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Specifically alkylated DNA fragments. Synthesis and physical characterization of d[CGC(O^6Me)GCG] and d[CGT(O^6Me)GCG]

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ABSTRACT

Two hexamer DNA fragments containing a carcinogenic modified base, O^6-methyl guanine, have been synthesized by a solid-phase phosphotriester method, in which the unmodified guanine residues present were O^6 protected with the 4-nitrophenylethyl group. These two alkylated oligonucleotides were found to have similar T_m's about 40° lower than the unmodified parent compound, $d(CG)_3$. Moreover, the presence of the (O^6Me)G appears to inhibit the B→Z transition, as determined by CD spectroscopy.

INTRODUCTION

Alkylation of the guanine O^6 oxygen atom in DNA has been shown to result in both a mutagenic[1] and a carcinogenic[2] lesion. The molecular basis for these effects may reside in the ambiguous coding properties of the O^6-alkylguanine base.[3] For example, polymers containing O^6-methyl guanine in both the ribo[4] and deoxyribo[5] series have been shown to direct RNA polymerase incorporation of uridine: A G→A mutation. In one case a low level of adenosine incorporation, corresponding to a G→T mutation, was also reported.[4] The latter type of mutation has recently been shown to be involved in the activation of a human bladder cancer oncogene.[6,7] Furthermore, the inability of the E. coli mismatch correction system to alter the rate of (O^6Me)G induced mutagenesis suggests that no good base pair may exist for (O^6Me)G.[8]

It is therefore of interest to determine the relative stabilities of (O^6Me)G:N base pairs to aid in understanding the biological effects of alkylation and to give additional insight into the mechanism of action of the polymerase and mismatch correction systems. We are currently engaged in a program designed to define the effects of guanine alkylation via synthesis and physical study of DNA fragments containing this modification.

At this time we wish to report the synthesis of two hexamers, one having a central (O^6Me)G:C base pair while the other has an (O^6Me)G:T base pair. In addition, the first use of another of the deoxyguanosine O^6

protecting groups we recently introduced[9] is reported, together with a modified deprotection procedure.

RESULTS AND DISCUSSION

Synthesis

The O^6-methyl-2'-deoxyguanosine was prepared by the method we reported previously.[10] By modification of our procedure 2-N-isobutyryl-5'-O-dimethoxytrityl-3'-O-levulinyl-2'-deoxyguanosine (1) was converted to the fully protected compound, 2-N-isobutyryl-6-O-(4-nitrophenylethyl)-5'-O-dimethoxytrityl-3'-O-levulinyl-2'-deoxyguanosine (2), in 60-75% yield. Protection of the 6-oxygen prevents the degradation of deoxyguanosine that is otherwise observed during condensation reactions.[11,12] This one-flask route is much more direct than the route we reported previously for O^6 protection with the 4-nitrophenylethyl group.[9] Moreover, 2 is suitable for elongation in either the 5' or 3' directions by removal of either the 5'-O-dimethoxytrityl group or the 3'-O-levulinyl group. The 4-nitrophenylethyl group, (or Uhlmann-Pfleiderer group) which was originally introduced as a phosphate protecting group,[13] is removed at the end of the synthesis by a base promoted β-elimination reaction. This is conveniently accomplished concurrently with phosphotriester deprotection by treatment with 2-nitrobenzaldoxime and excess 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU), in the first deprotection step. Thus no additional steps are required for cleavage of the nitrophenylethyl group, unlike the two step cleavage we reported for removal of the phenylthioethyl group from the O^6.[11]

The oligonucleotide synthesis itself was performed by the addition of monomers, beginning with attachment of the succinylated compound 2d to amino functionalized polystyrene.[14,15] The addition cycle consisted of: 1) detritylation with 2% benzenesulfonic acid in methylene chloride:methanol (70/30), 2) condensation with the next monomer using triisopropylbenzenesulfonyl chloride and N-methyl imidazole,[16] 3) capping with acetic anhydride. Between each reaction cycle the resin was washed alternately with portions of methylene chloride and methanol, then diethyl ether, and dried under vacuum. The yields for each condensation for both sequences are given in the Table. A single preparation of the trimer d[DMT-(O^6Me)GpCpG-Polymer] was divided into two equal portions and used for both sequences. Subsequent monomer condensations on the two trimer portions were carried out simultaneously, perhaps accounting for the similarity in yields for these steps. The anomolously low yield in the next to last condensation is not general for

CONDENSATION REACTIONS[a]

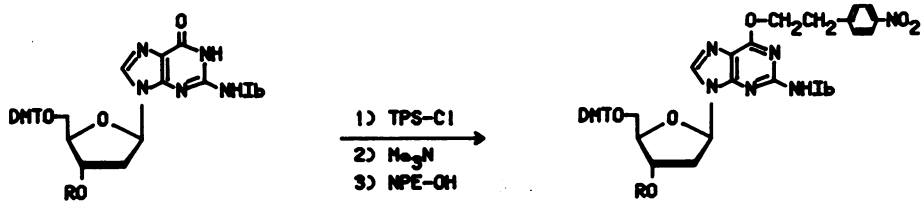
Phosphate Component (mmol)	Hydroxy Component (mmol)	TPS/MI (mmol)	Product (Yield)
DMT-Cp (6.0)	HO-G*-Polymer (1.5)	18.0/54	DMT-CpG*-Polymer (87)
DMT-(O^{6}Me)Gp (4.0)	HO-CpG*-Polymer (1.3)	12.0/36	DMT-(O^{6}Me)GpCpG*-Polymer (85)
DMT-Cp (4.0)	HO-(O^{6}Me)GpCpG*-Polymer (0.65)	12.0/36	DMT-Cp(O^{6}Me)GpCpG*-Polymer (83)
DMT-G*p (2.0)	HO-Cp(O^{6}Me)GpCpG*-Polymer (0.54)	6.0/18	DMT-G*pCp(O^{6}Me)GpCpG*-Polymer (55)
DMT-Cp (2.5)	HO-G*pCp(O^{6}Me)GpCpG*-Polymer (0.30)	7.5/22.5	DMT-CpG*pCp(O^{6}Me)GpCpG*-Polymer (74)
DMT-Tp (4.0)	HO-(O^{6}Me)GpCpG*-Polymer (0.65)	12.0/36	DMT-Tp(O^{6}Me)GpCpG*-Polymer (79)
DMT-G*p (2.0)	HO-Tp(O^{6}Me)GpCpG*-Polymer (0.51)	6.0/18	DMT-G*pTp(O^{6}Me)GpCpG*-Polymer (59)
DMT-Cp (2.5)	HO-G*pTp(O^{6}Me)GpCpG*-Polymer (0.30)	7.5/22.5	DMT-CpG*pTp(O^{6}Me)GpCpG*-Polymer (71)

[a]abbreviations are: DMT = 4,4'-dimethoxytrityl; C = 4-N-benzoyl-2'-deoxycytidine; T = thymidine; G* = 6-O-(4-nitrophenylethyl)-2-N-isobutyryl-2'-deoxyguanosine; p = 2-chlorophenylphosphate; TPS = 2,4,6-triisopropylbenzenesulfonyl chloride; MI = N-methyl imidazole. These abbreviations are based on the simplified scheme suggested by Reese.[22]

compounds containing O^6 protecting groups, since this has not been observed in other cases.[11,12] In principle this condensation could have been repeated to increase the overall yield of pentamer, but was not.

Deprotection of the hexamers was effected by treatment first with 2-nitrobenzaldoxime and DBU for 20 hours and then by treatment with methanol and DBU for three days, giving the 5'-O-dimethoxytrityl oligonucleotides. Since this was the first use of the nitrophenylethyl group for guanine O^6 protection, and because of the presence of the $(O^6Me)G$ residue, we carried out several small scale trial deprotections to determine the optimal conditions. The nitrophenylethyl group has a substantial effect on hplc retention, so that its clean removal by excess DBU was readily monitored. For deprotection of the amino groups we originally used the standard ammonia treatment, but at 65° for three days, because of the slow rate of ammonolysis of the isobutyryl group of O^6-alkyl guanosines. Each sample was then desalted on a Sephadex G-10 column, purified by reversed-phase hplc and detritylated with 80% acetic acid. Although each sample was homogeneous before detritylation, after detritylation two compounds were clearly present in each case. This situation is similar to the results reported in the synthesis of the tetranucleotide $d[T(O^6Me)GCA]$, in which two compounds were obtained upon deprotection by the standard methods; the major one proving to be the desired product while the minor one was not identified.[17] In the present case, while preparative resolution of the $d[CGT(O^6Me)GCG]$ mixture was not possible, the two components of the $d[CGC(O^6Me)GCG]$ mixture were sufficiently resolved to be separated.

Hplc analysis of the mixture obtained upon degradation with venom phosphodiesterase and alkaline phosphatase showed that the slower component



1 2

Figure 1. a, R=levulinyl; b, R=H; C, R=2-chlorophenylphosphate; d, R=succinyl.

contained the correct ratio of dC:dG:d(O^6Me)G of 3:2:1, while the faster compound contained dC and dG but had no d(O^6Me)G; instead 2-amino 2'-deoxyadenosine was found (identified by comparison with an authentic sample) again in the ratio of dC:dG:d(2-NH_2)A of 3:2:1. In addition, the hexanucleotide d[CGT(2-NH_2)ACG][12] was found to comigrate with the faster of the two components present in the d[CGT(O^6Me)GCG] mixture. Finally, treatment of a sample of 2-N-isobutyryl-6-O-methyl-2'-deoxyguanosine with aqueous ammonia at 65° was found to give rise to very slow formation of 2-amino-2'-deoxyadenosine.

Deprotection using concentrated ammonia at room temperature, rather than 65° appeared to avoid ammonolysis of the 6-O-methyl group while still slowly removing the isobutyryl function. However, since the presence of even a trace of d[CGT(2-NH_2)ACG], which has a much higher T_m,[12] in the d[CGT(O^6Me)GCG] sample would seriously alter any T_m observed, we elected to avoid entirely the use of ammonia. Instead the above mentioned solution of DBU in methanol was used. This gave clean deprotection for both sequences. Hplc profiles and the enzymatic degradation of each sequence are shown in Figures 2 and 3.

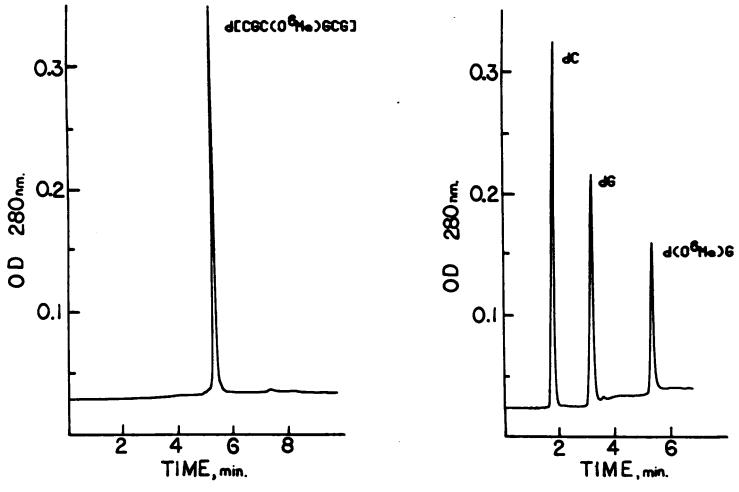


Figure 2. Hplc profiles of d[CGC(O^6Me)GCG] after purification (left), using a gradient of 5% to 20% CH_3CN:0.1 M triethylammonium acetate (TEAA) in 5 min at 4 mL/min, and after degradation with venom phosphodiesterase and alkaline phosphatase (right), using a gradient of 2% to 10% CH_3CN:0.1 M TEAA in 5 min at 4 mL/min.

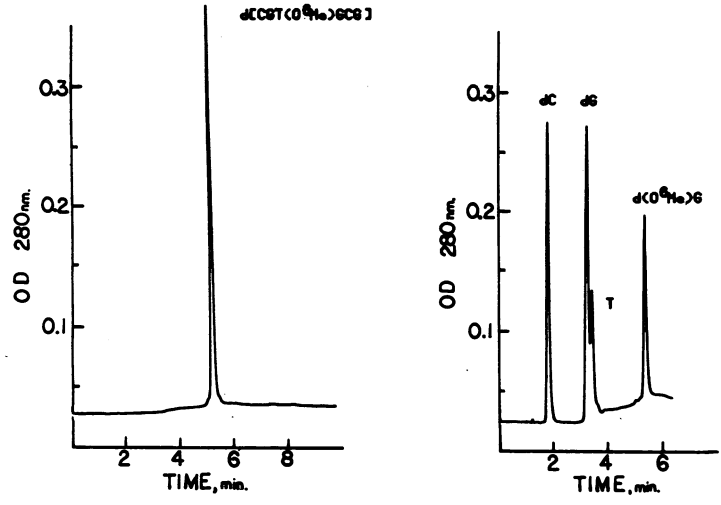


Figure 3. Hplc profiles of d[CGT(O^6Me)GCG] after purification (left) and after degradation with venom phosphodiesterase and alkaline phosphatase (right); in each case using the conditions given in Figure 2.

Physical Characterization

Figure 4 shows a plot of $1/T_m$ vs lnC for d[CGC(O^6Me)GCG], d[CGT(O^6Me)GCG], and the unmodified "parent" compound $d[CG]_3$. The destabilization of the duplex caused by the O^6-methyl group is evident in the

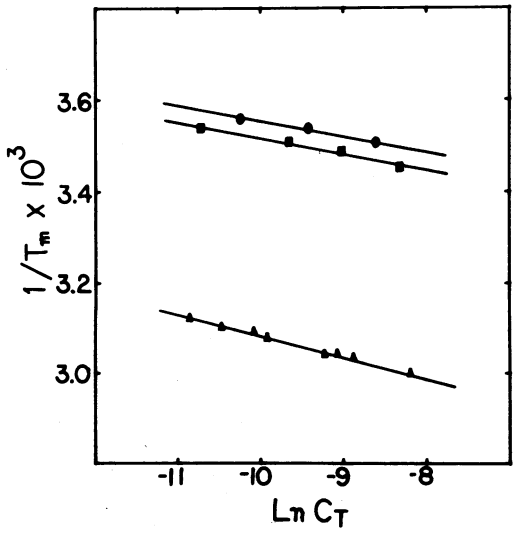


Figure 4. Plot of inverse melting temperature vs ln of concentration for d[CGC(O^6Me)GCG], (■); d[CGT(O^6Me)GCG], (●); and $d[CG]_3$, (▲).

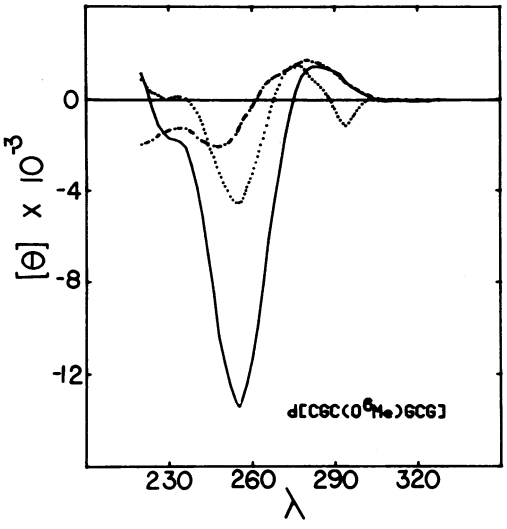


Figure 5. Circular dichroism spectra of d[CGC(O^6Me)GCG] at 0° in 1 M tetramethylammonium chloride (TMA) (——); 5 M TMA (•••); and at 50° (-•-•); [θ] is in degrees·cm^2/mmol of single strand.

40° T_m difference between $d[CG]_3$ and the methylated sequences. In addition, the "mutation" sequence, d[CGT(O^6Me)GCG], is seen to be little different in T_m, actually lower than d[CGC(O^6Me)GCG]. These results are consistent with the ambiguous coding reported for O^6MeG in biological studies.[3-5] Moreover, the low T_m observed for both methylated fragments supports the hypothesis that neither (O^6Me)G:C nor (O^6Me)G:T base pairing is very effective.[8]

Figure 5 shows the CD spectrum of d[CGC(O^6Me)GCG] in 1 M and 5 M tetramethylammonium chloride (TMA). The small negative band at long wavelength in the presence of 5 M salt is reminiscent of the long wavelength inversion observed with poly d[CG],[19] ascribed to the Z form of DNA.[20] In fact, the 5 M CD in Figure 5 is nearly identical to that of poly d[CG] at intermediate salt concentrations (3.5 M TMA or 2.5 M NaCl).[21] Thus the O^6MeG both substantially reduces the thermal stability of the B-DNA duplex and at the same time alters the salt dependent conformational behavior. The CD spectrum of d[CGT(O^6Me)GCG] (Figure 6) also displays a small negative band at long wavelength. In contrast, the CD spectra of d[CGTACG], reported earlier,[11] showed no trace of a long wavelength inversion.

The results reported above document the profound effect of guanine O^6 methylation on DNA duplex stability and conformational behavior. Moreover, now that we have developed deprotection procedures that avoid degradation of (O^6Me)G, it is feasible to synthesize larger alkylated DNA fragments to further clarify the effects of such alkylation. This is being done. In

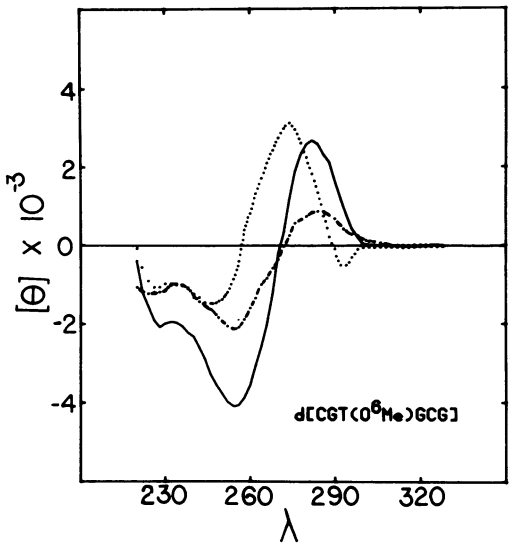


Figure 6. Circular dichroism spectra of d[CGT(O^6Me)GCG] at 0° in 1 M TMA (---); 5 M TMA (•••); and at 50° (-•-•); [θ] is in degrees•cm^2/mmol of single strand.

addition, we have improved our method for O^6 protection with the 4-nitrophenylethyl group and have demonstrated a deprotection procedure for this group that does not require additional steps.

EXPERIMENTAL

Chloromethylpolystyrene (1.33 meq/g) from Bio Rad Laboratories was derivatized according to the literature.[14] Reagents and procedures not described below were as previously reported.[15,18] The concentrations of d[CGC(O^6Me)GCG] and d[CGT(O^6Me)GCG] were determined using extinctions at 260 nm of 4.2×10^4 and 4.0×10^4, respectively, as determined from enzymatic hydrolysis. Physical measurements were obtained as described previously.[15]

2-N-Isobutyryl-5'-O-dimethoxytrityl-3'-O-levulinyl-2'deoxyguanosine (1)

To 3.4 g (10 mmol) of 2-N-isobutyryl-2'-deoxyguanosine and 100 mL of dry pyridine was added 5.1 g (15 mmol) of 4,4'-dimethoxytrityl chloride, 61 mg (0.5 mmol) of 4-dimethylaminopyridine and 2.5 mL (18 mmol) of triethylamine. The mixture was stirred at room temperature for one hour, poured into a 200 mL portion of 5% NaHCO and extracted with two 150 mL portions of ethyl acetate. The combined ethyl acetate layers were concentrated to a gum, which was dissolved in 100 mL of dry pyridine. To this solution was added, with filtration, an ethereal solution of levulinic anhydride (30 mmol) prepared by reaction of 6.2 mL (60 mmol) of levulinic acid with 6.2 g (30

mmol) of N,N'-dicyclohexylcarbodiimide in 50 mL of ether. The reaction mixture was then concentrated to remove the ether, and stirred at room temperature for one hour. The mixture was then poured into a 100 mL portion of 5% $NaHCO_3$, and extracted with two 100 mL portions of ethyl acetate. The combined ethyl acetate layers were concentrated to a gum which was dissolved in methylene chloride and purified by flash chromatography on silica gel. The product fractions were combined and evaporated to give a residue of 6.4 g (85%). ^1H NMR ($CDCl_3$) δ 1.00 ("t", 6, J_{app} = 6 Hz, $[CH_3]_2C$), 2.17 (s, 3, CH_3CO), 2.63 (m, 7, Me_2C-H, $-CH_2CH_2-$, $H_{2',2''}$), 3.33 (m, 2, $H_{5',5''}$), 3.73 (s, 6, 2 CH_3O-), 4.18 (m, 1, $H_{4'}$), 5.50 (m, 1, $H_{3'}$), 6.15 ("t", 1, J_{app} = 7 Hz, $H_{1'}$), 6.67 -7.83 (m, 14, H_8, Ar), 8.67 (brs, 1, N^2-H), 12.0 (brs, 1, N^1-H); UV_{max} (MeOH) 235 (ε 27 300); UV_{sh} 260, 280 (ε 19 400, 15 800).

Anal. Calcd. for $C_{40}H_{43}N_5O_9$: C, 65.11; H, 5.87; N, 9.49. Found: C, 64.94; H, 6.01; N, 9.29.

2-N-Isobutyryl-6-O-(4-nitrophenylethyl)-5'-O-dimethoxytrityl-3'-O-levulinyl-2'-deoxyguanosine {d[DMT-G*-Lev]} (2)

To 7.4 g (10 mmol) of 1 and 75 mL of methylene chloride was added 6.0 g (20 mmol) of 2,4,6-triisopropylbenzenesulfonyl chloride, 61 mg (0.5 mmol) of 4-dimethylaminopyridine and 5.6 mL (40 mmol) of triethylamine. Reaction was generally complete within two hours (tlc), whereupon 11.5 g (70 mmol) of 4-nitrophenylethanol was added and the mixture cooled to 0°. To this cold solution was added 10 mL of trimethylamine. After ten minutes DBU (6.0 mL, 40 mmol) was added. Reaction was allowed to proceed for thirty minutes and 10 mL of a mixture of acetic anhydride:pyridine (1:10) was then added. After ten minutes the mixture was poured into a 200 mL portion of 5% $NaHCO_3$. The mixture was extracted with two 100 mL portions of methylene chloride and the combined methylene chloride layers were concentrated to a gum which was dissolved in diethyl ether and purified by flash chromatography on silica gel (Et_2O). The product fractions were combined and evaporated to give a pure residue of 6.6 g (75%). ^1H NMR ($CDCl_3$) δ 1.18 (m, 6, $[CH_3]_2C$), 2.18 (s, 3, CH_3CO), 2.72 (m, 7, Me_2C-H,$-CH_2CH_2CO$, $H_{2',2''}$), 3.38 (m, 4, Ar$-CH_2-$, $H_{5',5''}$), 3.77 (s, 6, 2 CH_3O-), 4.25 (m, 1, $H_{4'}$), 4.85 ("t", 2, J_{app} = 6Hz, $-O-CH_2-$), 5.55 (m, 1, $H_{3'}$), 6.38 ("t", 1, J_{app} = 7Hz, $H_{1'}$), 6.78-8.28 (m, 18, H_8, Ar), 7.77 (brs, 1, N^2-H); UV_{max} (MeOH) 270, 235 nm (ε 32 300, 29 200); UV_{min} 247 nm (ε 24 600).

Anal. Calcd. for $C_{48}H_{50}N_6O_{11}$: C, 65.00; H, 5.68; N, 9.48. Found: C, 64.98; H, 5.93; N, 9.26.

2-N-Isobutyryl-6-O-methyl-5'-O-dimethoxytrityl-2'-deoxyguanosine {d[DMT-(O^6Me)G]}

To 1.75 g (5 mmol) of 2-N-isobutyryl-6-O-methyl-2'-deoxyguanosine and 50 mL of dry pyridine was added 2.54 g (7.5 mmol) of 4,4'-dimethoxytrityl chloride, 30 mg (0.25 mmol) of 4-dimethylaminopyridine and 1.25 mL (9 mmol) of triethylamine. The mixture was stirred at room temperature for one hour, poured into a 100 mL portion of 5% $NaHCO_3$, and extracted with two 100 ml portions of methylene chloride. The combined methylene chloride layers were concentrated to a gum which was dissolved in methylene chloride and purified by flash chromatography on silica gel. The appropriate fractions were combined and evaporated to give a pure residue of 2.9 g (89%). Crystallization of a sample from cyclohexane and methylene chloride gave diamond shaped plates, m.p. 105-110°, softens beginning at ca. 85°.
^{1}H NMR ($CDCl_3$) δ 1.20 (d, 6, J = 7Hz, $[CH_3]_2C$), 2.65 (m, 2, $H_{2',2''}$), 3.02 (m, 1, Me_2C-H), 3.40 (m, 2, $H_{5',5''}$), 3.80 (s, 6, 2 CH_3O-), 4.02 (m, 1, 3'-OH), 4.13 (s, 3, CH_3O-), 4.25 (m, 1, $H_{4'}$), 4.73 (m, 1, $H_{3'}$), 6.77 (m, 5, $H_{1'}$, Ar), 7.28 (m, 9, Ar), 8.02 (s, 1, H_8), 8.10 (brs, 1, N^2-H); UV_{max} (MeOH) 270, 234 (ε 20 400, 27 400); UV_{min} 251 (ε 17 000).

Anal. Calcd. for $C_{36}H_{39}N_5O_7 \cdot 3/4\ H_2O$: C, 64.80; H, 6.12; N, 10.50. Found: C, 64.81; H, 6.27; N, 10.31.

General Procedure for Detritylation

The resin bound oligonucleotide was treated with cold 2% benzenesulfonic acid in methylene chloride:methanol (70/30) for two minutes, the acid was removed by suction filtration and the resin washed with three portions of the methylene chloride:methanol mixture, two portions of methylene chloride, and again two portions of methylene chloride:methanol. This procedure was repeated two more times. The third acid treatment invariably produced little of the dimethoxytrityl cation color. The resin was then washed with pyridine, three portions of diethyl ether, and dried under vacuum.

General Procedure for Condensation

To the resin bound oligonucleotide, after detritylation, was added a dry pyridine solution of the 5'-O-dimethoxytrityl-3'-O-(2-chlorophenyl) phosphate derivative of the next deoxynucleoside, triisopropybenzenesulfonyl chloride, and N-methyl imidazole. The mixture was shaken at room temperature for ca. two hours. The solution was then removed by suction filtration and the resin washed successively with several portions of pyridine, methylene chloride and ether. After drying the resin under vacuum the extent of

reaction was determined by acid treatment of a weighed sample[15] and the resin was then capped with acetic anhydride and N-methyl imidazole.

Cleavage from Resin, Deprotection, and Purification

To ca. 500 mg of resin bound hexamer and 170 mg of 2-nitrobenzaldoxime was added 5 mL of THF and 0.5 mL of DBU. The mixture was shaken overnight and 5 mL of methanol was then added. The mixture was then shaken for three days, filtered, and the resin washed successively with pyridine, methanol, methylene chloride, methanol, and ether. The filtrate was then concentrated and applied to a Sephadex G-10 column. The combined product fractions were concentrated and the residue was purified on a semi-preparative C-18 Bondapak column, using a gradient of 20% to 50% CH_3CN:0.1 M triethylammonium acetate (TEAA) in thirty minutes at 2 mL/min. The combined product fractions were concentrated and the residue was treated with 80% acetic acid. After twenty minutes the acetic acid was removed by evaporation under reduced pressure and the residue was partitioned between equal volumes of water and ether. The aqueous layer was concentrated to a small volume and applied to the above semi-preparative column. A gradient of 5% to 20% acetonitrile:0.1 M TEAA in thirty minutes at 2 mL/min was employed. The combined product fractions were concentrated and applied to the Sephadex G-10 column to give ca. 450 OD of pure d[CGC(O^6Me)GCG] or d[CGT(O^6Me)GCG].

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