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A new approach to the synthesis of a protected 2-aminopurine derivative and its incorporation into oligodeoxynucleotides containing the Eco RI and Bam HI recognition sites

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ABSTRACT

A protected 2-aminopurine nucleoside suitable for incorporation into oligodeoxynucleotides using phosphite triester chemical synthesis procedures has been prepared via oxidation of a purine hydrazino derivative with silver (I) oxide. Five oligodeoxynucleotides containing Eco RI and Bam HI recognition sites have been prepared such that, in the double stranded form, the 2-aminopurine base has either a complementary thymine or cytosine nucleobase. The helix character and thermodynamic parameters for helix formation have been examined.

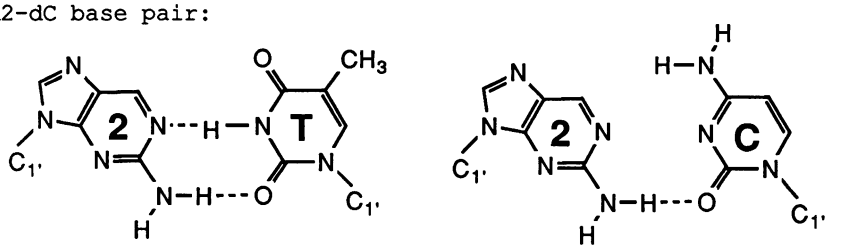
INTRODUCTION

Sequence specific recognition between proteins and nucleic acids can be generally understood in terms of contacts between the functional groups of the amino acid side chains and those associated with the nucleobase residues (1). It is likely in these cases that the protein interacts with a unique set of nucleobase functional groups characteristic of a specific DNA sequence. Recent reports have also suggested that specific contacts to the sugar-phosphate backbone may also play a significant role in observed sequence specific interactions (2,3).

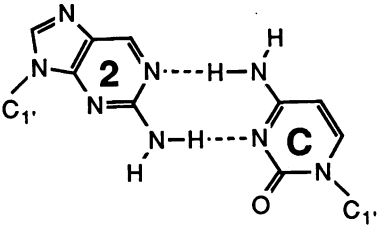
Small oligodeoxynucleotides can often be substituted for longer DNA molecules in order to probe such recognition processes. The incorporation of modified bases into oligodeoxynucleotides provides a useful technique for studying recognition phenomena at the functional group level. This approach has been employed with some success for DNA binding proteins (4-9), restriction endonucleases (10-17), modification enzymes (18) as well as RNA polymerase (19). The deletion of specific functional groups from a recognition site can often be most useful in this respect. The incorporation of uracil

for thymine represents the simplest deletion modification in which the thymine methyl group is removed.

The 2-aminopurine base analogue (d2) can be considered a derivative of 2'-deoxyguanosine from which the carbonyl at the 6-position has been deleted. It is also a structural isomer of 2'-deoxyadenosine in which the amino group at the 6-position has been displaced to the 2-position. In agreement with the latter analogy, the 5'-triphosphate derivative of the 2-aminopurine nucleoside is reported to function exclusively as an adenosine analogue in at least one enzymatic process (20). The possibility that 2-aminopurine could base pair with either thymine or cytosine has been suggested to account for its strong mutagenic properties (21). Watson-Crick base pairing schemes analogous to those of dA-dT and dG-dC provide two hydrogen bonds for the d2-dT base pair and only one for the d2-dC base pair:



The base pairing scheme in the former case has been confirmed by recent NMR studies (22,23). In the latter case little structural information has been reported. The possibility also exists for base "wobbling" to occur in the d2-dC base pair in order to take advantage of an alternate hydrogen bonding scheme:



In addition to its value for the study of nucleic acid structure and protein-nucleic acid interactions, the 2-aminopurine chromophore has also generated the attention of some investigators for its fluorescent properties (24-26).

The 2-aminopurine nucleobase as either the ribose or 2'-deoxyribose derivative has been previously prepared by hydrogenation

of the corresponding 6-chloro compound (27) as well as by cleavage of the 6-thio (28) or 6-seleno (29) derivatives using Raney nickel. The 2'-deoxyribonucleoside derivative has been additionally prepared enzymatically from 2-aminopurine and thymidine using a nucleoside deoxyribosyltransferase (30).

We wish to report a new approach to the synthesis of the 2-aminopurine heterocycle and the synthesis of the corresponding phosphoramidite derivative which allows incorporation of the modified nucleobase into oligodeoxynucleotides using chemical phosphite triester synthesis procedures (31,32). We have prepared four 2-aminopurine containing decadeoxynucleotides and one dodecadeoxynucleotide containing such that the 2-aminopurine residue has either a complementary thymine or cytosine nucleobase. The helix character as well as the thermodynamic stability of the DNA helices has been examined.

EXPERIMENTAL

Materials

Anhydrous hydrazine was a product of Aldrich (Milwaukee, WI). The 2'-deoxynucleosides were obtained from American Bionuclear (Emeryville, CA) and were converted to the protected phosphoramidite derivatives by known procedures. Thin layer chromatography (tlc) was performed using 5 x 10 cm glass backed plates of silica gel F60 (E.M. Science) and nucleoside derivatives were visualized by U.V. absorbance or by spraying with 10% aqueous sulfuric acid and heating. ^{1}H-NMR and ^{32}P-NMR spectra were obtained at 300 MHz and 121.5 MHz respectively using a Varian XL-300 multinuclear spectrometer.

Methods

2-Benzamido-6-hydrazino-9-(ß-D-3',5'-di-O-benzoyl-2'-deoxyribosyl) purine 2

To 4.05 g (7 mmol) of N-benzoyl-3',5'-di-O-benzoyl-2'-deoxyguanosine in 100 ml dichloromethane was added 4.75 ml (28 mmol) diisopropylethylamine, 4.24 g (14 mmol) triisopropylbenzene sulfonylchloride and 0.43 g (3.5 mmol) 4-(N,N-dimethyl)aminopyridine essentially as described elsewhere (33). After 2h stirring at ambient temperature the solvent was removed by rotary evaporation and the crude product isolated by chromatography on silica gel. The product eluted with 3% ethanol/dichloromethane.

To 4.85 g (5.7 mmol) of the crude sulfonyl derivative in 100

ml dry dioxane cooled on ice was added dropwise with a syringe through a rubber septum 0.32 ml (15 mmol) of anhydrous hydrazine. After the addition was complete, the ice bath was removed and the mixture stirred 1h at ambient temperature or until tlc analysis indicated that the reaction was complete. After removal of the solvent, the residue was partitioned between equal volumes (50 ml) of dichloromethane and 1M sodium bicarbonate. The organic phase was washed with water, dried (Na_2SO_4), the volume reduced and the mixture chromatographed on silica gel. The product (eluting with 6-8% ethanol/dichloromethane) was recrystallized from methanol. Yield: 2.51 g (61%) mp 105-107°C.

Elemental analysis calculated for $C_{31}H_{27}N_7O_6$: C:62.72, H:4.58, N:16.52; Found: C:62.70, H:4.68, N:16.47. ^{1}H-NMR (DMSO-d6 + Trace D_2O) δ= 2.2-2.9 (m, DMSO + H2',H2"), 3.54 (s, HOD), 4.6-4.8 (m, 3H, H4',H5',H5"), 5.9-6.0 (m, 1H, H3'), 6.50 (t, 1H, J = 7.0 Hz, H1'), 7.5-8.0 (m, 15H, ArH), 8.2 (s, 1H, H8).

2-Benzamido-9-(ß-D-3',5'-di-O-benzoyl-2'-deoxyribosyl) purine 3

To 2.07 g (2.48 mmol) of 2 in 125 ml of dioxane: water (95:5) was added 4.13 g (17.8 mmol) of finely divided silver(I) oxide. The reaction mixture was heated under reflux for 8 h. The reaction mixture was filtered (celite) and the solvents removed. The residue was taken up in 100 ml of dichloromethane and washed twice with an equal volume of a 10 % aqueous potassium iodide solution, followed with 100 ml of 10% thiosulfate, twice with water and dried (Na_2SO_4). Purification of the product was by chromatography on silica gel (eluting with 1-2 % ethanol in dichloromethane). Crystallization of the appropriate fractions from methanol yielded 1.08g (55 %) of product, mp 176-177°C.

Elemental analysis calculated for $C_{31}H_{25}N_5O_6$: C:66.07, H:4.47, N:12.43; Found: C:66.14, H:4.43, N:12.49. ^{1}H-NMR (DMSO-d_6) δ= 2.2-2.9 (m, DMSO + H2'H2"), 3.56 (s, HOD), 4.6-4.8 (m, 3H, H4',H5',H5"), 5.9-6.0 (m, 1H, H3'), 6.62 (t, 1H, J = 6.8 Hz, H1'), 7.4-8.1 (m, 15H, ArH), 8.69 (s, 1H, H8), 9.09 (s, 1H, H_6).

2-Benzamido-5'-O-(4,4'dimethoxytrityl)-9-(ß-D-2'-deoxyribosyl) purine 4

To 1.0 g (1.77 mmol) of 3 in 25 ml pyridine at -20°C was added 2.7 ml methanol and 2.5 ml 2N sodium hydroxide. After 15 min stirring the reaction was stopped with 10g Dowex 50WX8 (pyridinium form). The Dowex was filtered and washed with pyridine/water (1/4).

The filtrate was evaporated to dryness. Although this material could be crystallized from water at this point, generally the residue obtained upon evaporation was dissolved in dry pyridine and evaporated to dryness (3x). To the residue was added 20 ml dry pyridine and 0.6g (1.98 mmol) 4,4'- dimethoxytrityl chloride. After stirring overnight at ambient temperature the reaction was stopped with 5 ml methanol and subsequently evaporated to dryness. The residue was dissolved in 100 ml dichloromethane, washed with saturated aqueous sodium bicarbonate, water and dried (Na_2SO_4). After removal of the solvent and evaporation twice from toluene the product was isolated by chromatography on silica gel and precipitated into pentane. Yield: 0.76 g (65%).
^{1}H-NMR (DMSO-d6 + Trace D_2O) δ = 2.34 (m, 1H, H2'), 2.51 (m, DMSO), 3.01 (m, 1H, H2"), 3.43-3.52 (m, 2H, H5',H5"), 3.54 (s, HOD), 3.67 (s, 6H, methoxyl), 3.97 (m, 1H, 4H'), 4.64 (m, 1H, H3'), 6.46 (t, 1H, J = 6.2 Hz, H1'), 6.60-7.92 (m, 18H, ArH), 8.59 (s, 1H, H8), 9.05 (s, 1H, H6).

Preparation of the protected phosphoramidite 6

To the protected dimethoxytritylnucleoside derivative 4 (0.75g, 1.13 mmol) dissolved in 20 ml anhydrous dichloromethane containing 0.25 (1.5 mmol) diisopropylaminotetrazolide was added 0.75g, 2.5 mmol) of bis(diisopropylamino)-ß-cyanoethoxyphosphine, prepared essentially as described elsewhere (34). After stirring 3h at ambient temperature tlc analysis (ethylacetate/pet. ether/triethylamine, 49.5/49.5/1) indicated that the reaction was complete and it was stopped with 3 ml methanol. After 5 min the solvents were removed and the residue dissolved in a small quantity of dichloromethane/pet. ether/triethylamine (49.5/49.5/1), a small amount of activated charcoal was added and the mixture purified by column chromatography using 20g of silica gel. The desired column fractions were pooled, the volume reduced and the product filtered after precipitation into pet. ether.
Yield: 0.81g (83%). ^{31}P-NMR ($CDCl_3$): 146.0, 147.0 ppm.

Oligodeoxynucleotide Synthesis

Oligodeoxynucleotides were prepared using phosphite triester chemistry on an Applied Biosystems 380 DNA synthesis machine. The 2-aminopurine phosphoramidite was used in exactly the same manner as the four common nucleoside phosphoramidite derivatives and no

observable difference in coupling efficiency was observed. After deprotection in concentrated ammonia at 50 °C for 48 h the DNA fragments were purified by HPLC on a reversed-phase column. The presence of 2-aminopurine-2'-deoxynucleoside was confirmed by analysis of the nucleoside mixture obtained after hydrolysis of a small aliquot of the oligodeoxynucleotide with snake venom phosphodiesterase and bacterial alkaline phosphatase. The products of this hydrolysis were resolved on a 4.6 x 250 mm column of ODS-Hypersil (5 μm) using 50 mM KH_2PO_4, pH 5.5, and a gradient of 0-10% methanol in 60 min. Under these conditions the following retention times were observed: dC, 6.5 min; dG, 15.5 min; dT, 18.6 min; d2 24.8 min; dA, 29.5 min.

Determination of Tm Values

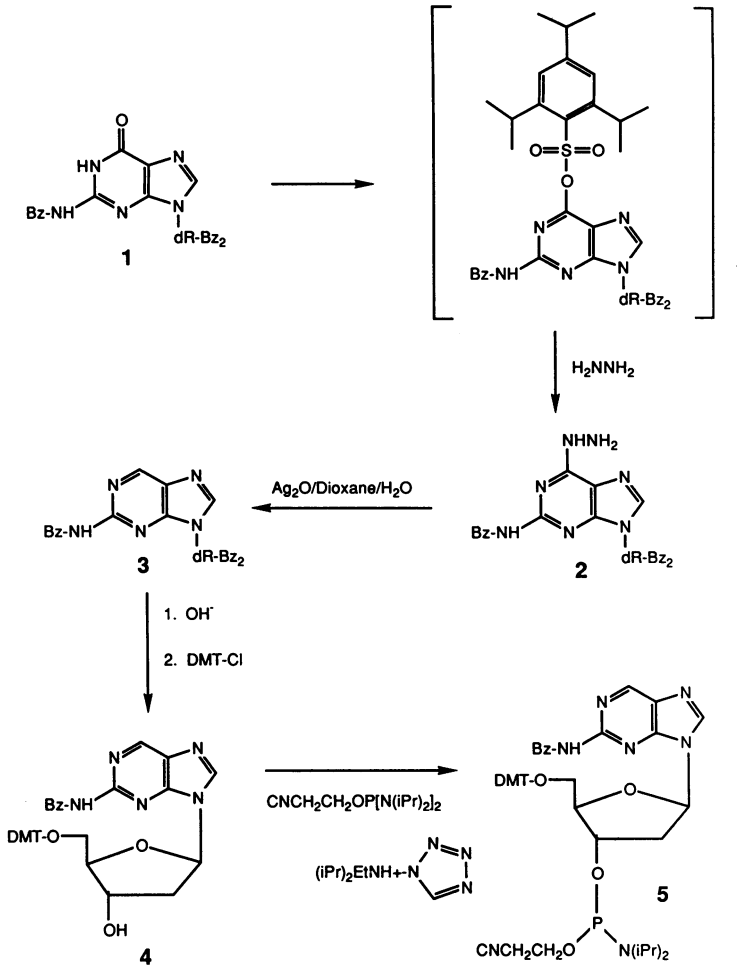
Thermal melting point (Tm) values were obtained in 40 mM Tris-HCl, pH 7.5, 20 mM $MgCl_2$, 200 mM NaCl, using a Perkin Elmer Lambda 3B spectrophotometer equipped with a C570-0701 digital temperature controller and temperature programmer. Solution temperature was measured directly using an immersible probe and a Telethermometer (Yellow Springs Instrument Co., Yellow Springs, OH) Absorbance and temperature data were collected and stored after analog to digital conversion (DT-2800, Data Translations, Marlboro, MA) using an IBM-XT personal computer employing the ASYST (ver.1.53) scientific package (MacMillian Software, New York, N.Y.). From absorbance vs temperature graphs, first and second derivatives were calculated to determine Tm values.

Circular Dichroism Experiments

CD Spectra were measured in 40 mM Tris-HCl, pH 7.5, 20 mM $MgCl_2$, 200 mM NaCl using the Auto-dichrograph Mark V (Jobin Yvon, Instruments S.A., N.J.) Solution temperature was maintained at 15°C with a refrigerated circulating water bath. Spectra were collected and stored using an Apple IIe computer and plotted with an Hewlett Packard 7574 plotter.

RESULTS

The sulfonation of the guanine O^6 is known to labilize the six position to addition/elimination reactions (33,35-39). The synthesis of 2,6-diaminopurine (33) and guanine-O^6-alkyl nucleoside derivatives (38) from sulfonated intermediates using amine or alcohol nucleophilic reagents has been reported. We have reacted the sul-



Scheme 1

fonated 2'-deoxyguanosine derivative, prepared in situ, with hydrazine in order to prepare the purine 6-hydrazino derivative 2 (Scheme 1). The hydrazino derivative 2 (Scheme 1) can be oxidized in the presence of silver (I) oxide to form the protected 2-aminopurine compound 3 (Scheme 1). This latter reaction is similar to that reported for arabinosyl adenosine (40) and various pyrimidine derivatives (41). Deprotection of the carbohydrate residue and subsequent preparation of the dimethoxytrityl phosphoramidite derivative 6 (Scheme 1), produced a compound suitable for incorporation

TABLE 1
Thermodynamic Parameters for Helix Formation for Native and 2-Aminopurine (d2) Containing Oligodeoxynucleotides

#	Sequence	-ΔH (kcal/mole)	-ΔS (cal/mole K)	-ΔG (37°C) (kcal/mole)	Tm(°C) 5μM
1	d(CTGAATTCAG)	72.4	206.2	8.5	41.4
	predicted*	(65.2)	(184.4)		(39.3)
2	d(CT2AATTCAG)	-	-	-	-
3	d(CTG2ATTCAG)	77.9	228.1	7.1	32.8
4	d(CTGA2TTCAG)	81.9	243.6	6.3	32.7
5	d(CTGGATCCAG)	84.9	240.4	10.4	48.0
	predicted*	(69.0)	(189.6)		(49.5)
6	d(CTG2ATCCAG)	56.6	168.0	4.5	21.7
7	d(CGCGAATTCGCG)	86.2	234.8	13.4	59.9
	predicted*	(107.8)	(281.6)		(79.3)
8	d(CGC2AATTCGCG)	58.0	163.9	7.2	35.4

* Predicted values derived according to reference 46.

into oligodeoxynucleotides using phosphite triester synthesis procedures (32).

We have found that silver oxide oxidation of the hydrazino derivative 2 (Scheme 1) is a simpler and more convenient route to the 2-aminopurine chromophore than previously described hydrogenation (27) or elimination reactions (28,29). Hydrazine reacts rapidly with the sulfonated nucleoside by attack at the 6-position. We observed no significant reaction at sulfur. It was therefore, unnecessary in this case to treat the intermediate sulfonyl derivative with trimethylamine as described previously with similar addition elimination reactions (33,37,38).

Six oligodeoxynucleotides were prepared using a controlled pore glass bead support and the appropriate nucleoside phosphoramidites. Initially the self complementary deoxynucleotide, d(CTGAATTCAG), containing the recognition site for the Eco RI restriction endonuclease (GAATTC) was prepared. Three additional fragments, each containing the 2-aminopurine base (d2) substituted for one of the purines within the six base pair canonical recognition site, were

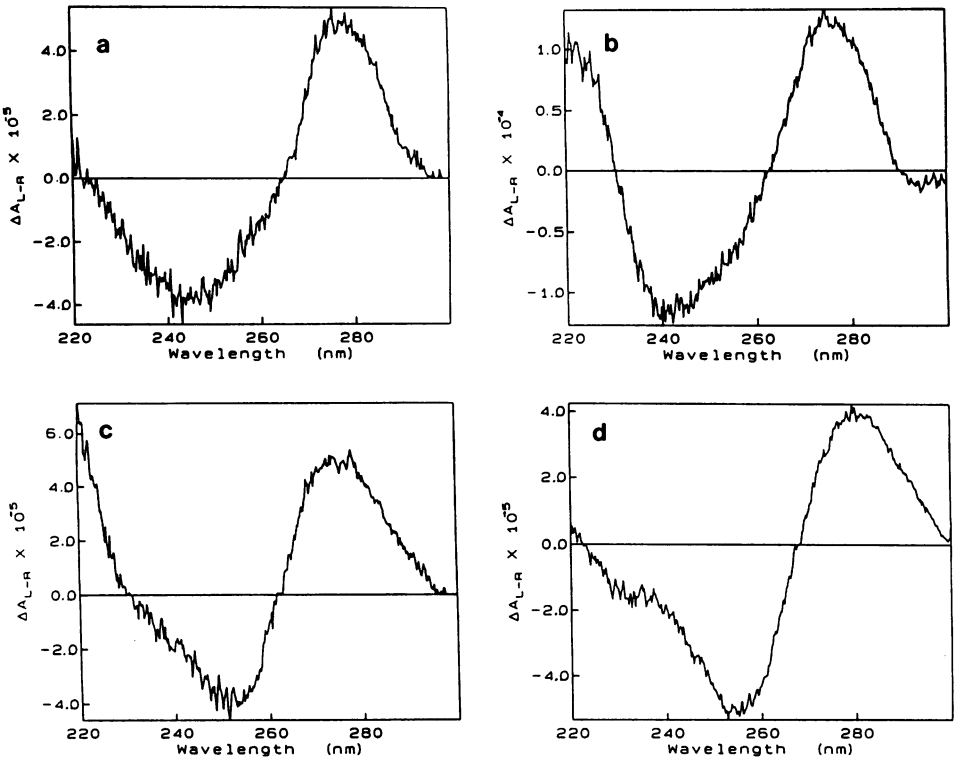


Figure 1. Circular Dichroism spectra of DNA fragments at 15°C: (a) d(CTGAATTCAG); (b) d(CTGA2TTCAG); (c) d(CT2AATTCAG); (d) d(CGC2AATTCGCG).

synthesized. Two of these modified self complementary fragments, d(CTG2ATTCAG) and d(CTGA2TTCAG), result in d2-dT base pairs in the double stranded form of the nucleic acid, while the third, d(CT2AATTCAG), produces d2-dC base pairs in the DNA duplexes. Preliminary thermal denaturation experiments indicated that although the d2-dT containing fragments exhibited a thermally induced helix to coil transition in the temperature range 15-80 °C, the d2-dC containing fragment did not (Table 1). In order to prepare a stable double stranded fragment containing d2-dC base pairs, we synthesized d(CTG2ATCCAG), and, for comparison, the native sequence d(CTGGATCCAG). These sequences, in contrast to the previous series, contain two additional dG-dC base pairs which will stabilize the double stranded form of the nucleic acid. Both of these derivatives

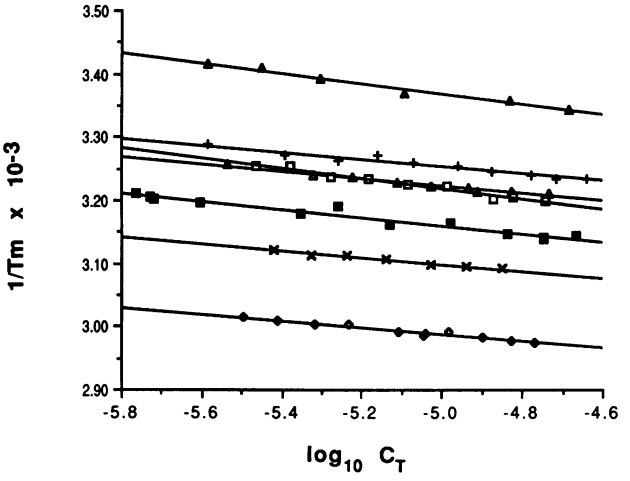


Figure 2. Plots of 1/Tm vs log c_T for d(CGCGAATTCGCG) (◇), d(CTGGATCCAG) (×), d(CTGAATTCAG) (■), d(CGC2AATTCGCG) (□), d(CTG2ATTCAG) (▲), d(CTGA2TTCAG) (+), d(CTG2ATCCAG) (▲).

exhibited thermally induced helix to coil transitions (Table 1).

The dodecamer, d(CGCGAATTCGCG), and a d2 containing fragment, d(CGC2AATTCGCG) were also prepared. The 2-aminopurine modified oligodeoxynucleotide also results in d2-dC base pairs substituted for dG-dC base pairs in the duplex form. Helix to coil transitions were observed in both cases (Table 1).

Circular dichroism experiments at 15°C indicated that all the native and 2-aminopurine containing sequences existed largely in the B-helical form (Fig. 1). This includes the sequence $[d(CT2AATTCAG)]_2$ which did not exhibit a thermally induced helix to coil transition under the assay conditions.

Thermodynamic parameters for the transitions were determined over the concentration range 1.6-25 μM single strand concentration (c_T) from plots of 1/Tm vs. log c_T. (Fig. 2) (42,43). Enthalpy (ΔH), entropy (ΔS) and free energy (ΔG) changes for helix formation have been derived assuming a two state model and are listed in Table 1. Determination of ΔH values from individual absorption vs temperature curves (43) agreed (within 15%) with those obtained from 1/Tm vs. log c_T plots. This is accepted as a reliable test of the two state helix to coil transition (43-46). For the native sequences the predicted values for solutions in 1M NaCl (44) are also reported.

DISCUSSION

Oxidation of nucleoside hydrazino derivatives provides a simple procedure for the conversion of R-$NHNH_2$ to R-H as demonstrated originally in the conversion of 8-hydrazino-ara A to ara A (40) and in the synthesis of a variety of pyrimidine derivatives (41). A similar procedure can be easily adapted for the preparation of 2-aminopurine derivatives as described in this report. Formation of the purine hydrazino derivative and its subsequent oxidation occurs under relatively mild conditions.

Although the purine hydrazino derivative described here was prepared solely as an intermediate in the synthesis of the desired 2-aminopurine chromophore, such nucleosides may additionally exhibit significant mutagenic activity as has been reported for pyrimidine hydrazino compounds (47,48).

The sequences of the two native decadeoxynucleotides differ in two positions. The sequence containing the Eco RI recognition site, d(CTGAATTCAG), contains dA-dT base pairs at positions four and seven while second sequence, containing a Bam HI recognition site, d(CTGGATCCAG), contains dG-dC base pairs at these positions. The thermodynamic parameters obtained for helix formation and and the measured Tm values confirm these differences. The Bam HI sequence exhibits a Tm 6.6 ^{o}C higher than the Eco RI sequence and the ΔG values differ by 1.9 kcal/mole. The ΔH values also suggest that the Bam HI helix has the larger number of hydrogen bonds as would be expected. The dodecamer containing the Eco RI site, d(CGCGAATTCGCG), exhibited a Tm of 59.9 ^{o}C and, ΔG and ΔH values for helix formation which were more negative than either of the decamers (Table 1). The predicted Tm and thermodynamic values for these three sequences are calculated for a higher salt concentration (1M NaCl) than that employed in this study (0.2 M). The lower salt concentration used in the present assays may account for the differences observed in the measured and calculated thermodynamic parameters of Table 1.

Incorporation of the 2-aminopurine base across from thymine in a DNA helix allows the formation of a stable double stranded structure (2 and 3; Table 1). This was not unexpected since recent NMR evidence (22,23) has indicated that two Watson-Crick hydrogen bonds will form in this base pair. However, the observed Tm values for oligodeoxynucleotides containing d2-dT base pairs indicate a de-

crease in helix stability compared with the fragment containing native dA-dT base pairs (Table 1). The ΔH values suggest similar hydrogen bonding characteristics for the native and 2-aminopurine containing sequences. Since two hydrogen bonds appear to be present with either the dA-dT or d2-dT (22,23) base pairs, the observed Tm values may reflect differences in base stacking for adenine vs. 2-aminopurine. Base stacking interactions in aqueous solution have been previously suggested to contribute significantly to helix stability (49).

A much larger decrease in helix stability is observed when a d2-dC base pair is substituted for a dG-dC base pair (1 vs. 2; 5 vs. 6; and 7 vs. 8; Table 1). Although the decamer, d(CT2AATTCAG), did not exhibit a thermally induced helix to coil transition over the temperature range examined (15-80°C), the CD spectrum at 15°C (Fig. 1) indicates the presence of the B-type DNA helix. This suggests that the Tm value for this fragment is near 15°C. This would be some 25°C lower than that observed with the native fragment, d(CTGAATTCAG). Both the decamer, d(CTG2ATCCAG), and the dodecamer, d(CGC2AATTCGCG), exhibited Tm values which were, respectively, 26.3 and 24.5 °C lower than the native dG-dC containing sequences. These differences in helix stability are largely the result of changes in enthalpy values and suggest that fewer hydrogen bonds are present in the DNA helices containing d2-dC base pairs.

The presence of the d2-dC base pairs in the duplex $[d(CTG2ATCCAG)]_2$ clearly results in a less stable structure than observed with $[d(CTG2ATTCAG)]_2$ containing d2-dT base pairs (Table 1). Differences in base stacking interactions present in these two sequences should be minimal since the flanking purines are the same in both cases (some minor stacking effects reflecting the difference between thymine or cytosine in the complementary strand may be present). The large difference in the observed Tm values and a $\Delta\Delta H$ value for these two sequences of about 21 kcal/mole are more likely to reflect differences in hydrogen bonding. These relatively large differences would argue against the presence of two hydrogen bonds in the d2-dC base pair analogous to the two bonds of the d2-dT base pair.

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