Synthesis and properties of defined DNA oligomers containing base mispairs involving 2-aminopurine

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ABSTRACT

DNA heptamers containing the mutagenic base analogue 2-aminopurine (AP) have been chemically synthesized and physically characterized. We report on the relative stabilities of base pairs between AP and each of the common DNA bases, as determined from heptamer duplex melts at 275 and 330nm. Base pairs are ranked in order of decreasing stability: AP•T > AP•A > AP•C > AP•G. It is of interest that AP•A is more stable than AP•C even though DNA polymerase strongly favors the formation of AP•C over AP•A base pairs. Comparisons of melting profiles at 330nm and 275nm indicate that AP•T, AP•A, and AP•C base pairs are annealed in heptamer duplexes and melt 2-3° prior to surrounding base pairs, whereas AP•G appears not to be annealed.

INTRODUCTION

2-Aminopurine (AP) is a well known mutagen in procaryotic systems, causing A•T→G•C, and G•C→A•T transitions (1-3). While AP most commonly forms base pairs with T (4-6), it can also mispair with C (7-8). The AP•C mispair can then serve as the intermediate in both transition pathways (6, 9-11). Since AP-induced mutation frequencies are typically 10-100 times greater than spontaneous mutation frequencies (3), one might expect base mispairs involving AP (e.g. AP•C) to be more stable than mispairs involving the common bases (e.g. A•C).

In this paper we report the stability of mispairs involving AP in relation to G•C, A•T, and A•C pairs in double-stranded oligomers of defined sequence. We present evidence that AP can also form base pairs with A but not with G. We have devised a technique in which the melting temperature of DNA in a highly localized region containing AP can be measured in order to evaluate the stability of the putative pair.

Part of this paper concerns the chemical synthesis of the DNA oligomers used in the study. We describe a modification of the "transient protection" procedure (12) employing an active ester as acylating agent. This technique yields protected AP nucleoside in good yields, sufficient for the preparation of AP-containing oligonucleotides for NMR studies (8). In addition, we

describe the synthesis and stability of an amidine derivative of AP deoxyribose. Methods reported here may prove invaluable for preparation of blocked derivatives of other biologically significant base analogues.

RESULTS

Heptanucleotides containing AP were synthesized by the phosphotriester method (13), as described in the Experimental Section. The key step was the preparation of the N-protected AP deoxyribonucleoside. We selected the isobutyryl group for protection of the 2-amino group in AP, by analogy with deoxyguanosine. The N-isobutyryl derivative was successfully prepared by using 2,4,5 trichlorophenyl isobutyrate instead of the commonly used isobutyryl chloride or anhydride. The anhydride failed to react, while the acid chloride resulted in unacceptably high levels of depurination. Another amino-protecting group, N,N-di-n-butylformamidinium, used to prepare depurination-resistant deoxyadenosine derivatives (14-16), was found to be roughly equivalent in stability. Both protecting groups were removed under the conditions usually employed for removal of amino-protecting groups after solid-phase oligonucleotide synthesis. The fully protected phosphotriester used for oligomer synthesis was N-isobutyryl-5'-dimethoxytrityl-3'-O-chlorophenyl, 2-cyanoethyl phosphate.

A homologous series of G•C-rich heptamer duplexes of the form, CCGXGGC
..... containing base pair X•Y = G•C, A•T, A•C, AP•T AP•C, AP•A or AP•G in GCCYCCG the center, were prepared by annealing equimolar mixtures of the synthesized heptamer strands. Thermal melting curves were determined spectrophotometrically by measuring UV absorbance (at 275 or 330nm) as a function of temperature. We use the melting data to address two questions: first, how does the presence of the four different base pairs involving AP affect the stability of the heptamer duplexes; second, under conditions in which surrounding G•C base pairs are annealed, can we determine whether or not the central base pair involving AP is also annealed?

Short DNA duplexes have relatively broad thermal melting profiles, making it difficult to identify the melting temperature (T_m). We have attempted to overcome this problem by computing first and second derivatives of absorbance vs. temperature and identifying T_m as the point of inflection where the first derivative with respect to temperature reaches a maximum or minimum and the second derivative changes sign. The technique is illustrated for the case of the AP•C base pair (Fig. 1). We find that the uncertainties in T_m values obtained by this method are less than 0.8°. The uncertainty in T_m is

Figure 1. Determination of T_m from absorbance changes at (a) 275 nm and (b) 330 nm for the 2-aminopurine-containing heptamer duplex whose absorption spectra are shown in Fig. 2. The solid curve in each case is the observed melting curve obtained by plotting absorbance vs. temperature. The dashed and dotted curves correspond to first and second derivatives of the melting curve, respectively. T_m is the point on the temperature axis where the first derivative shows (a) a maximum or (b) a minimum and the second derivative in each case changes sign (goes through 0).

estimated by varying the temperature interval over which the derivative is computed and analyzing the corresponding variation in the computed position of the point of inflection along the temperature axis.

When the G·C pairs in the duplexes are dissociated, a hyperchromic shift centered around 275 nm is observed (Fig. 2). On the other hand, base pairs containing AP give rise to a hypochromic shift around 330 nm. The absorption at 330 nm falls in the tail of the AP absorption peak located between 305 nm (neutral or basic pH) and 315 nm (acidic pH) (7). Annealing of AP-containing

Figure 2. Absorption spectra at 10°C (solid line) and 50°C (dashed line), showing a pronounced hyperchromic shift at 275 nm and hypochromic shift at 330 nm upon melting the heptamer duplex, $\begin{smallmatrix} \text{CGGPGGC} \\ \text{GCCCCCG} \end{smallmatrix}$, where P=2-aminopurine.

duplexes is accompanied by a spectral shift from 305 to 315 nm, possibly caused by protonation of base pairs involving AP (18). A hyperchromic shift at 330 nm occurs as the spectral shift is reversed by heat denaturation.

The solid curve in Fig. 1a shows the behavior of absorbance at 275 nm with temperature, for the G•C-rich duplex with AP•C in the center (whose absorption spectra are in Fig. 2). The computed first derivative (dashed curve) has a clear maximum around 25°C, indicating cooperativity in the dissociation of G•C pairs. A T_m of 24.9° is assigned to the duplex, based on where the second derivative (dotted curve) changes sign.

The corresponding melting curve at 330 nm (solid curve in Fig. 1b) is much noisier because absorbance at this longer wave length is 20-fold weaker. Nevertheless, the first derivative (computed over an interval wide enough to smooth the noise) shows a clear minimum between 23° and 24°C, indicating cooperativity in the dissociation of AP•C. A T_m of 23.3° is assigned in accordance with the change in sign of the second derivative.

Since normal bases have negligible absorption at 330 nm, the presence or absence of a cooperative hypochromic shift at 330 nm indicates whether or not AP mispairs are annealed in the duplex. Cooperativity is observed for AP•C and AP•A but not for AP•G (data not shown). These results indicate that when AP is surrounded by G•C pairs it can form annealed mispairs with C or A but not with G. It is evident these mispairs dissociate prior to the surrounding base pairs, since T_m at 330 nm is 1.5°-2° lower than at 275 nm (Table I).

Table I. Observed T_m values for heptamer duplexes with different central base pairs and mean standard ($\bar{T}_m$) values deduced for the corresponding central doublets of base pairs.

Heptamer duplex ^a	T_m (°C)		Central doublets ^c	$\bar{T}_m$ (°C) Deduced ^d
	Observed ^b			
	<u>275nm</u>	<u>330nm</u>		
$\begin{array}{c} \overrightarrow{\text{CGGGGC}} \\ \text{.....} \\ \overleftarrow{\text{GCCCCG}} \end{array}$	40.2	---	$\begin{array}{c} \overrightarrow{\text{GG}} \overrightarrow{\text{GG}} \\ \text{...} \\ \overleftarrow{\text{CC}} \overleftarrow{\text{CC}} \end{array}$	92
$\begin{array}{c} \text{A} \\ \text{.....} \\ \text{T} \end{array}$	34.6	---	$\begin{array}{c} \text{GA} \text{ AG} \\ \text{...} \\ \text{CT} \text{ TC} \end{array}$	72
$\begin{array}{c} \text{P} \\ \text{.....} \\ \text{T} \end{array}$	31.4	28.8	$\begin{array}{c} \text{GP} \text{ PG} \\ \text{...} \\ \text{CT} \text{ TC} \end{array}$	61
$\begin{array}{c} \text{P} \\ \text{.....} \\ \text{A} \end{array}$	28.0	25.9	$\begin{array}{c} \text{GP} \text{ PG} \\ \text{...} \\ \text{GA} \text{ AC} \end{array}$	49
$\begin{array}{c} \text{P} \\ \text{.....} \\ \text{C} \end{array}$	24.9	23.3	$\begin{array}{c} \text{GP} \text{ PG} \\ \text{...} \\ \text{CC} \text{ CC} \end{array}$	38
$\begin{array}{c} \text{P} \\ \text{.....} \\ \text{G} \end{array}$	20.4	n.c. ^e	$\begin{array}{c} \text{GP} \text{ PG} \\ \text{...} \\ \text{CG} \text{ GC} \end{array}$	22
$\begin{array}{c} \text{A} \\ \text{.....} \\ \text{C} \end{array}$	11.5	---	$\begin{array}{c} \text{GA} \text{ AG} \\ \text{...} \\ \text{CC} \text{ CC} \end{array}$	-9
$\begin{array}{c} \text{GGCPCGG} \\ \text{.....} \\ \text{CCGCGCC} \end{array}$	29.8	27.7	$\begin{array}{c} \text{CP} \text{ PC} \\ \text{...} \\ \text{GC} \text{ CG} \end{array}$	55

^aArrow indicates 5' → 3' direction in each DNA strand. Central base pair in each case is shown. P = 2-aminopurine.

^bObserved for heptamer duplex (in 0.05M Na phosphate buffer, pH7.2, adjusted to 0.15M Na⁺ with NaCl) by plotting absorbance (at 275 nm or 330 nm) vs temperature and using first and second derivatives to determine inflection point.

^cArrow indicates 5' → 3' direction in each strand.

^dMean standard value for the two central doublets, calculated from heptamer T_m at 275 nm by Eq. (4) in Appendix.

^eNo cooperativity (no clearly defined inflection point).

DISCUSSION

The main focus of this study was the chemical synthesis and physical characterization of defined oligonucleotides containing the mutagenic base analogue AP. Conventional methods for the preparation of suitably blocked nucleoside derivatives failed because of the extreme lability of the

glycosidic linkage in AP deoxyribonucleoside, as noted previously (19), and the low nucleophilicity of the 2-amino group. After trying several methods, it was found that amino protection with either 2,4,5-trichlorophenyl isobutyrate or N,N-di-n-butylformamidine dimethyl acetal provided the desired protected derivative in acceptable yields and both methods were practically equivalent in terms of yield and stability toward depurination and hydrolysis.

The N-isobutyryl APdR was transformed into a suitably protected monomer for standard phosphotriester oligonucleotide synthesis. The coupling yields of the protected APdR were similar to those obtained with similarly protected natural nucleotides. The presence of APdR in the synthetic oligonucleotides was verified by enzymatic degradation and subsequent analysis using HPLC (data not shown).

Heptamer oligonucleotides containing AP in the center were synthesized in order to measure the effect on oligomer duplex stability of pairing AP opposite each of the four common bases. Starting with G•C in the center, with G stacked between nearest-neighbor G's, the order of stability as the central pair is changed (Table I) is found to be G•C > A•T > AP•T > AP•A > AP•C > AP•G > A•C. Also shown in Table I (last row) is an increase in stability when AP is stacked in between nearest-neighbor C's instead of G's.

Base pairing in duplex DNA can be detected by the presence of a cooperative thermal melting profile. Heptamer T_m values are determined by locating the inflection point in melting curves after computation of first and second derivatives (Fig. 1). We have utilized the unusually long wavelength absorption of AP to observe the behavior of AP mispairing with common bases (Fig. 2). At 330 nm, in the tail of the AP absorption band, we observe a cooperative hypochromic shift for the case of AP•T, AP•C, and AP•A but not AP•G pairs. The hypochromicity of the melt at 330 nm is caused by a 10 nm spectral blue shift in the UV absorption band of AP when a transition is made from double- to single-stranded DNA (7). There is the usual cooperative hyperchromic shift in the melt at 275 nm (Fig. 2) associated with the UV absorption of the predominant G•C base pairs. The T_m values at 330 nm for AP•T, AP•C and AP•A are about 2° lower than those at 275 nm (Table I), suggesting that these central base pairs begin melting prior to the rest of the polymer.

Since T_m depends strongly on chain length for short duplexes, it is useful to relate heptamer T_m values to a set of standard T_m values for nearest-neighbor doublets of base pairs in native DNA (see Appendix). Standard T_m values are known at low ionic strength (0.02M) for all nearest

Figure 3. Base-pairing schemes for 2-aminopurine (AP) supported by NMR studies (8,23). While AP·T is a normal Watson-Crick base pair, AP·A is a wobble pair and AP·C is protonated. The proton in AP·C may be either on C or on AP, in equilibrium.

neighbor doublets in normal B DNA (20) and in B DNA having A·T replaced by AP·T (21). Using these data, we derive a formula (Eq. 4, Appendix) whereby heptamer T_m values can be used to calculate the mean standard value ($\bar{T}_m$) for the two central doublets, consisting of the central base pair and its two nearest neighbors (Table I, last column). The resultant $\bar{T}_m$ is the melting temperature expected for an infinitely long duplex composed of the central doublets only.

The doublet $\bar{T}_m$ values show that the A·C pair is so unfavorable that an alternating copolymer of G·C and A·C pairs would be unstable above -9°C . On the other hand AP·C would be stable up to 38°C when alternated with G·C and up to 55° when alternated with C·G. Previously, we deduced that AP·T also increases in stability (by about 10°) when stacked between C·G instead of G·C (21). Relative to the AP·C pair, we see that AP·A is more stable by about 11° while AP·G is less stable by 16° , when alternated with G·C.

NMR nuclear Overhauser measurements (8) on our heptamer duplexes containing AP demonstrate that AP pairs with T in a normal Watson-Crick structure as illustrated in Fig. 3. Two H-bonds are also present for the case of AP paired with C; however, unlike A·T which is neutral, AP·C appears to form to a protonated base pair at physiological pH (8). We want to emphasize that protonation rather than tautomerization is responsible for AP·C mispairs. This differs from earlier generally accepted proposals (9, 22) which suggested that AP (or possibly C) undergoes an amino to imino tautomeric

shift to form an electrically neutral mispair.

Our heptamer melting studies indicate that AP can anneal to A but not to G, since the hypochromic shift at 330 nm for the AP•G case shows no cooperativity (data not shown). Recently it has been verified by NMR (23) that AP•A forms a wobble base pair (Fig. 3), while in the case of AP•G, AP appears excluded from the helix.

An interesting question arises concerning the significance of the observation that AP•A is more stable than AP•C. The AP•C pair has been shown to form in the A•T → G•C transition pathway with a frequency of about 5% both in vitro (6, 11) and in vivo (5). On the other hand, the AP•A pair would result in the appearance of an A•T→T•A transversion, which does not appear to be a significant pathway for mutagenesis by AP. Using purified DNA polymerase, in vitro incorporation rates for AP•A pairs are at least an order of magnitude lower than for AP•C pairs (11). It seems likely that the reduced efficiency of the polymerase in accepting the more stable AP•A pair can be attributed to steric restrictions. A wobble base pair is believed to distort the helix (24), while a protonated AP•C base pair appears to be accommodated within the normal geometry of B-form DNA (8).

EXPERIMENTAL SECTION

Abbreviations: APdR = 2-aminopurine deoxyribonucleoside, Bz = benzoyl, Dbf = N',N'-di-n-butylformamidine, DMAP = dimethylaminopyridine, DMF = N,N-dimethylformamide, DMSO = dimethylsulphoxide, DMT = 4,4'-dimethoxytrityl, eq = equivalent, HOBT = 1-hydroxybenzotriazole, Ibu = isobutyryl, MMT = 4-methoxytrityl, p = internucleotide *o*-chlorophenyl phosphotriester.

Pyridine and acetonitrile were distilled and stored over CaH₂. DMT chloride was recrystallized from cyclohexane with 10% acetyl chloride (25). APdR was synthesized enzymatically (4). All other solvents and reagents were reagent grade and used without further purification.

Oligonucleotide syntheses were done in a Bachem manual synthesizer and in an automatic Microsyn 1460 synthesizer (Systec).

HPLC Conditions

In all cases solvent A was 0.01M ethylenediammonium diacetate (pH 7.5) and solvent B was a 1:1 mixture of solvent A and acetonitrile.

The reaction products shown in Table II were separated on a polystyrene PRP-1 column (10µm, Hamilton 20x0.5 cm) by elution at a flow rate of 1 ml/min. A 15 min linear gradient from 0 to 100%B was used and elution was

Table II. Composition of products obtained by reacting 3', 5'-O-bis(trimethylsilyl)-APdR with different acylating reagents as determined by HPLC.

Reagents		Conditions	Composition (%) [*]		
			I	II	III
(Ibu-) ₂ O	(15 eq)	25°C, 16hr	100	0	0
"	(15 eq) + DMAP (1 eq)	45°C, 16hr	25	67	8
"	(15 eq) + HOBt (1 eq)	45°C, 16hr	55	9	35
Ibu-Cl	(5 eq)	25°C, 2hr	2	93	5
"	(2eq)	0°C, 0.5hr	41	13	46
"	(2eq)	0°C, 1hr	14	28	57
Ibu-O-C ₆ H ₂ Cl ₃	(2 eq)	45°C, 4hr	31	3	66
"	(2 eq)	55°C, 7hr	16	11	73
"	(4 eq)	45°C, 16hr	6	34	60

^{*}The relative amounts of the following compounds were estimated from HPLC chromatograms in order of elution: (I) APdR, (II) 2-N-Ibu-AP, (III) 2-N-Ibu-APdR. The desired product (III) is the N-protected APdR derivative used for oligonucleotide synthesis.

monitored at 254 nm. The relative amounts of eluted compounds I, II, III (Table II) were estimated by measuring areas under absorption peaks in chromatograms and comparing with molar absorbances at 254 nm for purified compounds.

For small-scale (1 μ mol) purification of oligonucleotides we separated truncated sequences from desired DMT-oligonucleotides as follows. The polystyrene column was maintained at 50°C and eluted with a 10 min linear gradient from 20%B to 80%B at a flow rate of 1 ml/min. After removal of the

DMT group with 80% acetic acid, the product was rechromatographed under similar conditions, however, in this case a 10 min linear gradient from 0% to 40% was employed.

For large-scale (40 μ mol) purification of the polymers we used a polystyrene PRP-1 column (10 μ m, Hamilton 10x1.5cm) eluting with a flow rate of 2 ml/min and a 30 min linear gradient from 0% to 80%B. UV detection was at 300nm and the amount injected was approx. 200 O.D. units (260 nm).

2,4,5-trichlorophenyl isobutyrate

To a 100 ml round bottom flask containing 2 g of 2,4,5-trichlorophenol (10 mmol) dissolved in 10 ml of dichloromethane/pyridine (1:1) and cooled on ice, was added 1.05 ml of isobutyryl chloride (10 mmol). After 30 min of magnetic stirring at room temperature, the solution was evaporated and the residue dissolved with dichloromethane (30 ml) and water (30 ml). The solution was then transferred to a separatory funnel where the organic phase was washed with water (3x30 ml), saturated sodium bicarbonate (3x30ml) and water (3x30ml). After drying the organic phase with anhydrous sodium sulphate, the solution was evaporated and the resulting oil crystallized from ethanol upon addition of a few drops of water. Yield: 1.5 g (54%), m.p. 46°C. $^1\text{H-NMR}$ ($\text{Cl}_3\text{CD/TMS}$) δ (ppm) = 7.53 (s, 1H, arom.); 7.25 (s, 1H, arom.); 2.77 (quint., 1H, $\text{CH}(\text{CH}_3)_2$); 1.33 (d, 6H, $J=6.3\text{Hz}$, $\text{CH}(\text{CH}_3)_2$). IR(KBr, cm^{-1}) = 3050, 2990, 1760, 1450, 1340, 1120, 1090, 1070, 900. TLC: R_f = 0.67 in dichloromethane.

Acylation assay conditions

For the reactions described in Table II we used the following protocol. APdR (100 mg, 0.4 mmol) was first dried by coevaporation with pyridine. Pyridine (1 ml) and chlorotrimethylsilane (0.25 ml, 2 mmol) were added. After 15 min at room temperature the desired acylating agents were added and the mixture maintained under conditions described below. An aliquot of the mixture was evaporated, dissolved in methanol/water (1:1), centrifuged and injected into the HPLC column: The following compounds were identified in order of elution: (I) unreacted APdR, (II) 2-N-Ibu-AP, (III) 2-N-Ibu-APdR.

2-N-Ibu-APdR preparation

To a 50 ml round bottom flask 530 mg of APdR (2.1 mmol) were added, dried by coevaporation with pyridine and treated with 1.3 ml of chlorotrimethylsilane (10 mmol) in 10 ml of pyridine. After 15 min at room temperature a solution of 1.1 g of 2,4,5-trichlorophenyl isobutyrate (4.2 mmol in 6 ml of pyridine) was added and reaction allowed to proceed for 10 hours at 45°C. The reaction was then cooled in an ice bath and 2 ml of water added. After 5 min, 2.8 ml of

concentrated aqueous ammonia was added, the solution stirred for 15 minutes and evaporated to dryness. The residue was suspended in 5% MeOH/CH₂Cl₂ and purified directly on a silica gel column (30 g) eluted with 150 ml of 5% MeOH/CH₂Cl₂ and 400 ml of 10% MeOH/CH₂Cl₂ (all solvent mixtures were 0.1% pyridine). After analysis by HPLC, the fractions containing the desired product were pooled and evaporated. Yield: 210 mg (31%). UV_{max}(MeOH): 228, 255, 283. ¹H-NMR(DMSO-d₆, TMS) δ (ppm): 10.7 (s, 1H, NH); 9.2 (s, 1H, H₆); 8.9 (s, 1H, H₈); 6.7 (t, 1H, H₁); 5.6 (d, 1H, 3'-OH); 5.1 (t, 1H, 5'-OH); 4.7 (m, 1H, H₃); 4.1 (m, 1H, H₄); 3.8 (m, 2H, H₅); 3.0 (quint., 1H, CH(CH₃)₂); 2.4-3.0 (m, 2H, H₂); 1.3 (d, J=7.0 Hz, 6H, CH(CH₃)₂). TLC: R_f=0.35 in dichloromethane/methanol (85:15).

5'-O-DMT, 2-N-Ibu-APdR

The 2-N-Ibu-APdR preparation (210 mg, 0.65 mmol) was coevaporated twice with anhydrous pyridine. DMT chloride (220 mg, 0.65 mmol) and 2 ml of pyridine were added and the mixture stirred at room temperature. After two hours, 2 ml of methanol was added and the solvents evaporated. The residue was dissolved with dichloromethane (30 ml) and 5% sodium bicarbonate in water (30 ml) and the solution transferred to a separatory funnel. After separation of the phases, the organic layer was washed with water and dried (Na₂SO₄). Evaporation of the solvent gave a yellowish oil which was purified by column chromatography on silica gel (20 g) eluted with a gradient 0-5% MeOH in dichloromethane (containing 0.1% pyridine). Yield: 246 mg (61%). TLC: R_f = 0.5 in dichloromethane/ethanol (9:1). ¹H-NMR(acetone-d₆/TMS) δ (ppm) = 9.4 (s, 1H, NH); 8.8 (s, 1H, H₆); 8.4 (s, 1H, H₈); 7.1-7.4 (m, 9H, arom.); 6.7-6.8 (m, 4H, arom.); 6.5 (t, 1H, H₁); 4.9 (m, 1H, H₃); 4.1 (m, 1H, H₄); 3.7 (s, 6H, OCH₃); 3-3.5 (m, 3H, H₅, CH(CH₃)₂); 2.4-2.7 (m, 2H, H₂); 1.2 (m, 6H, CH(CH₃)₂).

5'-O-DMT, 2-N-Ibu-APdR-3'-O-Chlorophenyl, 2-Cyanoethyl Phosphate

245 mg of 5'-O-DMT, 2-N-Ibu-APdR (0.39 mmol) were phosphorylated with a mixture of cyclohexylammonium 2-chlorophenyl 2-cyanoethyl phosphate, 2,4,6-triisopropylbenzenesulfonyl chloride and 1-methylimidazole (26). Yield: 200 mg (59%) of a white solid. TLC: R_f = 0.54, 0.58 (2-diastereoisomers) in CH₂Cl₂/ethanol (9:1). ¹H-NMR (DMSO-d₆/TMS) δ (ppm) = 9.0 (s, 1H, H₆); 8.5 (s, 1H, H₈); 7.1-7.7 (m, 13H, arom.); 6.6-6.8 (m, 4H, arom.); 6.5 (m, 1H, H₁); 5.6 (m, 1H, H₃); 4.2-4.5 (m, 3H, CH₂-CH₂-CN, H₄); 3.7 (two s in ratio 2:3, 6H, 2 OCH₃); 3.3 (m, 2H, H₅); 2.7-3 (m, 5H, -CH₂-CH₂-CN, CH(CH₃)₂, H₂); 1.0 (m, 6H, CH(CH₃)₂, H₂); 1.0 (m, 6H, CH(CH₃)₂). ³¹P-NMR (DMSO-d₆, H₃PO₄) δ (ppm) = -7.01; - 7.09.

2-N-Dbf-APdR

100 mg (0.4 mmol) of APdR was dried by coevaporation with DMF. The residue was suspended in 2.5 ml of DMF and 0.16 ml (124 mg, 0.61 mmol) of the dimethylacetal of N,N-di-n-butylformamidine were added. The mixture was stirred until total dissolution and was maintained at 40°C for 48 hr. The reaction mixture was then evaporated to dryness, the residue treated with dichloromethane (20 ml) and 5% sodium bicarbonate in water (20 ml) and transferred to a separatory funnel. After separation of the phases, the residue was washed with 5% sodium bicarbonate, dried and evaporated to dryness. The residue was purified by column chromatography on silica gel (10 g) eluted with one step gradient 5-10% MeOH in dichloromethane.

In this purification the syn and anti isomers of the desired compound were separated and were obtained in a 15:85 ratio (faster/slower isomer). The fact that the fast-running isomer is the minor product, and the splitting of some signals in the proton NMR spectrum suggest that this compound may be the syn isomer. UV max (H₂O) 307, 278 nm. MS (FAB +) = 391 (M+1), 301, 275, 269, 140, 136.

Fast-running diastereoisomer (8.5 mg, 5%) ¹H-NMR (200MHz, DMSO-d /TMS) δ (ppm): 8.83 (d, J=1.2Hz, 1H, N=CH-N); 8.66(s, 1H, H₆); 8.49 (s, 1H, H₈); 6.40(t, 1H, H-1'); 5.35(d, 1H, 3'-OH); 5.06(t, 1H, 5'-OH); 4.41(m, 1H, H-3'); 3.88(m, 1H, H-4'); 3.57(m, 2H, H-5'); 3.50(t, 2H, N-CH₂); 3.39(t, 1H, 1N-CH₂); 3.34(t, 1H, 1N-CH₂); 2.70(m, 1H, 1H-2'); 2.30(m, 1H, 1H-2'); 1.56(m, 4H, N-CH₂-CH₂); 1.31(m, 4H, N-CH₂-CH₂); 0.91(m, 6H, CH₃).

Slow-running diastereoisomer (56 mg, 36%): ¹H-NMR(200 MHz, DMSO-d₆/TMS): δ (ppm): 8.83 (s, 1H, N=CH-N); 8.66(s, 1H, H₆); 8.50(s, 1H, H₈); 6.42(t, 1H, H-1'); 5.36(d, 1H, 3'-OH); 5.08(t, 1H, 5'-OH); 4.43(m, 1H, H-3'); 3.89(m, 1H, H-4'); 3.58(m, 2H, H-5'); 3.48(t, 2H, N-CH₂); 3.38 (t, 2H, N-CH₂); 2.71 (m, 1H, 1H-2'); 2.30 (m, 1H, 1H-2'); 1.55 (m, 4H, N-CH₂-CH₂); 1.30 (m, 4H, N-CH₂-CH₂-CH₂); 0.91 (m, 6H, CH₃).

We have also isolated in the first fractions the product resulting from the depurination reaction, 2-N-Dbf-AP: (0.9 mg, 8%) ¹H-NMR (200 MHz, DMSO-d₆/TMS: 8.8 (s, 1H, N=CH-N); 8.55(s, 1H, H-6); 8.25 (s, 1H, H-8); 3.35(d, 4H, -CH₂); 1.55(m, 4H, N-CH₂-CH₂); 1.30 (m, 4H, N-CH₂-CH₂-CH₂); 0.91 (m, 6H, CH₃).

Kinetics of depurination and hydrolysis

Depurination: Approx. 0.01M solutions of the nucleosides were prepared in total volume of 1 ml 2% dichloroacetic acid in dichloromethane. At different times 0.1 ml aliquots were taken, neutralized with 0.1 ml 5% triethylamine in dichloromethane and the solvents evaporated. The residue was

Table III. Relative stabilities (half-lives) of nucleosides with respect to (a) depurination in 2% DCA/CH₂Cl₂ at 25°C, and (b) hydrolysis in conc. NH₄OH at 65°C.

NUCLEOSIDE	Half-life (hr)
(a) Depurination:	
2-aminopurine deoxyribonucleoside (APdR)	16
Deoxyadenosine	3
2-N-(N',N'-di-n-butylformamidine)-APdR*	0.8
2-N-isobutyryl-APdR	0.5
(b) Hydrolysis:	
2-N-(N',N'-di-n-butylformamidine)-APdR	1.5
2-N-isobutyryl-APdR	0.6

*Both syn and anti isomers showed the same rates of depurination.

resuspended with MeOH/H₂O (1:1) and analysed by HPLC (Table IIIa).

Hydrolysis: Approx. 0.01M solutions of the nucleosides were prepared in 0.1 ml conc. NH₄OH in screw-cap Eppendorf tubes (10 for each nucleoside). The tubes were closed tightly, placed in a press to prevent ammonia leakage and heated at 65°C. Tubes were removed at fixed intervals, cooled with dry ice and the ammonia evaporated. The residue was dissolved in MeOH/H₂O (1:1) and the solution analyzed by UV spectroscopy (Table IIIb).

Solid phase oligonucleotide synthesis

The following heptadeoxynucleotides were synthesized and purified to homogeneity for use in duplex melting studies:

<u>a.</u> C-G-G-AP-G-G-C	<u>d.</u> G-C-C-A-C-C-G	<u>g.</u> C-G-G-G-G-G-C
<u>b.</u> G-C-C-C-C-C-G	<u>e.</u> G-C-C-G-C-C-G	<u>h.</u> C-C-G-C-G-C-C
<u>c.</u> G-C-C-T-C-C-G	<u>f.</u> C-G-G-A-G-G-C	<u>i.</u> G-G-C-AP-C-G-G

The synthesis of heptamers a and b was carried out manually on a 40 μmol scale using the phosphotriester solid-phase methodology (27, 28). For the synthesis of a we used as coupling units, the monomer dDMT-AP^{Ibu}_p, dimer dMMT-G^{Ibu}_pG^{Ibu}_p and trimer dDMT-CBz^pG^{Ibu}_pG^{Ibu}_p (synthesized using solution methods). The synthesis of b was performed using commercially available dimers. The efficiency of the coupling reactions was measured by comparison of DMT absorbance at 500 nm (ε = 88700) and MMT absorbance at 475 nm (ε = 60000). Overall yields: a, 90%; b, 78%. The oligonucleotidyl-resins were deprotected using published methods (28) and the oligonucleotides purified by semi-preparative reversed-phase HPLC. The syntheses of heptamers c through i

were performed on a 1 μ mol scale using standard phosphotriester and phosphoramidite solid-phase methodologies.

The homogeneity of the synthesized polymers was checked by gel electrophoresis (20% acrylamide, 7M urea). The composition of the polymers containing AP was confirmed by hydrolysis with snake venom phosphodiesterase and alkaline phosphatase followed by HPLC analysis (13) and by the wandering spot method (29) following polymer end-labelling with (γ 32) ATP and T4 polynucleotide kinase (30).

Melting studies

Heptamer duplexes were made by mixing equimolar amounts of the two heptamer strands in 0.05M phosphate buffer, pH 7.2, adjusted to 0.15M Na⁺ concentration with NaCl. Duplexes were annealed by slow cooling from 80° to 4°C. UV absorption spectra and melting curves (absorbance vs. temperature) were recorded in 1-cm path-length cells using a Perkin-Elmer Lambda 3B spectrophotometer having a temperature controller with programmed temperature increase of 1 deg/min. Melts were run on heptamer duplex concentrations of 3-6 μ M (42-84 μ M nucleotide) at either 275 nm or 330 nm.

APPENDIX

DNA duplex stability depends on stacking interactions between nearest-neighbor base pairs (20,21,31). In a duplex of N base pairs there are N-1 stacking interactions arising from the N-1 nearest-neighbor doublets. Each doublet of base pairs has a characteristic (standard) T_m value in native DNA at low ionic strength (20). The T_m value in °K for a duplex of N base pair is empirically related to doublets as follows.

$$T_m = \frac{a}{N} \sum_{n=1}^{N-1} T_m(n) \quad \text{Eq. (1)}$$

where n labels each doublet in the duplex (n=1, 2, .., N-1), $T_m(n)$ is the standard T_m value in °K for doublet n, and a is a scaling factor close to 1, which depends on DNA concentration and ionic strength.

For three of the heptamer duplexes (Table I) standard T_m values are known for all doublets. These are the duplexes with G•C, A•T, and AP•T in the center, and which have T_m (at 275 nm) = 40.2°C (313.3°K), 34.6°C (307.7°K) and 31.4°C (304.5°K), respectively. Using standard doublet values in native DNA at 0.02M ionic strength (20), we can evaluate factor a in each case to satisfy Eq. (1) with N=7. The resultant a values are very close to 1 (1.001, 0.995, 0.994).

Consider the heptamer duplex with central doublets, $\begin{smallmatrix} \text{GX} \\ \text{CY} \end{smallmatrix}$ and $\begin{smallmatrix} \text{XG} \\ \text{YC} \end{smallmatrix}$.

Compared to the case with $X \cdot Y = A \cdot T$ ($T_m = 35^\circ\text{C}$), according to Eq. (1) with $a=1$ and $N=7$, the heptamer T_m at 275 nm should be

$$T_m = 35^\circ\text{C} + \frac{1}{7} [T_m(\begin{smallmatrix} \text{GX} \\ \text{CY} \end{smallmatrix}) + T_m(\begin{smallmatrix} \text{XG} \\ \text{YC} \end{smallmatrix}) - T_m(\begin{smallmatrix} \text{GA} \\ \text{CT} \end{smallmatrix}) - T_m(\begin{smallmatrix} \text{AG} \\ \text{TC} \end{smallmatrix})] \quad \text{Eq. (2)}$$

The standard T_m values for the two central doublets are then predicted to have the average value

$$\overline{T_m}(\begin{smallmatrix} \text{GX} & \text{XG} \\ \text{CY} & \text{CY} \end{smallmatrix}) = \frac{1}{2} [T_m(\begin{smallmatrix} \text{GX} \\ \text{CY} \end{smallmatrix}) + T_m(\begin{smallmatrix} \text{XG} \\ \text{YC} \end{smallmatrix})] = \overline{T_m}(\begin{smallmatrix} \text{GA} & \text{AG} \\ \text{CT} & \text{TC} \end{smallmatrix}) + \frac{7}{2} (T_m - 35^\circ\text{C}) \quad \text{Eq. (3)}$$

Using the known $\overline{T_m}(\begin{smallmatrix} \text{GA} & \text{AG} \\ \text{CT} & \text{TC} \end{smallmatrix}) = 72^\circ\text{C}$, we find

$$\overline{T_m}(\begin{smallmatrix} \text{GX} & \text{XG} \\ \text{CY} & \text{YC} \end{smallmatrix}) = 3.5 (T_m - 14^\circ\text{C}) \quad \text{Eq. (4)}$$

Application of this formula to the observed heptamer T_m values at 275 nm yields the standard $\overline{T_m}$ values for central doublets shown in Table I (last column). One may regard $\overline{T_m}(\begin{smallmatrix} \text{GX} & \text{XG} \\ \text{CY} & \text{YC} \end{smallmatrix})$ as the melting temperature expected for an infinitely long duplex composed of alternating G·C and X·Y base pairs.

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