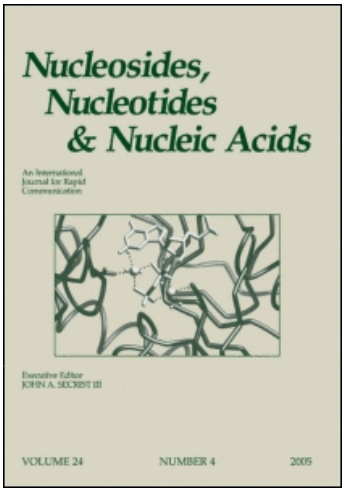

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SYNTHESIS OF PROTECTED 8-SUBSTITUTED DEOXYRIBONUCLEOSIDES AND ITS HELIX STABILITY IN OLIGODEOXYRIBONUCLEOTIDES CONTAINING THE Eco RI RECOGNITION SITE

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ABSTRACT: Octadeoxyribonucleotides with the sequences d(GGA*ATTCC), d(GGAA*TTCC), and d(GG*AATTCC) have been prepared by solid phase synthesis using the H-phosphonate units containing modified base moieties. These oligomers which have an isosterically altered recognition sequence of the restriction endodeoxyribonuclease Eco RI. The oligomers, with replacement to deoxy-7,8-dihyroadenosine-8-one (dA^{OH}), 8-methoxydeoxyadenosine (dA^{OMe}) and 8-methoxydeoxyguanosine (dG^{OMe}) from deoxyadenosine or deoxyguanosine were used for studying recognition phenomena at the functional group level. From thermodyamic data of these alternating octamers it was shown that the oligomer containing 8-methoxydeoxyadenosine in the center of the recognition sequence destabilizes such duplexes less strongly than the oligomers containing other 8-substituted nucleosides in the 5'-side of the recognition sequences. Further, the hydrolysis by Eco RI of the modified oligomers perfectly resisted compared to d(GGAATTCC).

INTRODUCTION

Restriction endonucleases catalyze the cleavage of specific sequences in double stranded DNA. Due to their high specificity for DNA sequences and simplicity of the DNA site recognized, they serve as convenient model systems for the investigation of specific protein-DNA interactions.[1] The incorporation of modified sugar and base moieties into oligodeoxyribonucleotides have been used to study the interaction of the restriction endonucleases with their substrates.[2-23] We have been interested in a comparison of the structural requirements needed for the recognition of the substrate by enzymes. Such a comparison proves an excellent opportunity to learn something about the fundamental principles governing specific protein/nucleic acid interactions. In order to study the interaction of the restriction endodeoxyribonuclease Eco RI with its substrate, we have synthesized different oligodeoxyribonucleotides which contain the Eco RI

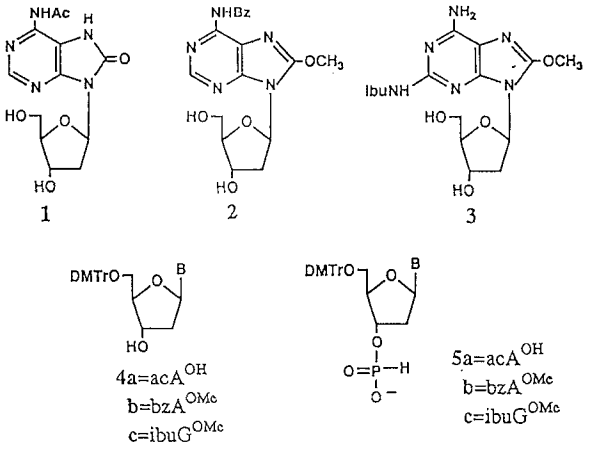
site, d(GGA^{OH}ATTCC) (**6**), d(GGAA^{OH}TTCC) (**7**), d(GGA^{OMe}ATTCC) (**8**), d(GGAA^{OMe}TTCC) (**9**), and d(GG^{OMe}AATTCC) (**10**), by the H-phosphonate approach[24]. Deoxy-7,8-dihydroadenosine-8-one was the major product eliminated directly from the DNA chain during irradiation, especially it is interesting for the interaction with enzymes.[25] On the other hand, the methoxy group has been shown to occur in the natural purine nucleoside spongosine[26], the isomer 8-methoxydexoyadenosine and guanosine were prepared.[27,28] The stabilities of these complementary octadeoxyribonucleotides were investigated by measuring temperature-absorbance profiles and circular dichroism (CD) spectra, since these duplexes had a right handed B-DNA like structure. We have also demonstrated that these modified DNA duplexes were strongly resistant to hydrolysis by the Eco RI.

RESULTS AND DISCUSSION

The deoxyribonucleoside 3'-H-phosphonate units are key intermediates in the synthesis of oligodeoxyribonucleotides by the H-phosphonate approach.

First, we examined the synthesis of the modified nucleosides, N^6-acetyldeoxy-7,8-dihyroadenosine-8-one (dacA^{OH}) (**1**) and N^6-benzoyl-8-methoxydeoxyadenosine (dbzA^{OMe}) (**2**), and N^2-isobutyryl-8-methoxydeoxyguanosine (dibuG^{OMe}) (**3**) as the starting materials for the preparation of the H-phosphonate units (**5**). Compounds **1-3** were synthesized using a modification of published procedure[27-29]. The dimethoxytritylated derivatives (**4**) were prepared by treatment of the modified nucleosides (**1-3**) with dimethoxytrityl chloride. The required nucleoside H-phosphonate building blocks (**5a-c**) were readily prepared by allowing the corresponding modified nucleoside derivatives (**4a-c**) to react with 1.1 molar equivalents of tris-(1,1,1,3,3,3-hexafluoro-2-propyl) phosphite (THFPP) in the presence of a catalytic amount of pyridine in CH_2Cl_2 at room temperature for 10 min, followed by a hydrolytic work-up and chromatography of the products.[30]

The H-phosphonate units were employed together with those of dA, dC, dG, and dT in solid phase synthesis of the oligomers **6-10** on a manual DNA synthesizer. As references compound **11** was also prepared. The synthesis followed a synthetic cycle of detritylation and condensation already described earlier[31]. After the synthetic cycle were over, the H-phosphonate product was oxidized to the phosphate with 0.1 M I_2 in THF-pyridine-H_2O (44:3:3, v/v), deprotected with 2.0% dichloroacetic acid in CH_2Cl_2 and treated with ammonia. The unblocked oligomers (**6-11**) were purified by TSKgel DEAE-2SW HPLC. Then the oligomers (**6-11**) were purified again by reverse phase HPLC.



dGCA^{OH}ATTCC	6
dGGAA^{OH}TTCC	7
dGGA^{OMe}ATTCC	8
dGGAA^{OMe}TTCC	9
dGG^{OMe}AATTCC	10
dGGAATTCC	11

Four of these modified self complementary fragments, d(GGA^{OH}ATTCC) (**6**), d(GGAA^{OH}TTAA) (**7**), d(GGA^{OMe}ATTCC) (**8**), and d(GGAA^{OMe}TTCC) (**9**), result in dA^{OH} or dA^{OMe}-dT base pairs in the double stranded form nucleic acid, while the fragment, d(GG^{OMe}AATTCC) (**10**), produces dG^{OMe}-dC base pairs in the DNA duplexes. The thermal denaturation experiments indicated that these derivatives exhibited thermally induced helix coil transition (Table 1). The modified sequences has major influence on the oligomer duplexes. In order to establish that the modified oligonucleotides adopted a B type helix, their circular dichroism (CD) spectra were recorded in low salt concentration at 18°C (Figure 1).[32] These includes the sequences, d(GGAA^{OH}TTAA) (**7**), d(GGA^{OMe}ATTCC) (**8**), and d(GGAA^{OMe}TTCC) (**9**) exhibited a thermally induced helix to coil transition under the Eco RI emzyme assay conditions.

Thermodynamic parameters for the transitions were determined over the concentration range 2-25 μM single strand concentration (C_T) from plots of 1/Tm vs. log C_T (Figure 2). This graph allows the calculation of the enthalpy (ΔH) from the slope and the entropy (ΔS) from the intercept for each particular oligomers.[33-35] The energetic contributions to ΔH from

Table 1: Enthalpy and entropy change values for double helix formation of the octamers **6–11**.

Compd.	Tm(°C)[a]	ΔH(kJ/mol)	ΔS(kJ/mol.K)	ΔΔG[b] (kJ/mol)
11	27.1	-154	-0.37	-
6	22.1	-597	-1.89	7.5
7	20.0	-479	-1.49	9.2
8	19.4	-327	-0.98	6.9
9	16.3	-778	-2.56	27.5
10	23.8	-275	-0.79	2.0

a) oligomer concentration was 5 μM; b) the values were calculated at 25°C.

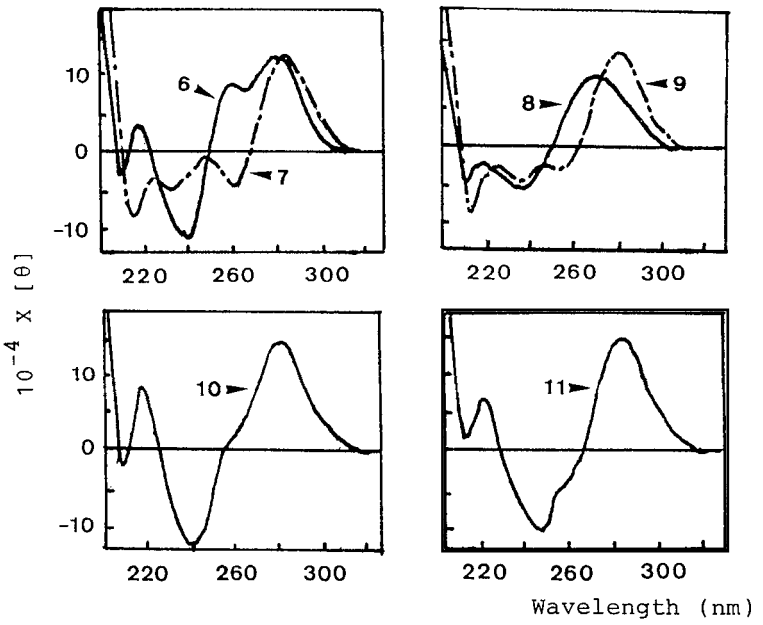


Figure 1. CD spectra of the oligodeoxyribonucleotides **6–11** in 10 mM TRIS/HCl (pH 7.6) buffer containing 80 mM NaCl and 20 mM $MgCl_2$ at 18°C.

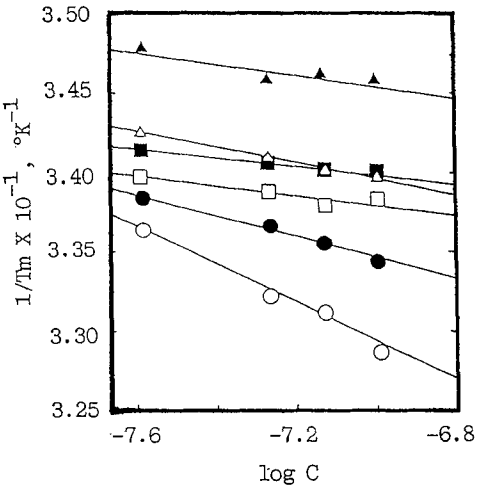


Figure 2. Plot of 1/Tm vs. log C for the oligomers **6** (□), **7** (■), **8** (△), **9** (▲), **10** (●), and **11** (○) in H_2O. All solutions contained 80 mM NaCl, 20 mM $MgCl_2$, and 10 mM TRIS/HCl, pH 7.6.

duplex formation comes both from hydrogen bonding and from stacking interactions with adjacent base pairs. The difference in Tm values between two different oligomers is proportional to the free energy difference ΔΔG between the two duplexes.[36] The free energy difference (ΔΔG) between the oligomer **11** and the octamers **6–10** reflects the stability of these duplexes in the environment of the aqueous buffer solution and is directly related to the Tm values. Due to the different slopes of the curves of Figure 2 the calculated values were all taken at a temperature of 25°C. The enthalpy change for the transition of the duplex to the single-stranded form was most pronounced for the oligomer **11**. The oligomers **6–10** containing 8-substituted adenosine or guanosine have a lower ΔH value compared to that of parent oligomer **11**. According to Table 1 some of the enthalpy and entropy values do not correspond to the Tm values. As can be seen, the oligomer **6** containing dA^{OH} in position 3 (from the 5'-end) has a lower ΔH and ΔS values compared to that containing dA^{OH} in position 4, but exhibits a higher Tm value. This suggests that the free energy difference value term plays an important part in the process of duplex formation of oligomers **6–7** containing dA^{OH}.

On the other hand, the enthalpy values for the octamers **8–10** containing dA^{OMe} and dG^{OMe} corresponds to the melting temperature. From the ΔH values of Table 1, it can be seen that the oligomer **9** which contain the hydrophobic

adenosine (**2**) in position 4 (from the 5'-end) exhibits the lowest ΔH value. The oligomers **8** and **10** which contains 8-methoxydeoxyadenosine (**2**) or 8-methoxydeoxyguanosine (**3**) in position 2 or 3 from the 5'-end show higher values. These results show that the oligonucleotides containing 8-substituted nucleosides (dA^{OH}, dA^{OMe}, dG^{OMe}) in the recognition sequence were more sensitive to helix stability. Further, the presence of the 8-substituted nucleosides in the center of the recognition sequences clearly results in a less stable structure than observed with oligonucleotides containing 8-substituted nucleosides in the 5'-side of the sequence.

Finally the relative rates of hydrolysis of the modified oligomers **6-10** were determined by HPLC technique. As expected, the modified oligomers **6-10** were not cleaved even after incubation for 36 h (Figure 3). This result shows that oligonucleotides containing 8-substituted adenine or guanine in the recognition sequences to Eco RI were completely resistant to this enzyme. A very important characteristic of these oligomers is their thermal stability, especially as Eco RI requires a double-strand structure as substrate. In addition to its value for the study of nucleic acid structure and protein-nucleic acid interactions, 8-substituted adenosine and guanosine have also generated the attention of some investigators for its mutagenic properties.[25,26]

EXPERIMENTAL

MATERIALS AND GENERAL METHODS

N^6-Acetyldeoxy-7,8-dihyroadenosine-8-one ($dacA^{OH}$) (**1**)[29] and N^6-benzoyl-8-methoxydeoxyadenosine ($dbzA^{OMe}$) (**2**)[27], and N^2-isobutyryl-8-methoxydeoxyguanosine ($dibuG^{OMe}$) (**3**)[28] were prepared according to the reported procedures. The other deoxyribonucleosides (dbzC=33 μmol/g and dbzA=36 μmol/g)-loaded CPG were prepared according to published procedure.[37]

Thin layer chromatography (TLC) was carried out on Merck Kiselgel $60F_{254}$ plates which were developed in system A (CH_2Cl_2-MeOH, 9:1, v/v) and system B (CH_2Cl_2-MeOH, 8:2, v/v). Reverse phase TLC, was carried out on Merck silanized gel; [RP-8F $60F_{254}$] plates with a mixture of acetone and 0.02 M triethylammonium acetate (TEAA) (6:4, v/v) as the eluting agent. Column chromatography was carried out on silica gel (BW-300; Fuji Davison Co.Ltd.). ^{1}H-NMR spectra were recorded on a JOEL JNMPS 100 spectrometer with TMS as an internal standard. Ultraviolet spectra were recorded on a Shimazu UV-160 spectrometer. Reverse phase HPLC was performed on a Shimazu LC 6A system using a TSKgel oligo-DNA RP. The solvent systems and gradients consisting of 0.1 M TEAA (pH 7.0) (A) and CH_3CN (B) were used in the following order: solvent system I, 20 min (5-25%); II, 20 min (5-18%). For anion exchange

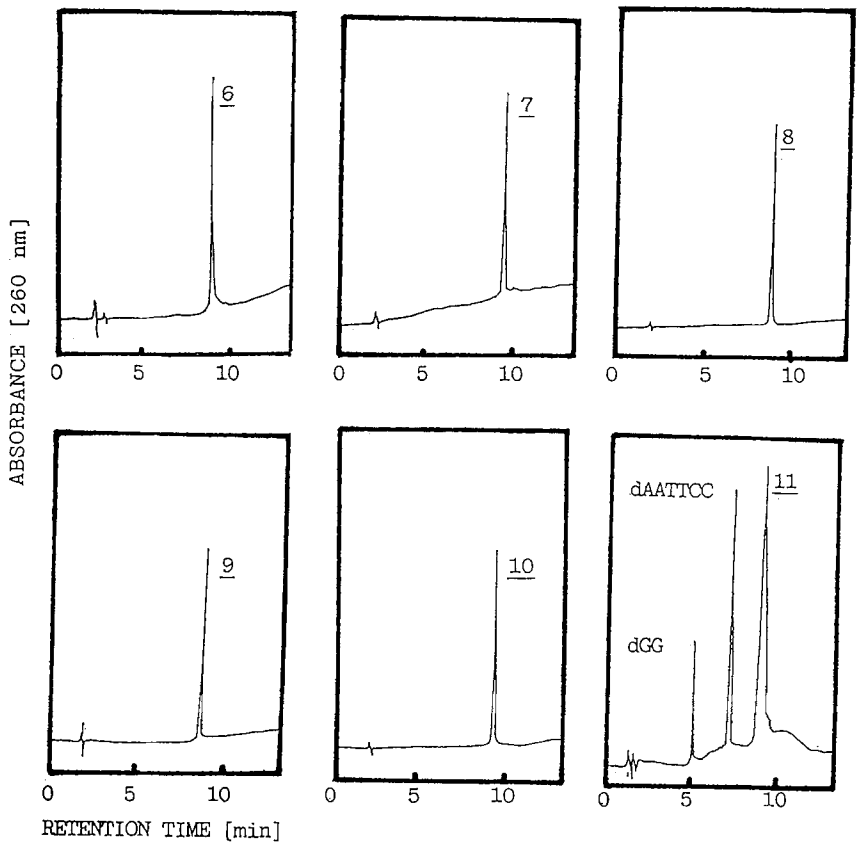


Figure 3. Reverse phase HPLC elution profile of the oligomers **(6-11)** after digestion with the endodeoxyribonuclease Eco RI for 36 h. Solvent system II.

HPLC, a TSKgel DEAE-2SW column was used with a linear gradient of ammonium formate in 20% aqueous acetonitrile.

Snake venom phosphodiesterase and alkaline phosphatase were purchased from Boehringer Mannheim. Eco RI was purchased from Takara Shuzou Co., Ltd.

Tritylation of nucleoside derivatives **(4)**

After coevaporation with pyridine, **1-3** (1.0 mmol) was dissolved in pyridine (10 ml) and a solution of dimethoxytrityl chloride (1.2 mmol) in pyridine (2 ml) was added dropwise at 0°C and the mixture was stirred for a further 12 h at room temperature. The solution was concentrated and the extracted with CH_2Cl_2 (50 ml). The solution was washed with water (20 ml X 2) and dried over Na_2SO_4. The CH_2Cl_2 layer was evaporated and the residue was applied to a silica gel column and eluted with a stepwise gradient of

MeOH (0-3%) in CH_2Cl_2. The appropriate fractions were pooled and evaporated in vacuo to give **4a-c** (87-90%).

Preparation of modified nucleoside 3'-H-phosphonate units (**5a-c**)

After coevaporation with dry pyridine, modified nucleosides (**4a-c**) (1.0 mmol) was dissolved in CH_2Cl_2 (4 ml) and tris-(hexafluoro-2-propyl) phosphite[30] (1.1 mmol) and pyridine (1.1 mmol) were added. The reaction was complete in 10 min, and a mixture of 1 M-triethylammonium bicarbonate (TEAB) and triethylamine (50:1, v/v) was added to the reaction mixture. After 30 min, the product was extracted with CH_2Cl_2 (25 ml X 2), washed with 1 M TEAB and dried (Na_2SO_4). The CH_2Cl_2 layer was evaporated and the residue was applied to a silica gel column and eluted with a stepwise gradient of MeOH (0-10%) in CH_2Cl_2 containing triethylamine (2%). The appropriate fractions were pooled and evaporated in vacuo to give the corresponding H-phosphonate units **5a-c** (85-95%). ^{31}P-NMR spectral data ($CDCl_3$, 85% H_3PO_4) were as follows: **4a**=3.62, 3.97 ppm, **4b**=4.51, 5.15 ppm, and **4c**=4.02, 4.41 ppm.

The other nucleoside H-phosphonate units (dT, dbzA, dbzC, and dibuG) were similarly obtained in good yields.[31]

Solid phase synthesis of the oligonucleotides (**6-11**).

The manual synthesizer was charged with the nucleosides-loaded CPG (5 μmol). The following synthetic cycle was used for oligomerization: (1) detritylation by treatment with 2.0% dichloroacetic acid in CH_2Cl_2 for 2 min; (2) washing step with CH_2Cl_2 (2 min), CH_3CN (3 min), and pyridine-CH_3CN (3 min); (3) coupling step with H-phosphonate units (30 molar equiv.) and pivaloyl chloride (150 molar equiv.) in pyridine-CH_3CN (1:1, v/v) for 5 min; (4) washing step with CH_3CN (3 min) and CH_2Cl_2 (2 min). After the chain assembly was complete, the resulting oligomer was oxidized to the corresponding oligomer by reaction with 0.1 M I_2 in THF-pyridine-H_2O (44:3:3, v/v, 2.0 ml) for 15 min. The resin was washed with pyridine, CH_2Cl_2, ether and then treated with conc. ammonia (4 ml) at 55°C for 12 h. The resin was filtered off and washed with H_2O. The filtrate was evaporated in vacuo and the residue was treated with 80% AcOH (3 ml) for 10 min. The solution was concentrated, the resulting oligomer was dissolved in H_2O (5 ml) and the solution was extracted with ether (10 ml X 2). The detritylated products were purified by a TSKgel DEAE-2SW with a linear gradient of ammonium formate (0.2-0.9 M during 20 min) in 20% CH_3CN. The main peak was further purified by reverse phase HPLC using a TSKgel-ODS 80TM with a solvent system I. The oligomers were purified, and 45-60 OD units were obtained. The chain length of the latter purified materials was established by electrophoresis on 20% polyacrylamide gel.

Melting temperature experiments

Melting temperature was measured by a Shimazu UV 160 spectrophotometer equipped with a temperature controller (TCC-240A, Shimazu) in 10 mM TRIS/HCl buffer (pH 7.6) containing 80 mM NaCl and 20 mM $MgCl_2$. The temperature of solution was increased linearly with time at a rate of 15°C/h.

CD spectra measurements

The CD spectra of octanucleotides were measured on a JASCO J-6 spectropolarimeter in the same buffer above described.

Hydrolysis of the oligonucleotides with the endodeoxyribonuclease Eco RI.

The oligonucleotides (0.5 A_{260} unit) were incubated at 18°C in 50 mM TRIS/HCl (pH 7.5), 100 mM NaCl, and 10 mM $MgCl_2$ with 120 unit endodeoxyribonuclease Eco RI in a total volume of 100 µl. The rate of phosphodiester bonds cleavage with octanucleotides was analyzed at different intervals of time by a reverse phase HPLC using a TSKgel oligo-DNA RP with a solvent system II at a flow rate of 1 ml/min.

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