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Synthesis and physical characterization of the self-complementary, alternating pyrimidine/purine hexanucleotide d[CGTACG]

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ABSTRACT

The hexanucleotide d(CGTACG) has been synthesized by a phosphotriester method in which, for the first time, an O-6 protected deoxyguanosine derivative has been used to avoid side reactions of the guanine residues. Conformational analysis by circular dichroism shows that d(CGTACG) maintains a B form under conditions (5 M NaCl) where the all C/G hexanucleotide $d(CG)_3$ adopts a Z form, even though d(CGTACG) is a fully alternating pyrimidine/purine molecule. The ΔH for the helix-to-coil transition has been measured.

INTRODUCTION

The left-handed conformation of poly d(CG), first observed by circular dichroism[1] and now known as the Z form of DNA,[2,3] has been detected in solution with oligonucleotides as small as $d(CG)_3$ [4,5] and $d[T(2\text{-aminoA})]_3$.[5] It has also been found in Drosophila polytene chromosomes[7] and in $d(GC)_n$ oligonucleotides incoroporated into a plasmid,[8] but in small oligonucleotides of mixed sequence the Z-form has proved to be more elusive. For example, neither $d(AT)_3(CG)_3$[4] nor d(CGCGAATTCGCG)[9] can be induced into the Z-form. However, neither contains the strictly alternating pyrimidine/purine sequence that may be a prime structural requirement for Z-DNA.[10] This seeming reluctance of small molecules to be induced into the Z form makes them ideal tools for precise analysis of the structural requirements and energetics of the B→Z transition. Accordingly, we have recently begun the synthesis and conformational analysis of a number of short oligonucleotides for this purpose.

In view of the importance of guanine to the B→Z transition such oligonucleotides will contain a substantial number of guanine residues. Since degradation of guanine is known to occur during oligonucleotide synthesis using conventional protecting methodologies,[11,12] we have been interested, in addition, in devising new strategies for the protection of deoxyguanosine.[13]

We now wish to report use of the 2-phenylthioethyl group for O-6 protection of deoxyguanosine during the synthesis of d(CGTACG). Circular

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dichroism and melting studies of this molecule are also reported.

RESULTS AND DISCUSSION

Synthesis

The synthesis was carried out in solution using a phosphotriester method. The dinucleoside phosphate 3a (Figure 1 and Table 1) was prepared by condensation of d[DMT-Cp] (1) with the N-2 and O-6 protected deoxyguanosine nucleoside 2[13] using 1-(triisopropylbenzenesulfonyl)-3-nitrotriazole as the condensing agent. As has been observed by others,[14,17] the small amount of 3'-3' joined material was readily removed by careful chromatography on silica gel. Because of the guanine O-6 protection the darkly colored reaction mixtures and fluorescent impurities normally observed during condensation reactions involving guanine were totally absent. Phosphorylation of 3a with 2-chlorophenyl phosphoroditriazolide gave the 5' terminal dinucleotide 3c, while, alternatively, reaction of 3a with benzoyl chloride followed by detritylation gave the 3' terminal dimer 8. Construction of the tetramer d[DMT-TpApCpG*-OBz] (9) was effected by condensation of 8 with d[DMT-TpAp] (7); 7 was prepared in analogous fashion to 3c. After detritylation of 9 the hexamer d[DMT-CpG*pTpApCpG*-OBz] (11) was obtained

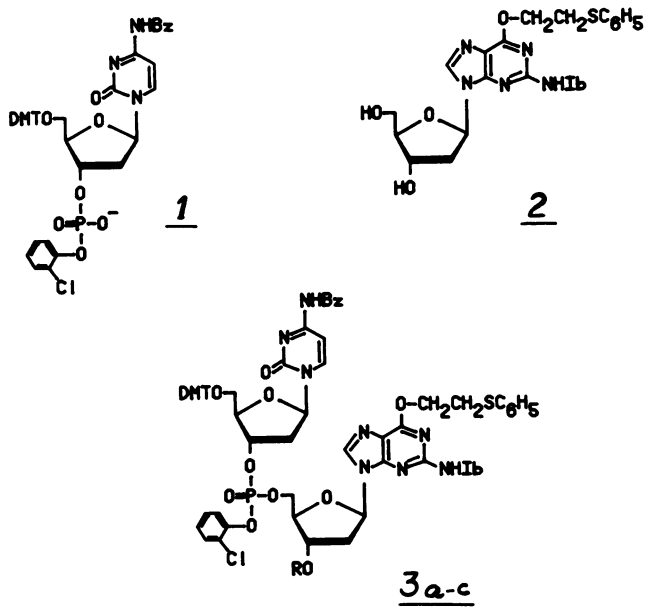

Figure 1. a, R = H; b, R = benzoyl; c, R = 2-chlorophenylphosphate

TABLE 1. CONDENSATION REACTIONS[a]

3'-phosphate (mmol)	5'-hydroxyl (mmol)	TPSNT	Reaction Time	Isolated Yield	Product
d[DMT-Cp] (1.00) 1	d[HO-G*-OH] (1.00) 2	3	1 hr	82%	d[DMT-CpG*-OH] 3a
d[DMT-Tp] (1.00) 4	d[HO-A-OH] (1.00) 5	3	1 hr	86%	d[DMT-TpA-OH] 6
d[DMT-TpAp] (0.80) 7	d[HO-CpG*-OBz] (0.80) 8	2.4	1 hr	75%	d[DMT-TpApCpG*-OBz] 9
d[DMT-CpG*p] (0.44) 3c	d[HO-TpApCpG*-OBz] (0.44) 10	2.6	2 hr	69%	d[DMT-CpG*pTpApCpG*-OBz] 11

[a]abbreviations are: DMT = 4,4'-dimethoxytrityl; C = 4-N-benzoyl-2'-deoxycytidine; G* = 6-O-phenthioethyl-2-N-isobutyryl-2'-deoxyguanosine; A = 6-N-benzoyl-2'-deoxyadenosine; T = thymidine, p = 2-chlorophenyl phosphate; Bz = benzoyl; TPSNT = 1-(triisopropylbenzenesulfonyl)-3-nitrotriazole. These abbreviations are based on the simplified scheme suggested by Reese.[16]

by condensation with d[DMT-CpG*p] (3c). Thus we were able to employ the same dinucleoside preparation (3a) to provide both the 5' and 3' terminal dimers. Protection of the 3' hydroxyl group was employed for all condensations above the dimer level, since incorrectly joined material undoubtedly would be more difficult or impossible to separate.

The deprotection was carried out in four steps. 1) Deprotection of the triesters was effected by treatment with dilute ammonia at room temperature overnight. 2) The phenylthioethyl groups were oxidized to the corresponding sulfoxide with sodium metaperiodate. 3) β-elimination of the sulfoxides, and complete N deprotection, was accomplished by heating with concentrated ammonia at 55° for 24 hours. 4) Detritylation was carried out using 80% acetic acid. The final product was then desalted on a Sephadex G-10 column and purified by hplc on a semi-preparative μ Bondapak C_{18} column (Figure 2). Degradation of a

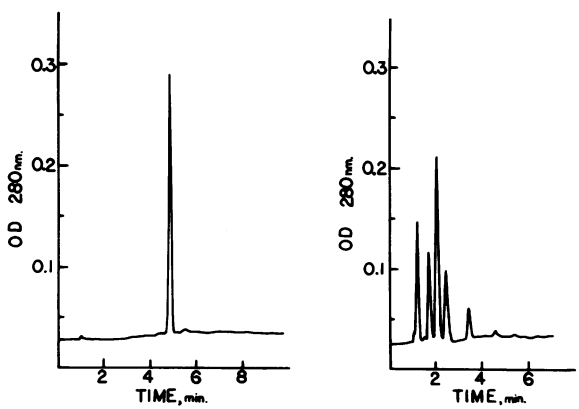

Figure 2. d(CGTACG) after purification (left) and after degradation with venom phosphodiesterase (right).

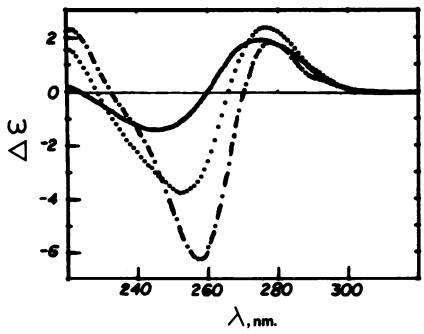

Figure 3. Circular dichroism spectra of d(CGTACG) at 0°C in 1 M NaCl (-•-•); 5 M NaCl (••••); and at 50°C (——).

sample of d(CGTACG) with venom phosphodiesterase gave the expected ratio of cytidine-5'-phosphate:thymidine-5'-phosphate:guanosine-5'-phosphate:cytidine: adenosine-5'-phosphate of 1:1:2:1:1 (Figure 3). In addition, the exclusive presence of 5'→3' linkages was established by complete degradation of a sample of d(CGTACG) with spleen phosphodiesterase.

The phenylthioethyl group employed for guanine O-6 protection eliminated guanine degradation during the condensation reactions. However, removal by the two step oxidation/elimination procedure does increase the time required for deprotection. Syntheses employing other O-6 protecting groups are being carried out and will be reported shortly.

Circular Dichroism and Stability

The circular dichroism spectra of d(CGTACG) in 1 M and 5 M NaCl are shown in Figure 3. In each case the spectrum is that of a normal B conformation, with no trace of a Z form evident. The slightly different intensities of the bands are due to destabilization of the helix form in high salt. At high temperature the CD spectra are identical for both 1 M and 5 M NaCl.

The melting curve shown in Figure 4 is indicative of a cooperative

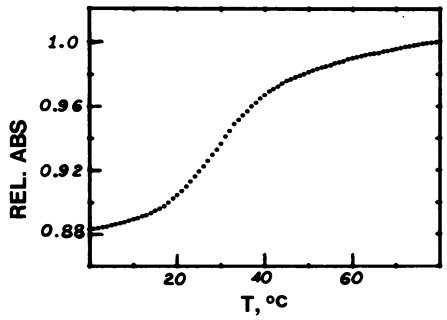

Figure 4. A typical absorbance vs temperature plot for d(CGTACG) in a buffer solution containing 10mM sodium phosphate, 1mM Na_2 EDTA, 1 M NaCl at pH = 7.0.

TABLE 2

C_T μM in single strands	T_m, °C
17.7	27.4
26.2	29.1
33.5	30.4
76.5	33.0
97.9	34.6
174	36.7

process with an average hypochromicity of 12%, and a T_m of 29.1° (26.2μM). A series of such experiments at different concentration gave the data listed in Table 2. The van't Hoff enthalpy may be determined from the slope of a plot of $1/T_m$ vs $\ln C_T$ since

$$\frac{1}{T_m} = \frac{R}{\Delta H} \ln C_T + \frac{\Delta S}{\Delta H}$$

as shown in Figure 5, giving a value of 46 kcal/mol. The two state melting process presumed in the van't Hoff equation has been confirmed by the excellent agreement of the van't Hoff ΔH of 46 kcal/mol with the ΔH measured directly, by differential scanning calorimetry, of 47 kcal/mol.[18]

The inability of the alternating pyrimidine/purine molecule d(CGTACG) to adopt a Z conformation in solution is consistent with the behavior of other small molecules of a mixed, if not a strictly alternating sequence. Yet the molecule d(CGCGCG) does adopt a Z form.[4,5] Evidently in molecules of sequence d(CGNNCG) the central base pair is critical to the ability to undergo a B→Z transition. Thus modification of the base residues of this central dimer may give other molecules which can be induced into the Z form and thereby more clearly define the structural requirements of Z-DNA. Such studies are under way and some results will be reported shortly.

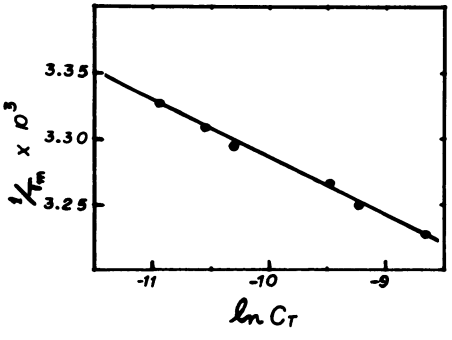

Figure 5. Plot of inverse melting temperature vs ln of concentration for d(CGTACG) in a buffer solution containing 10mM sodium phosphate, 1mM Na_2 EDTA, 1 M NaCl at pH = 7.0.

EXPERIMENTAL

Protected deoxynucleosides were prepared by procedures described elsewhere.[19] Other reagents and procedures used were as previously reported.[6] The concentration of d(CGTACG) was determined using an extinction coefficient of 5.89×10^4 at 260 nm and 80°C estimated from nearest neighbor interactions.[20] Circular dichroism spectra were obtained as described previously[6] except that a 1 cm pathlength cell was used.

General procedure for condensation reactions

To a solution of the appropriate 3'-O-(2-chlorophenyl)phosphate[6] and 5' hydroxyl components, dried three times by evporation of pyridine, is added 1-(triisopropylbenzenesulfonyl)-3-nitrotriazole (TPSNT). After the specified time (Table 1), the reaction is terminated by dropwise addition to water. Triethylamine (2mmol/mmol TPSNT) is then added and the mixture extracted with four portions of methylene chloride:methanol (70:30). The combined organic layers are concentrated and the residue purified by flash chromatography on silica gel. The quantities used and yields obtained are given in Table 1.

d[DMT-CpG*-OBz] (3b)

To 3a (1.3g, 1mmol) dissolved in 10mL of pyridine was added benzoyl chloride (0.7mL, 6mmol). After 1 hr the reaction mixture was added dropwise to 100mL of 3% aqueous sodium bicarbonate. The solution was extracted with three 75mL portions of methylene chloride:methanol (70:30). The combined organic layers were concentrated to a gum, which was not further purified.

d[HO-CpG*-OBz] 8

To 3b was added 40mL of a saturated solution of $ZnBr_2$ in nitromethane: water (99:1).[21] After 10 minutes 20mL of 1 M NH_4OAc was added and the mixture was extracted with two 100mL portions of methylene chloride. The combined organic layers were evaporated and the residue purified by flash chromatography to give 8 in an overall yield from 3a of 50%.

Deprotection and Purification

The hexamer 11 was dissolved in methanol:pyridine (1:1) and treated with an equal volume of aqueous ammonia at room temperature for 24 hours. The solution was then concentrated to remove ammonia and pyridine and a portion of the residue was then treated with excess aqueous $NaIO_4$ at room temperature for 24 hours. The solution was concentrated and the residue dissolved in concentrated aqueous ammonia. This solution was kept at 55° for 24 hours. It was then concentrated to remove ammonia and treated with 80% acetic acid for twenty minutes. The acetic acid was removed by coevaporation with water; the aqueous layer extracted once with ether and then desalted on a Sephadex

G-10 column. Appropriate fractions were combined, concentrated and the residue purified on a semi-preparative μ Bondapak C_{18} column to give 175 OD_{260} of d(CGTACG).

Treatment of a 1.0 OD_{260} sample with venom phosphodiesterase in 0.1 M phosphate buffer, pH 8.5 at 37° gave, by hplc analysis: deoxycytidine-5'-phosphate (1.2 min, 1.1 parts); thymidine-5'-phosphate (1.7 min, 0.8 parts); deoxyguanosine-5'-phosphate (2.0 min, 2.1 parts); deoxycytidine (2.5 min, 0.9 parts); and deoxyadenosine-5'-phosphate (3.4 min, 1.0 parts) using a μ Bondapak C_{18} cartridge (Waters Z module). Complete degradation was also effected by treatment with spleen phosphodiesterase in 0.1 M phosphate buffer, pH 6.5 at 37°.

ACKNOWLEDGMENTS

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