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Solid-phase synthesis of the self-complementary hexamer d(c^{7}GpCpc^{7}GpCpc^{7}GpC) via the O-3'-phosphoramidite of 7-deaza-2'-deoxyguanosine

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ABSTRACT

The synthesis of the O-3'-phosphoramidite of a suitably protected 7-deaza-2'-deoxyguanosine (c^{7}G) which is an isostere of 2'-deoxyguanosine is described. The phosphoramidite of the modified nucleoside was used in the synthesis of the self-complementary hexamer d(c^{7}GpCpc^{7}GpCpc^{7}GpC) on functionalized silica gel in a mini-reactor. As expected from the parent hexamer d(GpCpGpCpGpC) the isosteric d(c^{7}GpCpc^{7}GpCpc^{7}GpC) exhibits a rigid secondary structure (22% hypochromicity at 280 nm) and forms a duplex in 1 M aqueous sodium chloride solution. Due to the altered π-electron system of the pyrrolo[2,3-d]pyrimidine nucleobase, which affects base stacking and hydrogen bonding, the T_m of the modified duplex is decreased by 10°C compared to that of the parent purine hexamer. Moreover, it is expected that the incorporation of c^{7}G influences the pitch of the helix.

INTRODUCTION

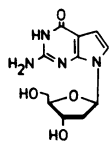
The rare nucleoside queuosine (2a)[1,2] and derivatives thereof[3] have been detected in the wobble position of the anticodon of tRNAs. In addition the monomerically occuring pyrrolo[2,3-d]pyrimidine nucleoside cadeguomycin (2b) has been isolated as a fermentation product of Streptomyces hygroscopicus[4]. The parent nucleoside of both naturally occuring compounds is 7-deazaguanosine (2c) which was synthesized in our laboratory several years ago[5]. Polymerisation of 7-deazaguanosine-5'-diphosphate with polynucleotide phosphorylase from Micrococcus luteus yielded a homopolymer[6] which forms a duplex with poly(C), but is not able to bind a second strand of poly(C) via Hoogsteen base pairing. Because of the interesting properties of the ribonucleoside 2c we initiated the synthesis of the 2'-deoxynucleoside 1[7], which is an isostere of 2'-deoxyguanosine (3).

Modern strategies of oligonucleotide synthesis which have been developed for regular nucleosides[8] can also be applied to rare nucleosides if appropriately protected monomers are available. Since this technique is performed on polymeric support[9] and makes use of nucleoside phosphoramidites[10], we decided to synthesize a suitably protected phosphoramidite of 7-deaza-2'-deoxy-

guanosine (1)[11], which was then used in the solid-phase synthesis of the self-complementary hexamer d(c^{7}GpCpc^{7}GpCpc^{7}GpC) (12).

RESULTS AND DISCUSSION

Starting material for the synthesis of the hexamer 12 was the nucleoside 1. It was obtained by phase-transfer glycosylation of 2-amino-4-methoxy-7H-pyrrolo[2,3-d]pyrimidine[7] with 1-chloro-2-deoxy-3,5-di-O-p-toluoyl-D-erythro-pentofuranose[12] via the methoxynucleoside 4a. In previous experiments, sodium p-thiocresolate was chosen for cleavage of the ether linkage. In later experiments we favored HBr/glacial acetic acid in absolute tetrahydrofuran[11]. This reaction had to be carried out under strictly anhydrous conditions to prevent cleavage of the glycosydic bond.



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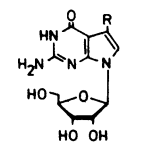
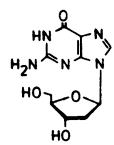


2a: R = CH_2NH–
b: R = CO_2H
c: R = H



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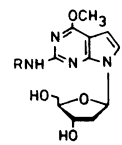


4a: R = H
b: R = iBu

From studies on N-1 alkyl-pyrazolo[3,4-d]pyrimidines[13] and their corresponding 2'-deoxynucleosides[14], it became evident that the methoxy group of the pyrimidine moiety is easily replaced by a hydroxyl function in a nucleophilic displacement reaction in dilute sodium hydroxide at room temperature. Since the 2'-deoxyribofuranoside moiety is stable under these conditions we employed similar reaction conditions on the pyrrolo[2,3-d]pyrimidine nucleoside 4a. Due to the lower reactivity of 4a this nucleophilic displacement afforded much more vigorous reaction conditions than that using the corresponding pyrazolo[3,4-d]pyrimidine. Quantitative conversion of compound 4a into the nucleoside 1 was achieved by treatment of compound 4a with 2 N sodium hydroxide at 100°C for 5 h. The crystalline nucleoside was isolated after neutralization of the alkaline reaction mixture without any chromatographic purification.

In order to use the nucleoside 1 in oligonucleotide synthesis, the 2 -amino group was first protected. As already described for the protection of 2'-deoxyguanosine the isobutyryl residue was considered for 2-amino protection of compound 1. This residue is sufficiently stable during oligonucleo-

Table 1. ^{13}C NMR chemical shifts of 7-deaza-2'-deoxyguanosines[a].

	1	5a	5b	5c
C-2	152.5	146.5	146.5	146.7
C-4	158.5	156.3	156.4	156.5
C-4a	100.1	104.2	103.4	104.2
C-5	102.1	103.0	102.3	102.7
C-6	116.7	118.8	119.2	119.0
C-7a	150.5	147.5	147.3	147.5
C-1'	82.2	81.0	82.4	82.5
C-2'	39.5	35.6	39.5	-
C-3'	70.8	74.0	70.7	70.6
C-4'	86.9	82.6	87.0	85.3
C-5'	61.9	63.3	61.8	64.0
CH_3	-	18.3/18.5	18.6	18.6
CH	-	34.0/32.9	34.5	34.5
C=O	-	179.6/175.5	179.6	179.8

a) Chemical shifts in δ ppm downfield from internal Me_4Si in Me_2SO-d_6 solution.

tide synthesis and can be split off under mild alkaline conditions.

For regular 2'-deoxynucleosides, like 2'-deoxyguanosine, a highly efficient method for regioselective acylation of the nucleobase amino groups has been developed[15,16]. Transient protection of the sugar hydroxyls with trimethylsilyl residues allows selective isobutyrylation of the 2-amino group of compound 3. The temporarily employed silyl residues are then removed with aqueous pyridine or ammonia in a one-flask reaction.

Application of this method to compound 1 failed and unchanged starting material was isolated after the work-up procedure. These results, which are due to the low nucleophilicity of the 2-amino group in pyrrolo[2,3-d]pyrimidines, complicated the protection of the nucleoside 1. In order to overcome this problem the classic Khorana procedure was employed[17]. Isobutyrylation of the nucleoside 1 with isobutyric anhydride in pyridine solution resulted in the formation of the triisobutyrylated compound 5a. The material was obtained in crystalline form after purification by silica gel chromatography. The ^1H and ^{13}C NMR spectra (table 1) of compound 5a confirmed that each of the sugar hydroxyls and the 2-amino group carried one isobutyryl residue. Selective removal of the hydroxyl protecting groups with ammonia failed. Monitoring the reaction on TLC (silica gel, solvent C) indicated simultaneous removal of all protecting groups.

If aqueous sodium hydroxide was used instead of ammonia (0°C, pH 12.5), selective removal of the sugar protecting groups took place without affecting the N^2-isobutyryl residue. This selective process is due to deprotonation of

the chromophore moiety of compound 5a at N-3 which stabilizes the amide function; the negatively charged chromophore is also less susceptible to hydroxyl ions.

The structure of 5b was confirmed from its ^{13}C NMR spectrum which exhibited similar chemical shifts for the sugar hydroxyls as the unprotected nucleoside 1, but showed chromophore signals which are located at similar positions as found for the isobutyrylated 5a.

In the course of protection of the 2-amino group in 2-amino-7H-pyrrolo-[2,3-d]pyrimidine 2'-deoxy-ß-D-ribofuranoside it was noted that the isobutyryl protecting group could not be hydrolyzed under mild alkaline conditions[18]. Since the hydrolysis in ammonia is a prerequisite for a successful oligonucleotide synthesis, we studied the kinetics of the deprotection reaction and compared it with isobutyrylated 2'-deoxyguanosine and the methoxy compound 4b. The latter was obtained from compound 4a[7] by a similar procedure as described for compound 5b.

The reactions were followed photometrically at wavelengths where the differences of the UV spectra of educt and product were most pronounced. As can be seen from figure 1a, deisobutyrylation of the deazapurine nucleoside 5b occured at only a slightly decreased rate compared to N^2-isobutyryl-2'-deoxyguanosine[19,20] in 1 N NaOH-MeOH (1:1, v/v) solution. In comparison the methoxy compound 4b is deprotected at a much lower rate. If the reaction is carried out in a more dilute sodium hydroxide solution (figure 1b) the deprotection rates of isobutyryl-2'-deoxyguanosine and of compound 5b are only slightly reduced, whereas the rate of the methoxy compound is strongly affected. These findings imply that the cleavage of the amide bond is controlled by the lactam anion of 5b. Since the methoxy nucleoside 4b cannot form an anion at N-3, we conclude that the N-3 anion of compound 5b controls this reaction rate. If the deprotonation were to occur at the nitrogen of the amide function of C-2 the pronounced differences between the kinetics of the nucleosides 4b and 5b at different sodium hydroxide concentrations could not be explained.

Treatment of compound 5b with 4,4'-dimethoxytrityl chloride in the presence of 4-dimethylaminopyridine yielded the protected nucleoside 5c. It was purified by flash chromatography[21] and obtained in amorphous form after lyophilisation. Regioselective 5'-tritylation was confirmed by ^{13}C NMR spectroscopy. The spectrum showed a downfield shift for carbon-5' and an upfield shift of C-4', both indicating a 5'-alkylation. Moreover, the triplet of OH-5' of the starting material 5b could not be detected in the ^{1}H NMR spectrum of

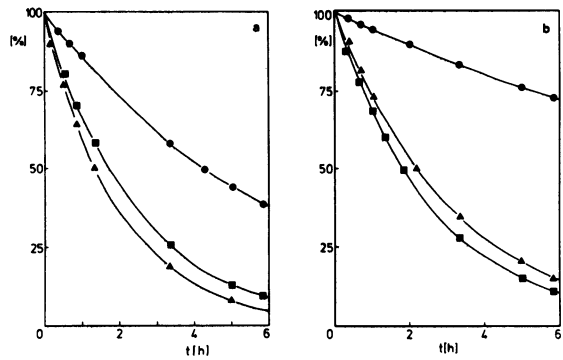
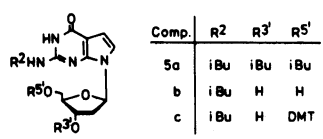


Fig. 1: Alkaline hydrolysis of the isobutyryl residues of isobutyryl 2'-deoxyguanosine (▲-▲), compound 5b (■-■), and 4b (●-●) in 1 N NaOH-MeOH (1:1,v/v) (a) and 0.2 N NaOH-MeOH (1:1,v/v) (b) at 25°C. The kinetics were UV-spectrophotometrically followed at 300 nm (isobutyryl 2'-deoxyguanosine), 310 nm (compound 5b), and 258 nm (compound 4b).

compound 5c. As expected, the cleavage of the 4,4'-dimethoxytrityl (DMT) group is accomplished under the same conditions (80% acetic acid) as employed for the corresponding purine nucleoside.

The conversion of the protected nucleoside 5c into the phosphoramidite 6 followed a procedure which was originally developed by Caruthers for regular nucleosides[10]. As phosphitylating reagent chlorodiisopropylamino-methoxyphosphine[10] was chosen which was obtained as a colorless liquid after distillation over cesium fluoride.



Comp.	R^2	$R^{3'}$	$R^{5'}$
5a	iBu	iBu	iBu
b	iBu	H	H
c	iBu	H	DMT

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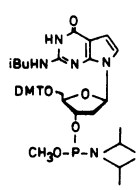

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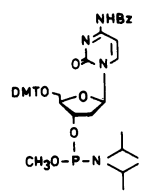

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Phosphitylation of compound 5c was carried out using a 1.5 fold excess of the reagent in anhydrous dichloromethane in the presence of ethyldiisopropylamine. Under these conditions the reaction was complete after half an hour. TLC (silica gel, solvent H) showed the two main spots of the diastereoisomers of 6 at about R_F 0.8 and a small amount of starting material (R_F 0.4). The latter was removed using flash chromatography (silica gel 60 H, solvent G)[22].

Table 2. ^{1}H NMR chemical shift data of phosphine derivatives[a].

	chlorodiisopropylaminomethoxyphosphine	compound 8
$(CH_3)_2C$	1.19 (s, broad)	1.25,1.26 (2d, J = 2.8 Hz)
HCN	3.68 (m)	3.49 (m)
CH_3OP	3.55 (d, J_P = 14 Hz)	3.65 (d, J_P = 12 Hz)
PH	-	6.84 (d, J_P = 630 Hz)

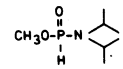
[a]All spectra were recorded in $CDCl_3$, chemical shifts are given as δ-values relative to TMS as internal standard; coupling constants in parantheses are given in hertz.

The material of the main zone was isolated as an amorphous solid, exhibiting a signal at 150.12 ppm in the ^{31}P NMR spectrum. A large excess of phosphitylating reagent gave rise to a side product exhibiting a ^{31}P NMR signal at 15.19 ppm. This signal increased if more phosphitylating reagent was used.

It has been suggested that the ^{31}P NMR signal around 15 ppm is due to a hydrolysis product of chlorodiisopropylaminomethoxyphosphine[23]. In order to establish the structure of this compound we isolated the material from the reaction mixture. The ^{1}H NMR spectrum of the crude reaction product implied that compound 8 was formed. Since its isolation in the presence of the phosphoramidite 6 was laborious, we decided to run the phosphitylating reaction in the absence of the nucleoside 5c. After the work-up procedure a colorless liquid was isolated and obtained pure after distillation. It exhibited a ^{31}P signal at 15.19 ppm which was located at the same position as the by-product from the phosphitylation reaction. The ^{13}C NMR spectrum of the compound showed a similar pattern as that of the starting material but with altered chemical shifts and coupling constants. The structure of compound 8 was finally confirmed by the ^{1}H NMR spectrum (table 2).

The phosphoramidite 6 was then used in the synthesis of the self-complementary hexamer 12 on polymeric support. As matrix macroporous silica (100 μm pore size) was used. It was functionalized by refluxing with 3-aminopropyltriethoxysilane in dry toluene. Succinic anhydride was next reacted with N^4-benzoyl-4,4'-dimethoxytritylcytidine and activated as the p-nitrophenylester. The latter was condensed with the functionalized matrix to give 9[24]. The content of the bound material was determined on the basis of liberated DMT-residues following a known procedure[24] and was 20 μmol/g of the polymer 9.

The polymer 9 was put into the oligonucleotide mini-reactor (see Experimental Section) connected with male-female Luer adapters. Five alternat-



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DMTd($c^7G^{iBu}pC^{Bz}pc^7G^{iBu}pC^{Bz}pc^7G^{iBu}pC^{Bz}$)-Suc-APSi

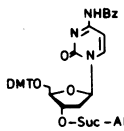
10

DMTd(c^7GpCpc^{7}GpCpc^{7}GpC)

11

d(c^7GpCpc^{7}GpCpc^{7}GpC)

12



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c^7G = 1; Suc = Succinyl; AP = Aminopropyl

ing reaction cycles, starting with the phosphoramidite 6 and continuing with the amidite 7[10] were carried out according to the procedure of Seliger[25]. Three distinct chemical transformations are then performed:

1) Demethylation of the polymer bound oligomer 10 with thiophenol-triethylamine-dioxane.
2) Removal of the oligonucleotide by the action of a 25 % solution of aqueous ammonia.
3) Deacylation of the amino protecting groups with conc. ammonia at 60°C.

Purification of the DMT-hexamer 11 was accomplished by reverse-phase HPLC (figure 2a). The 5'-protecting group of compound 11 was split off with

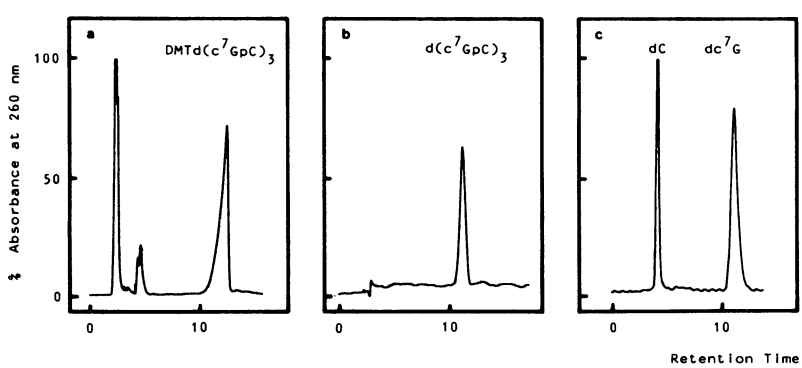

Fig. 2: HPLC elution profile of: (a) The crude reaction mixture of the deacylated DMT-hexamer 11 with gradient I as mobile phase; retention time 12.4 min. (b) The purified hexamer 12 with solvent system II; retention time 11.7 min. (c) The enzymatically hydrolized hexamer 12 with solvent system III; digestion of 12 was performed with snake venom phosphodiesterase followed by alkaline phosphatase (see experimental section); retention times 4.1 min (dC) and 11.4 min (dc^7G).

80% acetic acid, tritylcarbinol was extracted with ether, and after lyophilisation the hexamer 12 was obtained in 13.7 % yield on the basis of the polymer bound starting material 9.

As the HPLC pattern of compound 12 demonstrates (figure 2b) the resulting hexamer was pure. It exhibits a UV spectrum with a maximum at 265 nm.

The structure of the hexamer was confirmed by hydrolysis with snake venom phosphodiesterase to give the nucleoside 1, its 5'-monophosphate, and dCMP. Quantification was accomplished after subsequent digestion with alkaline phosphatase. From the HPLC pattern (figure 2c) it was shown that hydrolytic cleavage yields three equivalents of the nucleoside 1 and three equivalents of dC. Since the polymer 9 was used as starting material and alternating cycles of addition reactions of the phosphoramidites 6 and 7 were performed, the structure of the hexamer corresponds to formula 12.

The base composition of 12 implies that a rigid structure is formed in aqueous solution which is partially reflected by the hypochromicity. Complete digestion of 12 with snake venom phosphodiesterase resulted in an increase of its UV absorbance at 280 nm. From that a hypochromicity of 22% was calculated.

From the alternating sequence of the hexamer 12, duplex formation should occur under appropriate reaction conditions. Since duplex formation can be derived from thermal cooperativity, the melting of compound 12 was investigated. On recording the absorbance between 10°C and 80°C at 280 nm of compound 12 in 0.1 M TRIS-HCl buffer pH 8.5, a continuous increase was observed without showing a cooperative process.

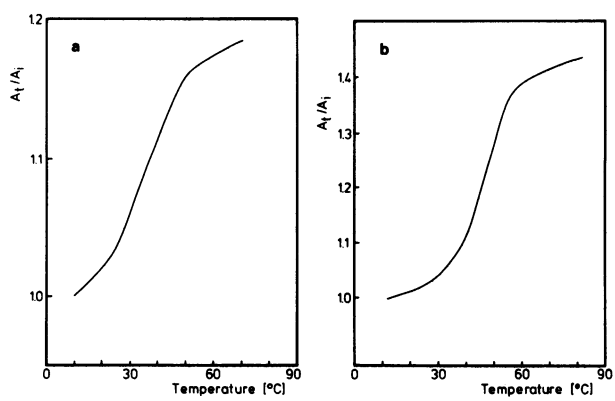

Fig. 3: Melting profiles of (a) $d(c^7GpC)_3$ and (b) $d(GpC)_3$ in H_2O, containing 1.0 M NaCl and 45 mM cacodylate, pH 7.0. A_t/A_i is the ratio of absorbance at a given temperature (t) to that at the initial temperature (i).

Earlier work on the conditions for double-helix formation of regular $d(GpC)_3$[26] has shown that a duplex is formed at high ionic strength. In 1.0 M sodium chloride in the presence of 45 mM sodium cacodylate (pH 7.0) a cooperative melting was observed (figure 3b), indicating a helix-coil transition from $(d(GpC)_3)_2$ to 2 $d(GpC)_3$. When the thermal melting of the hexamer 12 was followed under the same conditions, a similar melting profile was observed (figure 3a). However, the T_M of this compound was only 35°C, compared to 45°C for the duplex of $d(GpC)_3$.

The decreased T_M (10°C) of the duplex 12 compared to the regular hexamer can be explained by two facts: (i) an altered overlap of the π-electron systems of the modified GC base pairs in this duplex compared to that of the nonmodified hexamer. This may lead to an altered pitch of the hexamer helix; (ii) a destabilization of the Watson-Crick base-pair due to the differences in the abilities of compound 1 and 3 to act as a donator or acceptor of protons. A change in the hydrogen bonding scheme should be reflected by the pK values of compounds 1 and 3. The pK_a values were determined spectrophotometrically in Teorell-Stenhagen[27] buffer solution at 300 nm and were found to be 10.3 for the modified nucleoside 1 and 9.3 for dG 3. This shows that the 7-deaza purine nucleoside is a less efficient hydrogen donor at N-3 compared with the corresponding purine nucleoside. Since this would result in a destabilization of the Watson-Crick base-pair between compound 1 and dC a destabilization of the duplex would be the immediate consequence. To study this in more detail, investigations on other oligomers containing 7-deaza-2'-deoxyguanosine 1 as monomeric unit are in progress.

EXPERIMENTAL SECTION

Melting points were determined on a Linström apparatus (Wagner and Munz, FRG) and are not corrected. Elemental analyses were performed by Mikroanalytisches Labor Beller (Göttingen, FRG). NMR spectra were recorded on a Bruker WM 250 spectrometer; δ values are in ppm relative to tetramethylsilane as internal standard (^1H and ^{13}C) or to external 85% phosphoric acid (^{31}P). Chemical shifts are positive when downfield from the appropriate standard. UV spectra were recorded on a Uvicon 810 spectrometer (Kontron, Switzerland). 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) and Amberlite XAD-4 resin (Serva, FRG) were used for column chromatography, flash chromatography was performed with silica gel 60 H (Merck, FRG) at 0.5 bar (N_2). Solvent systems for TLC: (A) H_2O-isopropanol (9:1), (B) $CHCl_3$-MeOH

(98:2), (C) $CHCl_3$-MeOH (95:5), (D) $CHCl_3$-MeOH (9:1), (E) CH_2Cl_2-acetone (8:2), (F) CH_2Cl_2-acetone (7:3), (G) CH_2Cl_2-ethyl acetate-triethylamine (70:25:5), (H) CH_2Cl_2-ethyl acetate-triethylamine (70:30:5). Pyridine and diisopropylethylamine were distilled from KOH and stored over 4 Å molecular sieves. Dioxane was filtered through a bed of aluminum oxide (Woelm basic, grade I). Dichloromethane was distilled from P_2O_5 and then redistilled from CaH_2. Acetonitrile was pre-dried with K_2CO_3 and then distilled from CaH_2. Tetrahydrofuran was distilled under argon from Na-K alloy (5:1)/benzophenone.

Snake venom phosphodiesterase (EC 3.1.16.1, Crotallus durissus) and alkaline phosphatase (EC 3.1.3.1, E. coli) were products of Boehringer, Mannheim, FRG). 2'-Deoxycytidine was purchased from Pharma-Waldhof (FRG).

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 recording spectrophotometer connected with a Kipp & Zonen BD 90 recorder. The increase of absorbance at the appropriate wavelength as a function of time was recorded while the temperature of the solution was increased linearly with time at a rate of 30°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 reference cell with a Pt-resistor.

Determination of hypochromicity. The hypochromicity was calculated by the formula: $H = (\varepsilon_{monomer} - \varepsilon_{polymer})/\varepsilon_{monomer} \times 100$ by enzymatic cleavage. The oligomer was digested with snake venom phosphodiesterase in TRIS-HCl buffer (0.1 M, pH 8.5). Hypochromicity was calculated from the absorbance before and after the cleavage.

HPLC separation. High performance liquid chromatography was carried out on 4 x 250 (10 µm) or 7 x 250 (7µm) RP-18 LiChrosorb columns (Merck,FRG) using a LKB HPLC with two pumps (model 2150), a variable wavelength monitor (model 2152), and a controller (model 2151), connected with an integrator (Hewlett Packard 3390^A). The gradients consisting of 0.1 M triethylammonium acetate (A) pH 7.0 and acetonitrile (B) were used in the following order: Gradient I: 5 min (25% B), 5 min (25-30% B), 5 min (30% B); gradient II: 15 min (9-12% B); gradient III^a:15 min 5% B. Flow rates of gradient I were 4.5 mL min^{-1} (preparative column) and 1 mL min^{-1} (analytical column); the gradients II and III were run with 1 mL min^{-1}; [a] 0.1 M ammonium acetate.

Mini-reactor for oligonucleotide synthesis. Solid-phase synthesis was carried out in a HPLC LiChroCART-cartridge (25-4). This cartridge was placed in a manu-fix 25-4 device (Merck, Darmstadt, FRG) equipped with a male

and female Luer adapter. The unit can then be connected with a syringe and a needle.

2-Amino-7-(2'-deoxy-β-D-erythro-pentofuranosyl)-3,7-dihydro-4H-pyrrolo[2,3-d]-pyrimidin-4-one (1).

a) Preparation by ether cleavage

Compound 4a[7] (100 mg, 0.36 mmol) in abs. tetrahydrofuran (30 mL) was treated with 30% hydrobromic acid in acetic acid (2 mL) and heated under reflux for 10 min. After cooling the solution was neutralized with aq. ammonium hydrogen carbonate and evaporated. The excess of acid was removed by repeated coevaporation with methanol- water (1:1,v/v). The residue was dissolved in water, applied to an amberlite XAD-4 column (20 x 2 cm) and chromatographed with solvent (A). Isolation of the material of the main zone resulted in amorphous material (61 mg, 64%) which yielded colorless crystals by recrystallization from water; ^{1}H NMR (Me_2SO-d_6) δ 6.26 (d,1H, H-5, J = 3.6 Hz), 6.92 (d, 1H, H-6, J = 3.6 Hz). Spectral and TLC properties were identical to those of an authentic sample[7].

b) Preparation by nucleophilic displacement

Compound 4a (1 g, 3.6 mmol) in 2 N aqueous sodium hydroxide (25 mL) is heated under reflux for 5h. The solution was cooled and neutralized with 2 N acetic acid. The resulting precipitate was filtered off and recrystallized from water as colorless needles (661 mg). The filtrate was chromatographed on an Amberlite XAD-4 column with solvent (A). From the main zone another 254 mg were isolated and crystallized which resulted in a total yield of 96% of crystalline 1; mp 262-265°C[7].

7-(β-D-2'-Deoxy-erythro-pentofuranosyl)-O-3',5'-diisobutyryl-2-isobutyryl-amino-3,7-dihydro-4H-pyrrolo[2,3-d]pyrimidin-4-one (5a).

Compound 1 (500 mg, 1.9 mmol) was dried by repeated evaporation with anhydrous pyridine. The residue dissolved in pyridine-isobutyric anhydride (40 mL, 1:1, v/v) was heated under reflux for 3 h. The solvent was evaporated in vacuo and the residue chromatographed on a silica gel column (15 x 4 cm, solvent B). Separation of the main zone and evaporation of the solvent resulted in a crude reaction product which was crystallized from methanol; colorless crystals (787 mg, 87%) which sintered at 65°C and melted at 96°C; TLC (silica gel, solvent C), R_f 0.7; UV (methanol) λ_{max} 268, 294 nm (ε 13 900, 13 400); ^{1}H NMR ($CDCl_3$) δ 1.11, 1.12, 1.19 (18H, 3d, 6 CH_3, J = 6.9 Hz), 2.32 (1H, m, H-2'b), 2.85 (1H, m, H-2'a), 4.22 (2H, m, H-5'), 4.68 (1H, m, H-4'), 5.37 (1H, m, H-4'), 6.17 (1H, dd, H-1', J = 6 Hz), 6.58 (1H, d, H-5, J = 3.6 Hz), 8.87 (1H, s, NHCO), 11.75 (1H, s, NH).

Anal. Calcd for $C_{23}H_{32}N_4O_7$: C, 57.97; H, 6.77; N, 11.76. Found: C, 58.36; H, 6.58; N, 11.73.

7-(β-D-2'-Deoxy-erythro-pentofuranosyl)-2-isobutyrylamino-3,7-dihydro-4H-pyrrolo[2,3-d]pyrimidin-4-one (5b).

Compound 5a (1.0 g, 2.1 mmol) was dissolved in methanol (40 mL) and kept at 0°C while an ice cold solution of 2 N sodium hydroxide was added gradually with stirring until the pH value rose to 12.5. The reaction was stopped after 20 min by addition of ion exchanger (Dowex W X 8, pyridinium form). The neutralized solution was filtered, the ion exchange resin was washed with methanol and the filtrate and the washings were combined. The solvent was evaporated in vacuo and the residue crystallized from a small amount of water. Colorless crystals (560 mg, 79%), mp 208-213°C; TLC (silica gel, solvent D), R_f 0.4; UV (methanol) λ_{max} 269, 293 nm (ε 12 900, 12 800); ^{1}H NMR (Me_2SO-d_6) δ 1.11 (6H, 2 CH_3, J = 6.8 Hz), 2.13 (1H, m, H-2'b), 2.39 (1H, m, H-2'a), 2.75 (2H, m, CH), 3.50 (2H, m, H-5'), 3.78 (1H, m, H-3'), 4.32 (1H, m, H-4'), 4.91 (1H, t, OH-5', J = 5.4 Hz), 5.25 (1H, d, OH-3', J = 3.4 Hz), 6.41 (1H, dd, H-1', J = 6 Hz), 6.48 (1H, d, H-5, J = 3.6 Hz), 7.25 (1H, d, J = 3.6 Hz), 11.50, 11.80 (2H, 2s, NHCO, NH). Anal. Calcd for $C_{15}H_{20}N_4O_5$: C, 53.56; H, 5.99; N, 16.66. Found: C, 53.50; H, 6.06; N, 16.66.

7-(β-D-2'-Deoxy-erythro-pentofuranosyl)-5'-O-(4,4'-dimethoxytrityl)-2-isobutyrylamino-3,7-dihydro-4H-pyrrolo[2,3-d]pyrimidin-4-one (5c).

Compound 5b (500 mg, 1.5 mmol) was dried by coevaporation with anhydrous pyridine in vacuo. The material was dissolved in pyridine-dioxane (5 mL, 3:2, v/v), 4-dimethylaminopyridine (16 mg, 0.12 mmol) and 4,4'-dimethoxytrityl chloride (677 mg, 2 mmol) were added and the solution stirred for 3 h at room temperature. The reaction was monitored on TLC (silica gel, solvent F). To the solution 5% aqueous potassium hydrogen carbonate (50 mL) was added and the resultant was extracted twice with dichloromethane. The combined organic layers were dried with sodium sulfate, filtered, and the solvent was evaporated. The residue was applied to a 20 x 4 cm column (silica gel 60 H, solvent E) and separated by flash chromatography. Isolation of the material of the main zone and lyophilization (dioxane) yielded colorless, amorphous 5c (732 mg, 76%). TLC (silica gel, solvent F) R_f 0.7; UV (methanol) λ_{max} 272, 282, 294 nm (ε 15 100, 13 900, 12 500); ^{1}H NMR ($CDCl_3$) δ 1.05, 1.08 (6H, 2d, 2 CH_3, J = 3.6 Hz), 2.25 (2H, m, H-2'b, CH), 2.46 (1H, m, H-2'a), 2.88 (1H, d, OH-3', J = 2.1 Hz), 3.27 (2H, m, H-5'), 3.73 (6H, s, 2 OCH_3), 4.10 (1H, m, H-4'), 4.51 (1H, m, H-3'), 6.44 (1H, m, H-1'), 6.58 (1H, d, H-5, J = 3.5 Hz), 6.77, 7.28 (15H, m, arom. H and H-6), 11.75 (1H, s, NH).

Anal. Calcd for $C_{36}H_{38}N_4O_7$: C, 67.69; H, 5.99; N, 8.77. Found: C, 67.53; H, 6.19; N, 8.62.

7-(β-D-2'-Deoxy-erythro-pentofuranosyl)-3'-O-(diisopropylmethylphosphoramidite)-5'-O-(4,4'-dimethoxytrityl)-2-isobutyrylamino-3,7-dihydro-4H-pyrrolo-[2,3-d]pyrimidin-4-one (6).

Compound 5c (100 mg, 0.16 mmol) was dissolved in anhydrous dichloromethane and diisopropylethylamine (0.1 mL, 0.48 mmol) in a round bottom flask preflushed with argon. Chlorodiisopropylaminomethoxyphosphine (36 mg, 0.18 mmol) was added by syringe to the solution under argon at room temperature. After 1 h the solution was diluted with ethyl acetate and extracted with saturated aqueous sodium bicarbonate. The organic layer was washed with aqueous sodium chloride solution and dried over anhydrous sodium sulfate. After evaporation to a foam, the product was purified by flash chromatography on silica gel 60 H (column 10 x 3 cm, solvent G) affording 68 mg of colorless amorphous compound 6 in 53% yield. TLC (silica gel, solvent H) R_f 0.8; ^{31}P NMR ($CDCl_3$) δ 150.12 ppm.

N,N'-Diisopropylmethylphosphoamidous acid (8).

Chlorodiisopropylaminomethoxyphosphine (2.0 mL, 10.1 mmol) and ethyldiisopropylamine (1.3 g, 10.1 mmol) were cooled to 0°C and water (1 mL) was added. The solution was stirred for 30 min at that temperature, ethyl acetate was added and the mixture was transfered to a separatory funnel. The organic layer was washed with saturated aqueous sodium hydrogen carbonate and dried over sodium sulfate. After evaporation of the solvent the oily residue was distilled in vacuo yielding a colorless liquid (1.5 g, 83%) with bp 49°C/0.2 mm. ^1H NMR ($CDCl_3$), δ 1.25, 1.26 (12H, 2d, CH_3, J = 7 Hz), 3.49 (2H, m, CH), 3.65 (3H, d, CH_3O, J = 12 Hz), 6.84 (1H, PH, J = 630 Hz); ^{13}C NMR ($CDCl_3$) δ 22.5 (s, 4 CH_3), 44.8 (d, CH, J_P = 5.5 Hz), 49.3 (d, CH_3O, J = 7.6 Hz); ^{31}P NMR ($CDCl_3$) δ 15.19 ppm.

Anal. Calcd for $C_7H_{18}NO_2P$: C, 46.92; H, 10.12; N, 7.81. Found: C, 46.72; H, 10.15; N, 7.66.

Solid-phase synthesis of d(c^7GpCpc^{7}GpCpc^{7}GpC)(12).

The mini-reactor was charged with 100 mg of polymer support (prepared as described[25]) containing 20 μmol/g immobilized N^4-benzoyl-5'-O-(4,4'-dimethoxytrityl)-2'-deoxycytidine. The following five reaction cycles were used: (1) Detritylation by addition of 2 mL of a 2% solution of trichloroacetic acid in dichloromethane for 1 min; (2) washing step with dichloromethane (3x2 mL), dichloromethane/pyridine (4:1, 1 mL) and dichloromethane (5x2 mL); (3) drying with argon for 5 min; (4) coupling with a 20 fold excess of nucleoside-phosphoramidite in 0.5 mL of a saturated solution of tetrazol in

abs. acetonitrile for 10 min (15 min for the 7-deaza-phosphoramidite 6); (5) washing step with acetonitrile (3x2 mL) and THF/pyridine/water (40:20:1, 1 mL); (6) oxidation by addition of a 0.1 M solution of iodine dissolved in THF/pyridine/water (40:20:1, 1 mL) for 1 min; (7) washing step with THF/pyridine/water (40:20:1, 3x2 mL) and THF (5x2 mL); (8) drying with argon for 2 min; (9) capping of unreacted hydroxyl groups by addition of a solution (1 mL) of dichloromethane/acetic acid anhydride/triethylamine/N-methylimidazole (6:1:1:0.3) for 1 min; (10) washing step with dichloromethane (3x2 mL); (11) drying with argon for 5 min.

Step 11 completes the addition of one nucleotide. The growing oligomer is further elongated by beginning at step 1 and the last reaction cycle stops at step 8. For demethylation the reactor was filled with thiophenol/triethylamine/dioxane[28] (1:1:2, 1 mL) and rotated for 45 min. After washing with methanol (8 mL) and ether (8 mL) the silica gel was dried by passage of dry argon through the gel bed. Thus dried, the silica gel was poured into a 25 mL round-bottom flask and treated with 25% aqueous ammonia (5 mL) overnight. The solution containing the oligomer was decanted from the silica gel and the base-protecting groups were removed by storing at 60°C for 7 h. After filtration the solution was concentrated and the dimethoxytrityl oligomer 11 was purified by reverse-phase HPLC using system I. The 4,4'-dimethoxytrityl group was then completely removed by treatment with 80% acetic acid (2 mL) for 20 min. Acetic acid was removed by evaporation, the resulting oligomer 12 was dissolved in water (2 mL) and the solution extracted with ether (4x3 mL). After lyophilization 0.27 μmol (13.7%) of the hexamer 12 was obtained as triethylammonium salt . The purity of the oligomer was checked by HPLC using different acetonitrile concentrations in 0.1 M triethylammonium acetate as the eluting buffer. In all cases the detritylated material eluted from the column as one peak, indicating that the sample was homogeneous.

Hydrolysis of d(c^{7}GpCpc^{7}GpCpc^{7}GpC)(12) with snake venom phosphodiesterase and alkaline phosphatase.

The oligomer 12 (about 0.4 A_{260} unit) was dissolved in 1 mL of 0.1 M TRIS-HCl, pH 8.5, and digested with snake venom phosphodiesterase (5 μg) for 3 h at 37°C. Digestion was complete after this time and an aliquot was analysed by HPLC (system III) resulting in three peaks which were identified as compound 1, its monophosphate, and dCMP. To the remaining solution were added alkaline phosphatase (5 μg). After further incubation at room temperature overnight the mixture was again analysed by HPLC (system III). Quantification of the material was made on the basis of the peak areas of figure 2c which were

divided by the extinction coefficients of the two products. Compound 1 and dC were formed in a molar ratio of 1 : 1.

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