in anhydrous THF, N-ethyldiisopropylamine (138 mg, 1.07 mmol)

and β-cyanoethylchloro-diisopropylaminophosphine (118 mg, 0.5 mmol) was added (argon, r. t.). The precipitate was filtered off and the filtrate was poored into a mixture of CH_2Cl_2/5% aq. $NaHCO_3$ (50 mL, 1:1). The organic layer was separated, dried over Na_2SO_4, filtered, and concentrated. The solution (1 mL) was chromatographed on silica gel, the main zone was separated, and the solvent evaporated. Coevaporation with acetone afforded colorless amorphous 6a (268 mg, 82%). TLC (silica gel, solvent E) R_f 0.9. ^{31}P NMR ($CDCl_3$) δ 149.3, 149.6 ppm.

7-(2-Deoxy-β-D-erythro-pentofuranosyl)-2-[(formyl)amino]-4-methoxy-7H-pyrrolo[2,3-d]pyrimidine (3c):

A solution of 2 [4-8] (500 mg, 1.8 mmol) in DMF (5 mL) containing N,N-dimethylformamide diethylacetal (5 mL) was stirred for 2 h at 50°C. The solvent was evaporated in vacuo and the oily residue coevaporated with toluene (5 mL) and acetone (5 mL). Crystallisation from water afforded colorless needles (495 mg, 90%) with m. p. 169°C. TLC (silica gel, solvent F) R_f 0.3. UV(MeOH) λ_{max} 233, 279 (ε 19200, 14000). ^1H NMR ([D_6]DMSO) δ 2.17, 2.50 (2m, 2H, 2′-$H_{a,b}$), 3.52 (m, 2H, 5′-H), 3.82 (m, 1H, 4′-H), 4.03 (s, 3H, OCH_3), 4.36 (m, 1H, 3′-H), 4.89 (t, 1H, 5′-OH, J = 5.4 Hz), 5.27 (d, 1 H, 3′-OH, J = 3.8 Hz), 6.50 (d, 1H, 5-H, J = 3.7 Hz), 6.51 (m, 1H, 1′-H), 7.45 (d, 1H, 6-H, J = 3.7 Hz), 9.44 (d, 1H, NH, J = 9.7 Hz), 10.73 (d, 1H, HCO, J = 9.7 Hz). Anal. calcd. for $C_{13}H_{16}N_4O_5$ (308.3): C, 50.64; H, 5.23; N, 18.17. Found: C, 50.80; H, 5.18; N, 18.22.

7-[2-Deoxy-5-O-[bis(4-methoxyphenyl)phenylmethyl]-β-D-erythro-pentofuranosyl]-2-[(formyl)amino]-4-methoxy-7H-pyrrolo[2,3-d]pyrimidine (4b):

Compound 3c (500 mg, 1.6 mmol), dissolved in anhydrous pyridine, was stirred for 2 h with 4,4′-dimethoxytrityl chloride (610 mg, 1.8 mmol) and N-ethyl diisopropylamine (300 µL, 1.7 mmol) at room temperature. The solvent was evaporated in vacuo and coevaporated with toluene (5 mL).

The residue was redissolved in CH_2Cl_2 and chromatographed on silica gel (column: 15x4 cm, solvent C). The main zone was concentrated (3 mL) and added to a mixture of n-hexane-diethyl ether (1:1). The colorless precipitate (772 mg, 78%) was isolated. TLC (silica gel, solvent E) R_f 0.3. UV (MeOH) λ_{max} 234, 277 (ε 38400, 17700). ^1H NMR ([D_6]DMSO) δ 2.23, 2.51 (2m, 2H, 2'-$H_{a,b}$), 3.14 (m, 2H, 5'-$H_{a,b}$), 3.72 (s, 6H, 2 OCH_3), 3.92 (m, 1H, 4'-H), 4.03 (s, 3H, OCH_3), 4.37 (m, 1H, 3'-H), 5.45 (d, 1H, 3'-OH, J = 4.2 Hz), 6.49 (d, 1H, 5-H, J = 3.7 Hz), 6.51 (m, 1H, 1'-H), 6.81, 7.25 (2m, 13 aromat. H), 7.29 (d, 1H, 6-H, J = 3.7 Hz), 9.43 (d, 1H, OCH, J = 9.8 Hz), 10.78 (d, 1H, NH, J = 9.8 Hz). Anal. calcd. for $C_{34}H_{34}N_4O_7$ (610.6): C, 66.87; H, 5.61; N, 9.18. Found: C, 66.98; H, 5.63; N, 9.15.

7-[5-O-[Bis(4-methoxyphenyl)phenylmethyl]-[3-O-(diisopropylamino)methoxyphosphino]-2-deoxy-β-D-erythro-pentofuranosyl]-2-[(formyl)amino]-4-methoxy-7H-pyrrolo[2,3-d]pyrimidine (5b):

Compound 5b was prepared as described for 5a, starting with 4b (250 mg, 0.4 mmol). The reaction product was purified by flash chromatography on silica gel (column: 15x2 cm, solvent D). From the main zone (268 mg, 85%) was obtained. TLC (silica gel, solvent G) R_f 0.7. ^{31}P NMR ($CDCl_3$) δ 147.17 ppm.

7-[5-O-[Bis(4-methoxyphenyl)phenylmethyl]-[3-O-β-cyanoethoxy-(diisopropylamino)phosphino]-2-deoxy-β-D-erythro-pentofuranosyl]-2-[(formyl)amino]-4-methoxy-7H-pyrrolo-[2,3-d]pyrimidine (6b):

Compound 6b was prepared as described for 6a, except that 4b (250 mg, 0.38 mmol) was used. Flash chromatography on silica gel (column, 10x2 cm, solvent C) afforded colorless amorphous 6c (268 mg, 81%). TLC (silica gel, solvent E) R_f 0.9. ^{31}P NMR ($CDCl_3$) δ 147.06, 147.23 ppm.

Oligonucleotide Synthesis.

The oligomers 7 -14 were synthesized on an Applied Biosystems DNA-synthesizer (Model 680 B) employing

methoxyphosphoramidites (5a and 5b) or cyanoethyl amidites (6a and 6b) together with the regular phosphoramidites [11,12]. Synthesis was carried out on solid support (CPG) in a 1 μmol scale. The reaction cycle of detritylation, coupling, oxidation, and capping followed the user manual (Edition 1986). Cleavage of the CH_3-protecting group was accomplished by the action of thiophenol. Removal of the oligonucleotide from the solid support was carried out with concentrated ammonia at room temperature on the stage of the 5′-tritylated oligomers. This procedure also removed the cyanoethyl protecting group. Further incubation (60^0C, 96 h in case of compounds 5a and 6a, and 24 h for the compounds 5b and 6b) cleaved the nucleobase protecting groups. The 5′-protected oligomers 15 - 22 were purified by HPLC (solvent I). Detritylation was performed by 80% acetic acid for 5 min. After removal of the acid the oligomer was dissolved in water (5 mL) and extracted with diethyl ether. The deprotected oligomers were then purified by reverse-phase HPLC employing solvent system II. Samples of the main HPLC zones were lyophilized and desalted by reverse phase HPLC with solvent system IV. After lyophilization the oligomers (15-20 A_{260} units) were dissolved in water (100 μL) and stored frozen at -20^0C. The following oligomers were obtained (HPLC data are given in brackets).

d(CGCGCG)	(7)	[11.0 min]
d(CGCm^{6}c^{7}GCG)	(8)	[11.0 min]
d(GCGCGC)	(9)	[11.1 min]
d(GCm^{6}c^{7}GCGC)	(10)	[11.4 min]
d(CGCGAATTCGCG)	(11)	[11.8 min]
d(Cm^{6}c^{7}GCGAATTCGCG)	(12)	[11.8 min]
d(CGCm^{6}c^{7}GAATTCGCG)	(13)	[11.8 min]
d(CGCGAATTCm^{6}c^{7}GCG)	(14)	[11.8 min]
5′-DMT d(CGCGCG)	(15)	[13.0 min]
5′-DMT d(CGCm^{6}c^{7}GCG)	(16)	[13.2 min]
5′-DMT d(GCGCGC)	(17)	[13.1 min]
5′-DMT d(GCm^{6}c^{7}GCGC)	(18)	[13.2 min]
5′-DMT d(CGCGAATTCGCG)	(19)	[10.5 min]
5′-DMT d(Cm^{6}c^{7}GCGAATTCGCG)	(20)	[10.8 min]
5′-DMT d(CGCm^{6}c^{7}GAATTCGCG)	(21)	[10.8 min]
5′-DMT d(CGCGAATTCm^{6}c^{7}GCG)	(22)	[10.9 min]

HPLC Separation.

HPLC was carried out on 4x250 mm (10 µm) RP-18 LiChrosorb column (Merck, FRG) which was connected with a 4x25 mm RP-18 LiChrosorb precolumn (Merck, FRG) using a Hitachi HPLC with one pump (model 655A-12), a variable wavelength monitor (model 655A), and a controller (model L-5000), connected with an integrator (Hitachi, model D-2000). The following gradients containing triethylammonium acetate, pH 7.0/5% CH_3CN (A), CH_3CN (B), water (C), and CH_3OH-H_2O (3:2) (D) were used: I: 15 min (15 - 60 % B) in A; II: 15 min (0 -25 % B) in A; III: 30 min (0-20 % B) in A; IV: 15 min C, 10 min D.

Enzymatic Hydrolysis of the Oligomers 7 - 14:

The oligomers (appr. 0.3 A_{260} units) dissolved in 0.1 M TRIS/HCl buffer, pH 8.5 (500 µL) were digested with snake venom phosphodiesterase (2 µg) for 1 h at 37°C. Further incubation with alkaline phosphatase (1 µg, 25°C, 30 min) yielded a mixture of the nucleosides. After separation on HPLC (solvent system III) quantification was made on the basis of the peak areas and the extinction coefficients of the nucleosides (dC 7300, dG 11400, dA 15400, dT 8800, 1 9600).

Melting Curves.

The melting curves were measured in Teflon stoppered cuvettes with 1 cm light path length in a thermostatically controlled cell holder with a Shimadzu 210-A spectrophotometer connected with a Kipp & Zonen BD 90 recorder. The increase of absorbance at the appropriate wavelength was recorded while the temperature of the solution was increased linear at a rate of 20°C/h using a Lauda PM-350 programmer and a Lauda RCS-6 bath equipped with a R 22 unit (MWG Lauda, FRG). The actual temperature was measured in the probe cell with a Pt-resistor.

Phosphodiester Hydrolysis of the Oligomers 11 - 14 with the Endodeoxyribonuclease Eco RI.

The oligonucleotides (2.5 µM single strand concentration), dissolved in water (1 mL), containing 10 mM TRIS/HCl,

20 mM $MgCl_2$, and 80 mM NaCl at pH 7.5 were hydrolyzed with the endodeoxyribonuclease Eco RI (100 units). [One unit is the enzyme activity that completely cleaves 1 µg λ DNA in 1 h at 37^0 in the incubation buffer (100 mM TRIS/HCl, 50mM NaCl, and 10 mM $MgCl_2$, pH 7.5 at 37^0) in a total volume of 50 µL]. The reaction mixture was incubated at 30^0C. Aliquots were analysed at different intervals of time by HPLC (solvent system III). The amount of the reaction was calculated according to Dwyer-Hallquist et al [23].

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