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Phosphoramidites of base-modified 2′-deoxyinosine isosteres and solid-phase synthesis of d(GCI*CGC) oligomers containing an ambiguous base

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ABSTRACT

Novel phosphoramidites (1,2) of appropriately protected 2′-deoxyinosine isosteres (I*) such as allopurinol 2′-deoxyribofuranoside (4a) and 7-deaza-2′-deoxyinosine (4b) have been synthesized. They were employed together with the phosphoramidite of 2′-deoxyinosine in solid-phase synthesis of d(GCI*CGC) hexamers (12a-d). From thermodynamic data of these alternating hexamers it was shown that allopurinol 2′-deoxyribofuranoside destabilizes such duplexes less strongly than 2′-deoxyinosine. Additionally, the phosphoramidite of 7-deaza-2′-deoxyinosine (2) exhibits an extraordinary stability of the N-glycosylic bond. Since the new phosphoramidites are structurally related to 2′-deoxyinosine, they can be used in the construction of hybridization probes containing an ambiguous base.

INTRODUCTION

The degeneracy of amino acid codons is a severe problem if synthetic oligonucleotides carrying the information of a certain protein are used in a DNA cloning experiment. Mixed DNA probes which contain a mixture of bases at the ambiguous position are used to overcome this difficulty [1]. Alternatively,the incorporation of a modified base at the ambiguous position which allows Watson-Crick base pairing with all four bases has been employed [2,3].

From RNA codon-anticodon interaction it is known that inosine is able to form base pairs with adenosine, cytidine,and uridine [4,5]. These findings have been applied to 2′-deoxyinosine which was incorporated into oligonucleotides which were then used as hybridization probes [2,3].

Due to the lack of the 2-amino group a dI-dC base pair is less stable than dG-dC [6]. As a consequence oligomers containing 2′-deoxyinosine exhibit a lower melting temperature when

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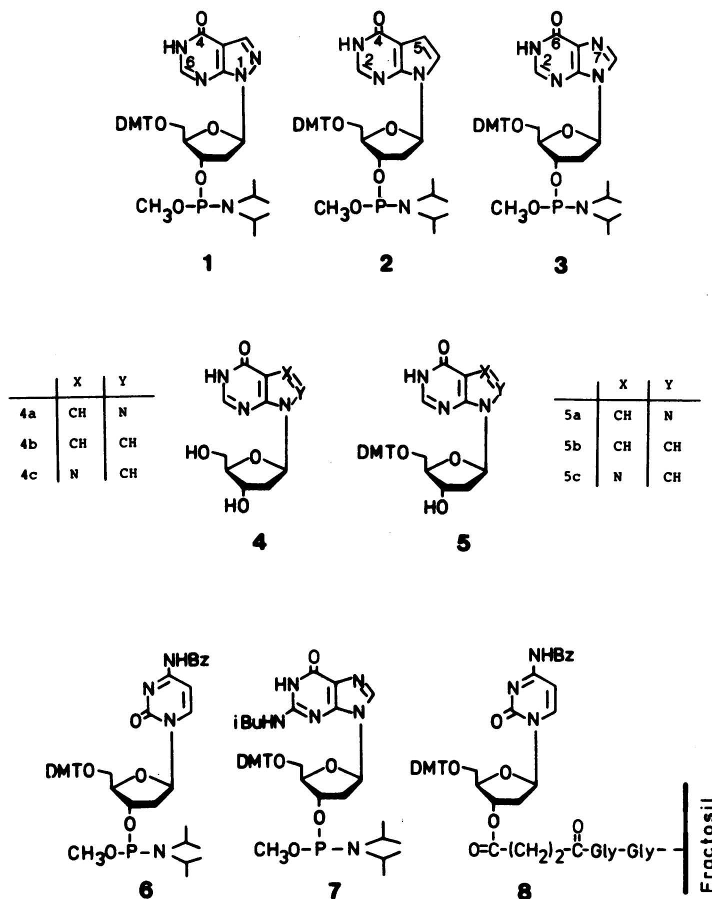
used in hybridization probes [2]. Therefore, it is of interest to search for other 2'-deoxyinosine analogues which can give rise to an enhanced base pair stability or have other advantages over 2'-deoxyinosine.

In order to study this problem in detail we decided to synthesize novel phosphoramidites of 2'-deoxyisosteres of 2'-deoxyinosine. The protecting groups of these new phosphoramidites were actually the same as those of regular 2'-deoxynucleosides so that they can be used in automatic DNA-synthesizers.

In the following we present the synthesis of such phosphoramidites and their incorporation into alternating d(G-C) duplexes. Furthermore, we compare thermodynamic data of several hexamers containing such an ambiguous base.

RESULT AND DISCUSSION

Recently we have reported on the synthesis of the two novel deoxyinosine isosteres 4a [7] and 4b [8]. One is the deoxyribofuranoside of allopurinol the other of 7-deazahypoxanthine. The nucleosides 4a and 4b have been synthesized via the stereoselective glycosylation procedure which was developed in our laboratory [9,10] on the basis of earlier observations on the equilibration of halogenose [11,12] employed in nucleoside syntheses [13]. As key intermediates 4-methoxy-1H-pyrazolo[3,4-d]pyrimidine and 4-methoxy-2-methylthio-7H-pyrrolo[2,3-d] pyrimidine have been used in a phase-transfer glycosylation reaction [7,8]. The original low glycosylation yield of the latter due to the loss of protected nucleoside during crystallization has now been increased to 56 % of the toluoylated glycosylation product employing phase-transfer techniques under the following conditions: 4-methoxy-2-methylthio-7H-pyrrolo[2,3-d]pyrimidine (500 mg) was dissolved in dichloromethane (30 mL) in the presence of tetrabutylammonium hydrogen sulfate (70 mg). After addition of 50 % aqueous NaOH (5 mL) 1-chloro-2-deoxy-3,5-di-O-(p-toluoyl)-α-D-erythropentofuranose (1.47 g) [14] in dichloromethane (10 mL) was gradually added under thorough mixing with an Ultra-Turrax dispersion apparatus for 5 min. The work-up was the same as originally described [8].



The purine- and the pyrazolo[3,4-d]pyrimidine 2'-deoxyribofuranosides exhibit very similar UV absorption maxima in water (4a: λ_{max} 250 nm, ε 8300; 4c: λ_{max} 248 nm, ε 13000). The spectrum of 4b was bathochromically shifted by about 10 nm (4b: λ_{max} 260 nm, ε 10000) which is a common feature of pyrrolo[2,3-d]-pyrimidine nucleosides. The ^{13}C NMR data of the nucleosides are almost identical to the non-base moieties (Table 1). According to the amount and positioning of the nitrogens in the nucleosides 4a-c these compounds exhibit different mobilities on TLC (silica gel, E): R_f(4a) 0.45; (4b) 0.4; (4c) 0.2. These values indicate that compound 4c (dI) is the most hydrophilic nucleoside of the three different isosteres whereas dc^{7}I (4b) and

Table 1: ^{13}C NMR shifts of inosine derivatives in Me_2SO-d_6 [a]; the first carbon number relates to pyrazolo[3,4-d]pyrimidine-, the second (in paranthesis) to pyrrolo[2,3-d]pyrimidine- and the third [in brackets] to purine numbering.

	C-6 (2) [2]	C-7a (7a) [4]	C-3a (4a) [5]	C-4 (4) [6]	- (6) [8]	C-3 (5) -
4a	148.1	153.3	106.2	156.8	-	134.9
4b	143.7	147.4	108.4	158.2	120.7	102.5
4c	145.4	147.6	124.4	156.2	138.2	-
5a	147.9	152.2	106.1	156.7	-	134.6
5b	143.5	147.1	108.3	157.9	120.3	102.3
5c	145.1	147.7	124.5	156.2	138.2	-
	C-1'	C-2'	C-3'	C-4'	C-5'	OCH_3
4a	84.0	38.1	70.9	87.7	62.3	-
4b	83.2	40.2	71.0	87.4	62.0	-
4c	83.5	39.8	70.4	87.8	61.5	-
5a	83.8	38.1	70.6	85.4	64.1	54.7
5b	82.8	40.4	70.6	85.3	64.0	54.9
5c	83.4	39.9	70.4	85.8	63.8	54.8

[a] Chemical shifts (δ) are relative to tetramethylsilane as internal standard, signals of DMT-residues are not shown.

dc^7z^8I (4a) show similar hydrophobic behaviour.

A common feature of the deoxynucleosides 4a-c is their identical pyrimidine ring structure. It can be therefore expected that 4a and 4b will show the same ambiguity as 2'-deoxyinosine with respect to base pairing. However, the interchange of nitrogens as in compounds 4a and 4c or the lack of one nitrogen as in compound 4b, can alter interactions between adjacent bases in a DNA fragment both in the single-stranded form and in the duplex structure. Moreover, the different polarity of these bases can influence the amount of tightly bound water to oligomers containing these 2'-deoxyinosine derivatives [15].

In order to study these phenomena and to show the effectiveness of the phosphoramidites in oligonucleotide synthesis,

we decided to synthesize hexamers with an alternating d(G-C)-sequence in which one of the novel nucleosides replaced a 2'-deoxyguanosine. From the thermodynamic data of d(GCICGC), d(GCc^{7}ICGC) as well as of d(GCc^{7}z^{8}ICGC) and from comparison with d(G-C)$_3$ it should be possible to decide which of the novel 2'-deoxyinosine isosteres has advantages over 2'-deoxyinosine.

As a result of recent advances in oligonucleotide synthesis such as the use of solid supports and the application of phosphoramidite methodology [16], the synthesis of the phosphoramidites 1 and 2 was a prerequisite. For the synthesis of the known phosphoramidite 3 [17] the conditions of preparation were studied and spectroscopic data were evaluated. In order to employ the nucleosides 4a-c in DNA-synthesizers their protecting groups had to be compatible with those of regular 2'-deoxynucleosides. The DMT-residue was therefore chosen for 5'-OH protection. This was achieved in pyridine solution with an excess of 4,4'-dimethoxytrityl chloride in the presence of 4-dimethylaminopyridine. The tritylated compounds 5a and 5b were purified by flash chromatography [18] with dichloromethane-acetone. In the case of 5c flash chromatography was omitted due to the immediate crystallization of 5c from the reaction mixture by addition of a small volume of dichloromethane. As shown in Table 1 the DMT-derivatives exhibit very similar chemical shifts for the non-base moiety in the ^{13}C NMR spectrum. The downfield shift of C-5' and the upfield location of C-4' of compounds 5a-c in relation to the unprotected nucleosides 4a-c confirm OH-5'-dimethoxytritylation in all three cases. The purity of the materials was confirmed by TLC and elemental analysis.

The conversion of the protected nucleosides 5a-c into the phosphoramidites 1-3 followed a procedure which was originally developed by Caruthers for regular nucleosides [19]. The phosphitylation was carried out using an equimolar ratio of chlorodiisopropylaminomethoxyphosphine in anhydrous acetonitrile in the presence of N-ethyldiisopropylamine. Under these conditions the reaction was complete after one hour and the products were purified from starting material by flash chromatography (silicagel 60H, solvent D). The materials of the main zone were isolated as amorphous solids exhibiting signals at 150.01 ppm for

compound 1, 149.8 ppm for 2 and 150.25 ppm for 3 in the ^{31}P NMR spectrum. Table 2 shows the ^{1}H NMR chemical shifts of the phosphoramidites 1-3, confirming their structure. Since all three phosphoramidites exhibit the same structure for the non-base moiety it was expected, that the chemical shifts of this part of the molecule would not differ significantly. As can be seen from Table 2 this is the case for the signals of 2'-H/5'-H and for those of the phosphoramidite residue. However, the H-1' signals exhibit differences in shielding; in particular,the chemical shift of the purine compound 3 differs from those of the isosteres 1 and 2, indicating that the different nucleobases influence the anomeric centre of the molecule. All three phosphoramidites exhibit two peaks due to the diastereoisomeric location of

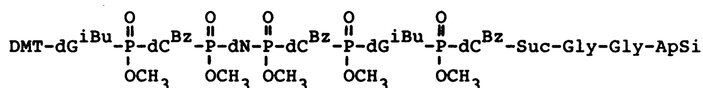
Table 2: ^{1}H NMR chemical shifts of the phosphoramidites 1-3 in $CDCl_3$.

	1	2	3
CH_3-diisopropyl	1.15 (m, 6H)	1.15 (m, 6H)	1.13 (m, 6H)
H-C(2')	2.46 (m, 2H)	2.56 (m, 2H)	2.66 (m, 2H)
CH_3O-P	3.28 (d, 3H, J = 13 Hz)	3.28 (d, 3H, J = 13 Hz)	3.29 (d, 3H, J = 13 Hz)
	3.37 (d, 3H, J = 13 Hz)	3.37 (d, 3H, J = 13 Hz)	3.37 (d, 3H, J = 13 Hz)
H-C(5')	3.24 (m, 2H)	3.26 (m, 2H)	3.26 (m, 2H)
CH-diisopropyl	3.55 (m, 1H)	3.55 (m, 1H)	3.56 (m, 1H)
CH_3O	3.73 (s, 6H)	3.75 (s, 6H)	3.74 (s, 6H)
H-C(4')	4.27 (m, 1H)	4.22 (m, 1H)	4.29 (m, 1H)
H-C(3')	4.80 (m, 1H)	4.67 (m, 1H)	4.68 (m, 1H)
H-C(1')	6.70 (m, 1H)	6.63 (m, 1H)	6.38 (m, 1H)
arom. H	6.64-7.4 (m)	6.75-7.4 (m)	6.74-7.3 (m)
H-C(3/5/7)	7.96 (s, 1H)	6.64 (m, 1H)	-
H-C(6/2/2)	8.01 (s, 1H)	7.89 (s, 1H)	8.01 (s, 1H)
H-C(2/6/8)	-	7.13 (m, 1H)	7.93 (s, 1H)

Chemical shifts (δ) are relative to tetramethylsilane as internal standard.

the P-OCH_3 groups. These signals are further split by the ^3J-phosphorus-proton coupling with a coupling constant of 13 Hz. The phosphoramidites 1-3 are fairly stable compounds and can be stored as solid foams for months.

The incorporation of compounds 1-3 into the oligomers 12b-d followed a method which has been already used in the synthesis of the hexamer d(c^7GpC)$_3$ [20]. As solid support the modified silica gel 8, carrying 5'-dimethoxytritylated cytidine via an alkaline labile succinate-glycine linker, was used. Two glycine residues were inserted between the 3-aminopropylsilyl-functionalized macroporus silica and the 3'-succinyl residue in order to ensure a high coupling yield during the first oligomerization step [21]. Five alternating reaction cycles, beginning with the phosphoramidite 7 and continuing with the amidite 6 were carried out for the synthesis of the protected hexamer 9a. In the case of the oligomers 9b, 9c, and 9d the third reaction cycle was passed through by using the phosphoramidites 1, 2, or 3, instead of 7. After deacylation and demethylation the purification of the



9a-d

10a-d

11a

11b

11c

11d

12a 12b 12c 12d

N = position of modification

c^7z^8I = allopurinol 2'-deoxyribofuranoside

c^7I = 7-deaza-2'-deoxyinosine

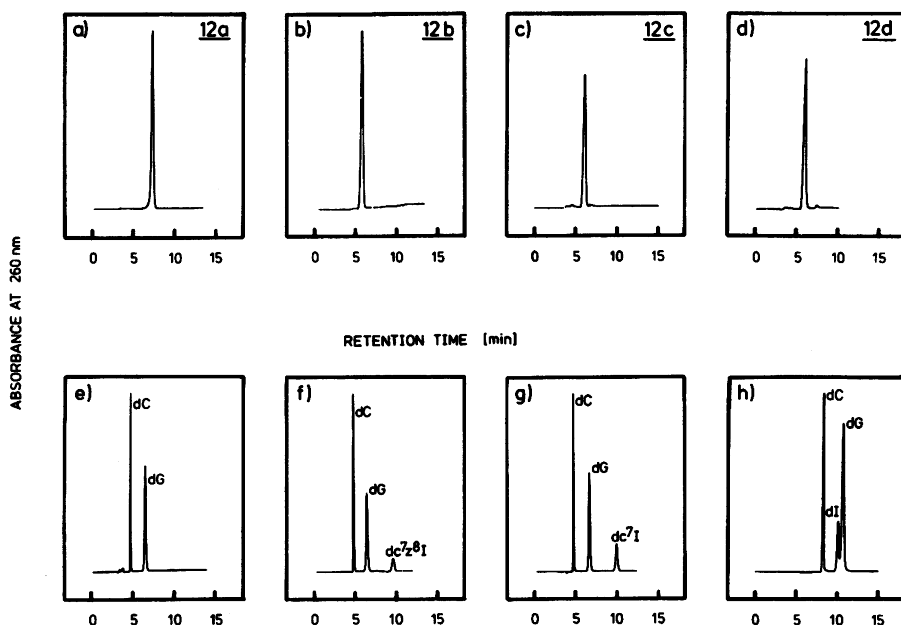


Figure 1. HPLC elution profiles of the purified oligomers 12a (a, gradient III), 12b (b, gradient IV), 12c (c, gradient IV), and 12d (d, gradient IV) and the enzymatically hydrolysed oligomers 12a (e, solvent I), 12b (f, solvent I), 12c (g, solvent I) and 12d (h, solvent II). Digestion was performed with snake venom phosphodiesterase followed by alkaline phosphatase (see Experimental Section).

DMT-protected oligomers 11a-d was accomplished by reverse-phase HPLC (see experimental conditions). Removal of the DMT-residues occured upon treatment with acetic acid for 20 min. The resulting oligomers 12a-d were purified again by reverse-phase chromatography and obtained in the following yields: 12a (18%), 12b (16%), 12c (20%) and 12d (13.5%). Figure 1a-d show the HPLC-pattern of the oligomers 12a-d. The structure of the oligomers was confirmed by hydrolysis with snake venom phosphodiesterase followed by alkaline phosphatase (see Figure 1e-h). Quantification of the nucleoside content was made on the basis of the extinction coefficients at 260 nm (see experimental procedures). The values obtained by this calculation confirmed the contents expected according to the structures of 12a-d.

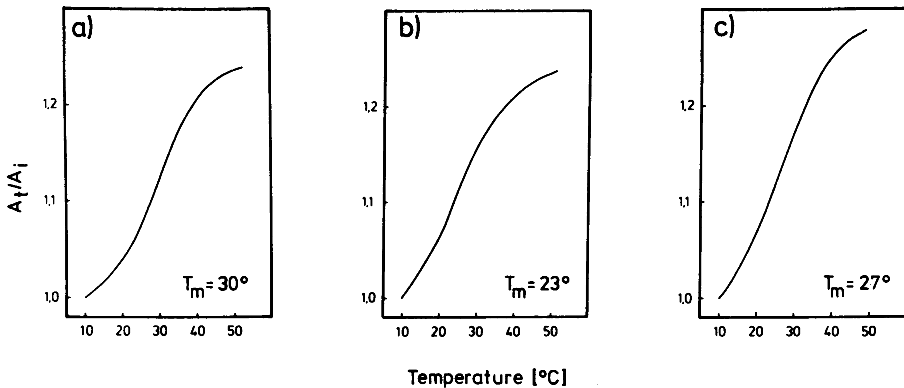


Figure 2. Normalized melting profiles of (a) d(GpCpc^{7}z^{8}IpCpGpC) (12b), (b) d(GpCpc^{7}IpCpGpC) (12c), and (c) d(GpCpIpCpGpC) (12d) in H_2O, containing 1.0 M NaCl, 0.1 M $MgCl_2$, and 60 mM Na-cacodylate at pH 7.0. Strand concentration was 20 µM, A_t/A_i is the ratio of absorbance at 280 nm at a given temperature (t) to the initial temperature (i).

Melting profiles and thermodynamic data.

In order to confirm that the oligomers 12b-d form duplexes under appropriate conditions melting profiles were determined in aqueous 0.1 M NaCl in the presence of 60 mM sodium cacodylate and 0.1 M $MgCl_2$. As Figure 2 shows, cooperative melting was observed for these oligomers indicating that a helix-coil transition takes place. As expected, all three oligomers exhibited a lower T_m than that of $[d(GpC)_3]_2$ (T_m= 46°C) at 20 µM oligomer concentration. It is interesting to note that the T_m of $[d(GCc^7ICGC)]_2$ is lower (23°C) than that of $[d(GCICGC)]_2$ (27°C). However, the oligomer $[d(GCc^7z^8ICGC)]_2$ containing allopurinol 2'-deoxyribofuranoside shows an increased T_m value (30°C) compared to the parent purine compound (27°C). To obtain a more complete picture of the melting process, the T_m values were measured at different sodium chloride concentrations. These data are summarized in Table 3 and show that a high sodium chloride concentration reduces the melting temperature for all four oligomers [22]. Due to the low melting temperature of the modified oligomers a full set of data could be only obtained up to a concentration of 3 M sodium chloride. Various reasons can ac-

Table 3: Melting temperatures of the hexamers 12a-d in water containing 0.1 M $MgCl_2$ and 60 mM Na-cacodylate at various NaCl concentrations, helix coil transitions were measured at 280 nm.[a)]

	T_m (°C)					
	1.0	2.0	3.0	4.0	5.0	M NaCl
12a	46	43	41	37	34	
12b	30	26	22	19	-	
12c	23	21	17	-	-	
12d	27	24	21	17	-	

[a)] oligomer concentration was 20 μM.

count for this decrease in the melting temperature. A high concentration of salt ions can remove tightly bound water from the duplexes by solvation. Also salt ions can compete with the proton acceptor- and donator substituents of Watson-Crick base pairs causing a break of hydrogen bonds. From the data of Table 3 it can also be concluded that an increase of the salt concentration has a similar effect on the structure of all four oligomers. According to earlier findings [23,24] that the octamer $d(GC)_4$ does not show a B - Z transition at high salt concentration we expected a similar behaviour for the hexamers (12a-d). Preliminary CD-experiments (data not shown) support this interpretation.

In order to determine thermodynamic data of the oligomers 12a-d the concentration-dependent changes of the T_m-values were measured . The concentration was determined spectrophotometrically at 260 nm using the following extinction coefficients for the monomers: dG (11700), dC (7300), dc^7I (10000), dc^7z^8I (6500), and dI (7700). Summation of these values according to the sequence and correction by the hyperchromicity values yielded the actual extinction coefficients of the oligomers which were then used for the determination of the concentrations.

There are several different ways to analyse the T_m values of the oligonucleotides in order to obtain thermodynamic parameters. A common procedure is the measurement of the T_m value as a function of oligomer concentrations [25]. We have measured

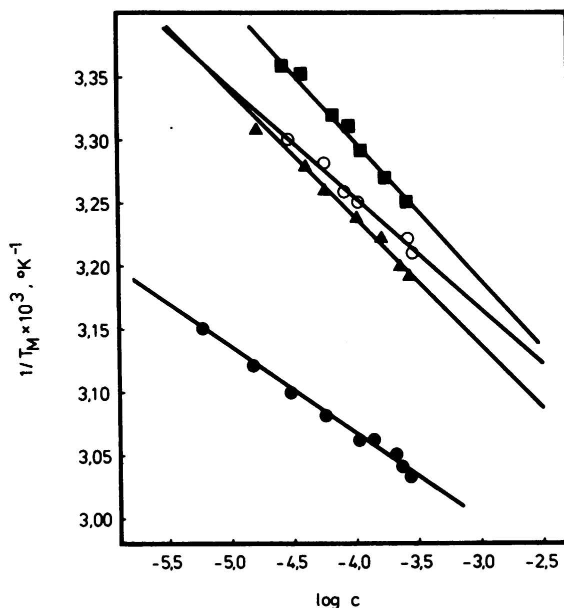


Figure 3. Plot of $1/T_m$ vs. log c for the oligomers 12a (●-●), 12b (▲-▲), 12c (■-■), and 12d (O-O) in H_2O. All solutions contain 1.0 M NaCl, 0.1 M $MgCl_2$, and 60 mM Na-cacodylate, pH 7.0.

the T_m values of 12a-d at about 8 different concentrations within a range of 10 - 250 µM. Reciprocal T_m values have then been plotted vs. log of oligomer concentration. Figure 3 shows a graph obtained from the oligomers 12a-d. This graph allows the calculation of the enthalpy ΔH from the slope and the entropy ΔS from the intercept for each particular oligomer according to equation (i): $1/T_m = 2.3\ R\ \log(c)/\Delta H + \Delta S/\Delta H$ [26, 27, 28]. The energetic contributions to ΔH from duplex formation comes both from hydrogen bonding and from stacking interactions with adjacent base pairs. Since the difference in T_m values between two different oligomers is proportional to the free energy difference $\Delta\Delta G$ between the two duplexes, the $\Delta\Delta G$ values can be calculated according to equation (ii): $\Delta\Delta G = 2.3\ RT\ \Delta\log(c)$ [29]. The principal here is that RT times the difference in the logarithm of total concentration results in the same T_m for the two different oligomers.

Table 4: Enthalpy and entropy values for double helix formation and data of hypochromicity of the hexamers 12a-d

Compd.	T_m (°C) a)	ΔH kcal/mol	ΔS cal/mol·deg	ΔΔG b) kcal/mol	H (%) (280 nm)
12a	46	-65	-183	-	37
12b	30	-45	-129	-3.2	19
12c	23	-41	-118	-3.9	17
12d	27	-51	-149	-3.6	18.5

a) oligomer concentration was 20 μM; b) the values were calculated at 42 °C.

The free enthalpy change (ΔΔG) between the oligomer 12a and the hexamers 12b-d (Table 4) reflects the stability of these different duplexes in the environment of the aqueous buffer solution and is directly related to the melting temperature. Due to the different slopes of the curves of Figure 3 the calculated values were all taken at a temperature of 42 °C. The enthalpy change for the transition of the duplex to the single-stranded form is most pronounced for the purine/pyrimidine oligomer $d(GC)_3$. Our ΔH value obtained from the melting of the oligomer 12a and determined in the presence of 0.1 M $MgCl_2$ (Table 4) is higher than that in the absence of Mg^{2+} ions [27, 28]. As one would expect the corresponding purine/pyrimidine oligomer d(GCICGC), lacking one exocyclic 2-amino group exhibits a much lower enthalpy value. According to Table 4 some of the enthalpy values do not correspond to the melting temperatures. As can be seen, the oligomer 12b containing allopurinol 2'-deoxyribofuranoside has a lower ΔH value compared to that containing 2'-deoxyinosine, but exhibits a higher melting temperature. This clearly indicates that the entropy term plays an important part in the process of duplex formation of 12b-c. From the ΔS values of Table 4 which are obtained according to equation (i) it can be immediately seen that the oligomer 12c which contains the most hydrophobic inosine isostere (4b) exhibits the lowest ΔS value. The oligomers 12b and 12d which have two polar nitrogen atoms either at N-7/9 or N-8/9 show higher values. The low value of 12c compared to 12d may be due to a reduced amount of tightly

bound water to this hydrophobic oligomer both in the single-stranded form and in the duplex state of 12c. From the data of Table 4 it can be also concluded that the relatively large ΔH value together with a relatively large ΔS term accounts for the high melting temperature of the oligomer 12b, containing allopurinol 2'-deoxyribofuranoside as ambiguous nucleoside.

Our findings demonstrate that the phosphoramidite 1 may be an useful synthon for the preparation of oligodeoxynucleotides employed as hybridization probes. It should be also emphasized that the phosphoramidite 2 has a built-in stability of its N-glycosylic bond. As could be shown from hydrolysis experiments the deoxynucleoside 4b is not even cleaved in 1 N hydrochloric acid at elevated temperature [30]. This finding can be related to the fact that repair enzymes acting via transglycosylation also do not cleave the glycosylic bond of 4b in high molecular DNA [31]. As a result the phosphoramidite 2 is not only a synthetic building block for incorporation of an ambiguous base into DNA, but it has the extra potential of minimizing enzymatic repair.

EXPERIMENTAL SECTION

Elemental analyses were performed by Mikroanalytisches Labor Beller (Göttingen, FRG). NMR spectra were recorded on a Bruker WM 250 spectrometer; values are in ppm relative to tetramethylsilane as internal standard (^{1}H 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). Flash chromatography was performed with silica gel 60H (Merck, FRG) at 0.9 bar (N_2). Solvent systems for TLC: (A) CH_2Cl_2-acetone (9:1), (B) CH_2Cl_2-acetone (7:3), (C) CH_2Cl_2-acetone (1:1), (D) CH_2Cl_2-ethylacetate-triethylamine (45:45:10), (E) $CHCl_3$-CH_3OH (85:15). Pyridine and N-diisopropylethylamine were distilled from KOH and stored over 4Å molecular sieves. Dioxane was filtered through a bed of aluminium oxide (Woelm basic, grade I). Dichloromethane was distilled from P_2O_5 and then redistilled from CaH_2. Acetonitrile

was predried with K_2CO_3 and then distilled from CaH_2. Tetrazole, 4-dimethylaminopyridine and trichloroacetic acid were sublimated under reduced pressure. 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 and 2'-deoyxguanosine were purchased from Pharma-Waldhof (FRG). 2'-Deoxyinosine was purchased from Sigma (St. Louis, USA). Fractosil (50 µmol of immobilized protected 2'-deoxynucleoside/g of solid support) was purchased from Biosyntech (FRG).

Melting experiments. The melting experiments were carried out 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 as a function of time was recorded while the temperature of the solution was increased linearily with time 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 (MGW Lauda, FRG). The actual temperature was measured in the reference cell with a Pt-resistor. The melting curves of Figure 2 and the salt-dependent experiments were measured in Teflon-stoppered cuvettes with 1 cm light path length. The melting temperatures at various oligomer concentrations were determined in cuvettes with 0.2 cm light path length.

HPLC separation. High performance liquid chromatography was carried out on a 4x250 mm (10 µm) RP-18 LiChrosorb column (Merck) using a LKB HPLC apparatus 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 solvent systems and gradients consisting of 0.1 M triethylammonium acetate,pH 7,(A) and acetonitrile (B) were used in the following order: gradient I: 10 min (25-40 % B), 5 min (40 % B); gradient II: 15 min (20-35 % B), 5 min (35 % B); gradient III: 10 min (10-20 % B); gradient IV: 10 min (10-25 % B); flow rates of gradients I-IV: 1 mL min^{-1}. Solvent I: 6 % B, flow rate: 0.7 mL min^{-1}; solvent II: 6 % B, flow rate: 0.4 mL min^{-1}.

Mini-reactor for oligonucleotide synthesis. Solid-phase synthesis was carried out in a HPLC LiChroCart-cartridge (25-4). This cartridge was placed into 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.

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.

1-(ß-D-2'-Deoxy-erythro-pentofuranosyl)-5'-O-(4,4'-dimethoxytrityl)-1,5-dihydro-4H-pyrazolo[3,4-d]pyrimidin-4-one (dc^7z^8I, 5a). Allopurinol 2'-deoxyribofuranoside (4a) (100 mg, 0.4 mmol) [7] was dried by coevaporation with anhydrous pyridine. The material was dissolved in pyridine (30 mL), 4-dimethylamino-pyridine (27 mg, 0.2 mmol) and 4,4'-dimethoxytritylchloride (270 mg, 0.8 mmol), dissolved in dioxane (10 mL), were added and the solution was stirred for 2 h under nitrogen at room temperature. The reaction was monitored by TLC (silica gel, solvent C). To the solution 5 % aqueous potassium hydrogen carbonate (50 mL) was added and the resultant was extracted three times with dichloromethane (100 mL). The combined organic layers were dried with sodium sulfate, filtered, and the solvent was evaporated. The residue was applied to a 9x3 cm column (silica gel 60H, solvent B) and separated by flash chromatography. Isolation of the material of the main zone yielded colorless, amorphous 5a (150 mg, 68 %). TLC (silica gel, solvent C) R_f 0.75 ; UV (methanol) λ_{max} 235, 265 nm (ε 26200, 11100) ; ^1H NMR (Me_2SO-d_6) δ 2.27 (1H, m, H-2'a), 2.76 (1H, m, H-2'b), 3.05 (2H, m, H-5'), 3.71 (6H, s, 2 OCH_3), 3.94 (1H, m, H-4'), 4.52 (1H, m, H-3'), 5.32 (1H, d, OH-3', J = 4.5 Hz), 6.55 (1H, dd, H-1', J = 3 Hz), 6.73 - 7.32 (m, arom. H), 8.07 (1H, s, H-3), 8.15 (1H, s, H-6). Anal. Calcd. for $C_{31}H_{30}N_4O_6$: C, 67.14; H, 5.45 ; N, 10.10. Found: C, 67.18; H, 5.44 ; N, 10.20.

7-(ß-D-2'-Deoxy-erythro-pentofuranosyl)-5'-O-(4,4'-dimethoxytrityl)-3,7-dihydro-4H-pyrrolo[2,3-d]pyrimidin-4-one (dc^7I, 5b). The synthesis of compound 5b was carried out in the same way as described for 5a. Compound 4b (250 mg, 1 mmol) [8] was reacted with 4,4'-dimethoxytritylchloride (670 mg, 2 mmol) in the presence of 4-dimethylaminopyridine (65 mg, 0.5 mmol) in

anhydrous pyridine. Compound 5b (450 mg, 81 %) was obtained as colorless, amorphous material. TLC (silica gel, solvent C) R_f 0.55; UV (methanol) λ_{max} 235, 260 nm (ε 24700, 15200); ^1H NMR (Me_2SO-d_6) δ 2.11 (1H, m, H-2'a), 2.29 (1H, m, H-2'b), 3.14 (2H, m, H-5'), 3.72 (6H, s, 2 OCH_3), 3.93 (1H, m, H-4'), 4.34 (1H, m, H-3'), 5.35 (1H, d, OH-3', J = 4.4 Hz), 6.49 (1H, dd, H-1', J = 3.7 Hz, J = 7.2 Hz), 6.51 (1H, d, H-5, J = 3.6 Hz), 6.82 - 7.39 (m, arom. H), 7.18 (1H, d, H-6, J = 3.5 Hz), 7.9 (1H, s, H-2), 11.98 (1H, s, NH). Anal. Calcd. for $C_{32}H_{31}N_3O_6$: C, 69.43; H, 5.64; N, 7.59. Found: C, 69.25; H, 5.76; N, 7.40.

2'-Deoxy-5'-O-(4,4'-dimethoxytrityl)-inosine (dI, 5c). The synthesis of 5c followed the same route as described for compound 5a. Purification employing flash chromatography was omitted. Instead compound 5c was isolated from the oily reaction mixture by addition of a small volume of dichloromethane. By using 4c (250 mg, 1 mmol) and 4,4'-dimethoxytritylchloride (670 mg, 2 mmol) in the presence of 4-dimethylaminopyridine (65 mg, 0.5 mmol) colorless 5c (430 mg, 77 %) was obtained. TLC (silica gel, solvent C) R_f 0.25 UV (methanol) λ_{max} 236, 275 nm (ε 30000, 7800) ^1H NMR (Me_2SO-d_6) δ 2.31 (1H, m, H-2'a), 2.76 (1H, m, H-2'b), 3.16 (1H, m, H-5'), 3.72 (6H, s, 2 OCH_3), 3.98 (1H, m, H-4'), 4.42 (1H, m, H-3'), 5.37 (1H, m, OH-3'), 6.33 (1H, m, H-1'), 6.77 - 7.33 (m, arom. H), 7.99 (1H, s, H-8), 8.19 (1H, s, H-2). Anal. Calcd. for $C_{31}H_{30}N_4O_6$: C, 67.14; H, 5.45; N, 10.10. Found: C, 67.06; H, 5.49; N, 10.18.

General procedure for the preparation of the phosphoramidites 1-3. The 5'-tritylated nucleoside was dissolved in anhydrous acetonitrile (5 mL) in a round bottom flask preflushed with argon. Chlorodiisopropylaminomethoxyphosphine and diisopropylethylamine were added by a syringe to the solution kept under argon at room temperature. After 1 h the solution was added to 5 % aqueous potassium hydrogen carbonate (50 mL) and extracted three times with dichloromethane. The organic layer was dried over anhydrous sodium sulfate and evaporated, yielding a foam. This product was purified by flash chromatography on silica gel 60H (column 10 x 3 cm, solvent D) affording the colorless, amorphous phosphoramidite.

1-(ß-D-2'-Deoxy-erythro-pentofuranosyl)-3'-O-[(N,N-diisopropylamino)methoxyphosphino]-5'-O-(4,4'-dimethoxytrityl)-1,5-dihydro-4H-pyrazolo[3,4-d]pyrimidin-4-one (1). The reaction of 5a (300 mg, 0.55 mmol) with chlorodiisopropylaminomethoxyphosphine (0.12 mL, 0.6 mmol) in the presence of diisopropylethylamine (0.35 mL, 1.65 mmol) yielded colorless, amorphous 1 (220 mg, 56 %). TLC (silica gel, solvent D) R_f 0.15 ; ^{31}P NMR ($CDCl_3$) δ 150.01 ppm.

7-(ß-D-2'-Deoxy-erythro-pentofuranosyl)-3'-O-[(N,N-diisopropylamino)methoxyphosphino]-5'-O-(4,4'-dimethoxytrityl)-3,7-dihydro-4H-pyrrolo[2,3-d]pyrimidin-4-one (2). The preparation of 2 was identical to the synthesis of 1. Compound 2 (200 mg, 51 %) was obtained as colorless foam. TLC (silica gel, solvent D) R_f 0.3 ; ^{31}P NMR ($CDCl_3$) δ 149.8 ppm.

2'-Deoxy-3'-O-[(N,N-diisopropylamino)methoxyphosphino]-5'-O-(4,4'-dimethoxytrityl)-inosine (3). Using the starting material 5c (300 mg, 0.55 mmol) and following the same route as described for 1,colorless, amorphous 5c (200 mg, 51 %) was obtained. TLC (silica gel, solvent D) R_f 0.1 ; ^{31}P NMR ($CDCl_3$) δ 150.25 ppm.

Solid-phase synthesis of the oligomers 12a-d. A mini reactor was charged with 50 mg of the modified silica gel 8 containing 50 µmol immobilized 2'-deoxynucleoside/gram of solid support. The following reaction cycle was used for oligomerization: (1) Detritylation by addition of 10 mL of a 3 % solution of trichloroacetic acid in dichloromethane for 5 min.; (2) washing step with dichloromethane (10 mL) and acetonitrile (5 mL); (3) drying with argon for 5 min; (4) coupling with a 20-fold excess of nucleoside phosphoramidite in 0.6 mL of a 0.1 M solution of tetrazole in abs. acetonitrile for 15 min; (5) washing step with dichloromethane (5 mL); (6) capping of unreacted hydroxyl groups by addition of 2 mL of a mixture (1:1, v/v) of (a) dimethylaminopyridine (600 mg) in abs. tetrahydrofurane (10.7 mL) and (b) acetic acid anhydride/tetrahydrofurane/2,6-lutidine (8:1:1, v/v/v) for 5 min; (7) washing step with dichloromethane (5 mL) and tetrahydrofurane/pyridine/water (40:20:1, v/v/v, 1 mL); (8) oxidation by addition of a 0.1 M solution of iodine dissolved in tetrahydrofurane/pyridine/water (40:20:1, v/v/v, 1 mL) for 1 min; (9) washing step with tetrahydrofurane/pyridine/water (40:20:1,

v/v/v, 5 mL), tetrahydrofurane (5 mL),and dichloromethane (10 mL). Step 9 completed the addition of one nucleoside. The oligomer was then elongated by beginning at step 1. The last reaction cycle ends at washing step 9 omitting the capping procedure at step 6. After drying with argon for 10 min,the reactor was supplied with thiophenol/triethylamine/dioxane (1:1:2, v/v/v) for demethylation and rotated for 90 min. After washing with methanol (20 mL) and diethylether (20 mL) the polymer support was poured into 50 mL of a 25 % solution of ammonia in water and treated for 16 h at room temperature. The solution was then decanted from the solid support and the base-protecting groups were removed by storing at 60 ^{o}C for 10 h. After filtration the solution was concentrated to 1 mL and the dimethoxytritylated oligomers 11a-d were purified by reverse-phase HPLC using the gradients I or II. The collected fractions containing the 5'-protected oligomers (second main zone) were evaporated and the dimethoxytrityl residue was completely removed by treatment with 80 % acetic acid (5 mL) for 30 min. The acid was then removed by evaporation under reduced pressure. The residue was dissolved in 5 mL of water and the solution was extracted with diethylether (5 mL) for 5 times. After lyophilisation the hexamers 12a-d were obtained as triethylammonium salt. The purity was checked by HPLC using acetonitrile - 0.1 M triethylammonium acetate as the eluting buffer (Figure 1). The purified products were dissolved in 1 mL of water and stored frozen at -20 ^{o}C.

Hydrolysis of the nucleotide oligomers with snake venom phosphodiesterase. The nucleotide oligomer (about 1 A_{260} unit) was dissolved in 1 mL of a 0.1 M TRIS-HCl buffer pH 8.5 and treated with snake venom phosphodiesterase (5 µg) for 2 h at 37 ^{o}C. After further incubation with alkaline phosphatase (5 µg) at 37 ^{o}C for 30 min the mixture was analysed by HPLC with solvent I or II as eluent. Quantification of the material has been made on the basis of the peak areas divided by the extinction coefficients of the corresponding nucleosides (ε_{260}: dG 11700, dC 7300, $dc^{7}I$ 10000, $dc^{7}z^{8}I$ 6500, dI 7700).

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REFERENCES

1. Suggs, S. V., Wallace, R. B., Hirose, T., Kawashima, E. H., and Itakura, K. (1981) Proc. Natl. Acad. Sci. U.S.A., 78, 6613-6617.
2. Ohtsuka, E., Matsuki, S., Ikehara, M., Takahashi, Y., and Matsubara, K. (1985) J. Biol. Chem., 260, 2605-2608.
3. Takahashi, Y., Kato, K., Hayashizaki, Y., Wakabayashi, T., Ohtsuka, E., Matsuki, S., Ikehara, M., and Matsubara, K., (1985) Proc. Natl. Acad. Sci. U.S.A., 82, 1931-1935.
4. Crick, F. H. C. (1966) J. Mol. Biol., 19, 548-555.
5. Topal, M. D., and Fresco, J. R. (1976) Nature, 263, 289-293.
6. Wells, R. D., Larson, J. E., Grant, R. C., Shortle, B. E., and Cantor, C. R., (1970) J. Mol. Biol., 54, 465-497.
7. Seela, F., and Steker, H. (1985) Helv. Chim. Acta, 68, 563-570.
8. Seela, F., and Menkhoff, S. (1985) Liebigs Ann. Chem., 1360-1366.
9. Winkeler, H. D., and Seela, F. (1983) J. Org. Chem., 26, 3119-3122.
10. Winkeler, H. D., and Seela, F. (1980) Chem. Ber., 113, 2069-2080.
11. Hubbard, A. J., Jones, A. S., and Walker, R. T., (1984) Nucl. Acids Res., 12, 6827-6833.
12. Kotick, M. P., Szantay, C., and Bardos, T. J. (1969) J. Org. Chem., 34, 3806-3813.
13. Seela, F., Driller, H., Kehne, A., Menkhoff, S., Ott, J., and Winkeler, H. D. (1986) Chemical Synthesis in Molecular Biology (Blöcker, H., Franck, R., and Fritz, H. J., Eds.) Verlag Chemie, Weinheim.
14. Hoffer, M. (1960) Chem. Ber., 93, 2777-2781.
15. Saenger, W. (1984) Principles of Nucleic Acid Structure (Cantor, C. S., Eds) chapter 17, Springer Verlag.
16. Matteucci, M. D., and Caruthers, M. H. (1981) J. Amer. Chem. Soc., 103, 3185-3191.
17. Caruthers, M. H., unpublished results.
18. Still, W. C., Kahn, M., and Mitra, A. (1978) J. Org. Chem., 43, 2923-2925.
19. McBride, L. J., and Caruthers, M. H. (1983) Tetrahedron Lett., 24, 245-248.
20. Seela, F., and Driller, H. (1985) Nucl. Acids Res., 13, 911-926.
21. Gait, M., Matthes, W. D., Singh, M., Sproat, B. S., and Titmas, R. C. (1982) Chemical and Enzymatic Synthesis of Gene Fragments (Gassen, H. G., and Lang, A., Eds.) Verlag Chemie, Weinheim.
22. Uesugi, S., Shida, T., and Ikehara, M. (1981) Chem. Pharm. Bull., 29, 3573-3585.

23. Cosstick, R., and Eckstein, F. (1985) Biochemistry, 24, 3630-3638.
24. Thomas, G. A., and Peticolas, W. L. (1984) Biochemistry, 23, 3202-3207.
25. Bloomfield, V. A., Crothers, D. M., and Tinoco, I. (1974) Physical Chemistry of Nucleic Acids (Woods, J. A., Eds.) pp 349, Harper and Row, New York.
26. Borer, P. N., Dengler, B., Uhlenbeck, O. C., and Tinoco, I. (1974) J. Mol. Biol., 86, 843-853.
27. Albergo, D. D., Marky, L. A., Breslauer, K. J., and Turner, D. H. (1981) Biochemistry, 20, 1409-1413.
28. Pohl, F. M. (1974) Eur. J. Biochem., 42, 495-504.
29. Gralla, J., and Crothers, D. N. (1973) J. Mol. Biol., 73, 497-511.
30. Seela, F., and Steker, H. (1985) Chem. Soc. Perkin Transaction I, in press.
31. Jiricny, J., Ubasawa, A., and Wood, S. (1985) Nucleosides, Nucleotides, 4, 205-207.