Studies on *N*4-(2-Deoxy-D-pentofuranosyl)-4,6-diamino-5-formamidopyrimidine (Fapy•dA) and *N*6-(2-Deoxy-D-pentofuranosyl)-6-diamino-5-formamido-4-hydroxypyrimidine (Fapy•dG)†

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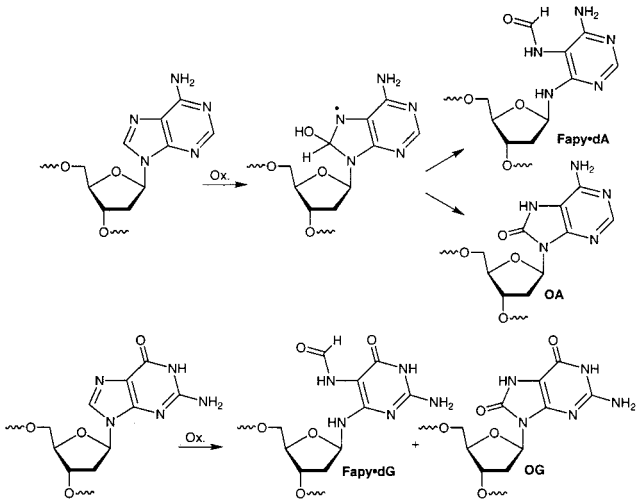
ABSTRACT: Exposure of DNA to oxidative stress produces a variety of DNA lesions including the formamidopyrimidines, which are derived from the purines. These lesions may play important roles in carcinogenesis. We achieved the first chemical syntheses of a monomeric form of Fapy•dA (**1**) and oligonucleotides containing this lesion or Fapy•dG at a defined site. Monomeric Fapy•dA readily epimerized at 25 °C in phosphate buffer (pH 7.5). The β-anomer was favored by a ratio of 1.33:1.0, and equilibration was achieved in less than 7 h. Deglycosylation of Fapy•dA in the monomer follows first-order kinetics from 37 to 90 °C. The rate constants for deglycosylation of Fapy•dA in the monomeric and oligonucleotide substrates were measured at a common temperature (55 °C) and found to be the same within experimental error ($t_{1/2}$ = 20.5 h). Implementation of the activation parameters measured for the deglycosylation of **1** indicates that the half-life for deglycosylation of Fapy•dA at 37 °C is approximately 103 h. Analysis of the rate constant for deglycosylation of Fapy•dG in an oligonucleotide, revealed that this lesion is ∼25 times more resistant to hydrolysis than Fapy•dA at 55 °C. These results indicate that Fapy•dA and Fapy•dG will be sufficiently long-lived in DNA so as to warrant investigation of their genotoxicity, and both anomers will be present during this time.

DNA damage is important in a variety of diseases, such as cancer and in aging. The formamidopyrimidine lesions *N*4-(2-deoxy-D-erythropentofuranosyl)-4,6-diamino-5-formamidopyrimidine (Fapy•dA) and *N*6-(2-deoxy-D-erythropentofuranosyl)-6-amino-5-formamido-4-hydroxypyrimidine (Fapy•dG) are produced in DNA as a result of oxidative stress (*1*−*4*). Although the mechanism(s) for formation of these imidazole ring opened purine lesions is uncertain, they result from the formal addition of hydroxyl radical (OH•) to the C8-position of deoxyadenosine and deoxyguanosine (Scheme 1). The respective C8-hydroxyl radical adducts are also believed to be precursors of 8-oxodeoxyguanosine (OG) and 8-oxodeoxyadenosine (OA). The former is often described as the most mutagenic DNA lesion and gives rise to G → T transversions (*5*, *6*). The structural basis of the effects of OG on polymerase enzyme activity are explained by rotation about the glycosidic bond, which enables the molecule to hydrogen bond to adenine (*7*). Cleavage of the purines' imidazole rings in the formamidopyrimidine lesions provides these molecules with a greater number of degrees of freedom and increased number of possible hydrogen bonding patterns. The mutagenic potential of Fapy•dA is uncertain, but studies of damaged DNA in *Escherichia coli* suggest that it results in A → G transitions (*8*). Conclusions regarding the mutagenicity of Fapy•dG could not be drawn from these experiments. Oligonucleotides containing *N*-methyl Fapy•dG have been prepared. This lesion blocks polymerase activity and increases G → T and G → C transversions (*9*−*12*). However, the additional methyl substituent could significantly alter the conformation about the formamido group, and it is uncertain whether one can extrapolate observations made using *N*-methyl Fapy•dG to the parent lesion.

Scheme 1: Formation of Formamidopyrimidine and 8-Oxopurine Lesions



The impact of a modified nucleotide on polymerase enzyme activity is affected by the lifetime of the lesion in DNA. Fapy•dA and Fapy•dG are excised by the base

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excision repair (BER) enzyme that bears their name, formamidopyrimidine DNA glycosylase (Fpg protein), also known as mutM (*1*, *2*). More recently, Fapy•dA was reported to be the first purine lesion recognized by *E. coli* endonuclease III (*13*). This report was followed shortly thereafter by the observation that *N*-methyl Fapy•dG is also recognized by Endo III (*14*). The biological effects of DNA lesions can also be affected by increased lability of their glycosidic bonds to adventitious hydrolysis resulting in their transformation into abasic sites, which induce polymerase enzymes to incorporate deoxyadenosine translesionally (*15*−*18*). Determinations of the rate constants for hydrolysis of Fapy•dA and Fapy•dG have been hindered due to synthetic limitations. It has not been possible to incorporate these lesions at defined sites in oligonucleotides. In addition, studies on monomeric compounds were limited by synthesis due to the predominant existence of formamidopyrimidine nucleosides in their pyranose forms (*19*, *20*). We developed synthetic methods that overcome these obstacles and report herein the rates of epimerization and deglycosylation of a Fapy•dA nucleotide (**1**) and deglycosylation of both formamidopyrimidine lesions in single-stranded oligonucleotides (*21*, *22*).

EXPERIMENTAL PROCEDURES

Materials. Oligonucleotides were prepared on an ABI 394 Oligonucleotide synthesizer as previously described (*21*, *22*). All other oligonucleotides were prepared and purified using standard procedures. DNA synthesis reagents were obtained from Glen Research Inc., Sterling, VA. Uracil DNA glycosylase (UDG) and γ-^{32}P-ATP were obtained from Amersham Pharmacia. T4 polynucleotide kinase was from New England Biolabs. NMR spectra were collected on either a Varian Inova 400 or 500 MHz instrument.

Synthesis of the 3′-Silylated Nitropyrimidine Phosphate Triester (3). Alcohol **2** (*22*) (490 mg, 1.3 mmol) was dried by azeotroping with pyridine. It was then dissolved in CH_2C1_2 (20 mL) and pyridine (0.3 mL, 3.7 mmol) and dimethyl chlorophosphate (200 μL, 3.7 mmol) were added. The mixture was stirred for 30 h at room temperature. The solvent was then removed in vacuo, and the residue was chromatographed (2:1 EtOAc/hexanes, then EtOAc) to provide **3** (320 mg, 51%). ^{1}H NMR ($CDCl_3$) δ 9.89 (d, J = 8.7 Hz, 1 H), 8.49 (br s, 1 H), 8.08 (s, 1 H), 6.65 (br s, 1 H), 6.51−6.44 (m, 1 H), 4.53 (d, J = 5.3 Hz, 1 H), 4.29−4.23 (m, 1 H), 4.11−4.03 (m, 1 H), 3.98−3.89 (m, 1 H), 3.81 (s, 3 H), 3.77 (s, 3 H), 2.43−2.32 (m, 1 H), 2.04 (d, J = 13.8 Hz, l H), 0.94 (s, 9 H), 0.18 (s, 3 H), 0.16 (s, 3H); ^{13}C NMR δ 159.7, 159.2, 156.6, 113.2, 86.1 (d, J = 8.2 Hz), 82.3, 74.2, 67.0 (d, J = 5.7 Hz), 54.7 (d, J = 6.2 Hz), 40.4, 26.1, 18.6, −4.4, −4.6; ^{31}P NMR δ 1.8; IR (thin film) 3448, 3336, 2955, 2856, 1580, 1520 cm^{-1}; HRMS calcd for $C_{17}H_{33}N_5O_8$-PSi (MH^+) 494.1836, found 494.1834.

Synthesis of the 3′-Silylated Formamidopyrimidine Phosphate Triester (4). Phosphate **3** (272 mg, 0.55 mmol) was reduced in ethanol (15 mL) in the presence of 10% palladium on carbon at 50 psi overnight. The mixture was filtered through Celite and concentrated in vacuo. The crude amine was dried by azeotroping with pyridine and then dissolved in THF (10 mL). The solution was cooled to 0 °C, and pyridine (1.5 mL, 19 mmol) was added, followed by acetic formic anhydride (1.13 g, 12.8 mmol). The mixture was allowed to warm to room temperature overnight. It was then concentrated and chromatographed (EtOAc, then 10:1 EtOAc/MeOH) to furnish formylated compound **4** (178 mg, 66%) as a mixture of anomers. ^{1}H NMR (CD_3OD) δ 8.27−8.24 (m, 1 H), 7.99−7.93 (m, 1 H), 6.27−6.13 (m, 1 H), 4.49−4.38 (m, 1 H), 4.15−3.89 (m, 3 H), 3.84−3.70 (m, 6 H), 2.50−2.36 (m, 1 H), 2.14−1.94 (m, 1 H), 0.98−0.92 (m, 9 H), 0.17 (s, 3 H), 0.13 (s, 3 H); ^{13}C NMR δ 163.9, 163.7, 161.0, 159.2, 158.9, 157.5, 157.1, 85.5 (d, J = 7.0 Hz), 85.1 (d, J = 7.2 Hz), 83.4, 83.2, 74.6, 73.9, 68.9 (d, J = 6.0 Hz), 68.4 (d, J = 6.0 Hz), 56.2, 55.5, 55.4, 41.1, 29.7, 26.6, 26.4, 19.1, 19.0, −4.3, −4.5; ^{31}P NMR δ 5.3, 5.2; IR (thin film) 3322, 2956, 2857, 1694, 1592 cm^{-1}; HRMS calcd for $C_{18}H_{35}N_5O_7PSi$ (MH^+) 492.2043, found 492.2026.

*Synthesis of the Formamidopyrimidine Phosphate Diester (**1**)*. Formamidopyrimidine **4** (83 mg, 0.17 mmol) was dissolved in THF (4 mL). Triethylamine trihydrofluoride (300 μL, 1.84 mmol) was added, and the mixture was stirred overnight. Triethylamine (0.5 mL 3.6 mmol) was added, the mixture was concentrated, and the residue chromatographed (10:1 EtOAc/MeOH) to provide the desilylated product (52 mg, 82%) as a mixture of anomers. ^{1}H NMR (CD_3OD) δ 8.30−8.25 (m, 1 H), 7.98−7.93 (m, 1 H), 6.22−6.14 (m, 1 H), 4.37−4.30 (m, 1 H), 4.16−3.94 (m, 3 H), 3.86−3.72 (m, 6 H), 2.44−1.96 (m, 2 H); ^{13}C NMR δ 168.6, 164.1, 163.9, 161.0, 159.4, 159.1, 157.6, 157.2, 156.9, 96.0, 85.2 (d, J = 7.0 Hz), 84.9 (d, J = 7.1 Hz), 83.4, 73.1, 72.6, 70.7, 69.4 (d, J = 6.3 Hz), 69.0 (d, J = 5.9 Hz), 55.6, 55.5, 55.4, 40.6, 40.5, 9.4; ^{31}P NMR δ 0.9, 0.6; IR (thin film) 3330, 2958, 1682, 1597, 1503 cm^{-1}; HRMS calcd for $C_{12}H_{21}N_5O_7P$ (MH^+) 378.1179, found 378.1181. The desilylated material (26.7 mg, 70.8 mmol) and sodium iodide (102 mg, 0.68 mmol) were refluxed overnight in acetone (5 mL). The precipitated solid was triturated thoroughly with acetone to provide a quantitative yield of **1**. ^{1}H NMR (CD_3OD) δ 8.27 (s, 1 H), 7.96−7.92 (m, 1 H), 6.24−6.09 (m, 1 H), 4.54−4.38 (m, 1 H), 4.16−3.63 (m, 3 H), 3.61−3.48 (m, 3 H), 2.49−1.90 ppm (m, 2 H); IR (KBr) 3338, 2951, 1624, 1600, 1499 cm^{-1}; ESI-MS calcd for $C_{11}H_{17}N_5O_7P$ (M-H$^+$) 362.0, found 362.1. HRMS calcd for $C_{11}H_{18}N_5O_7PNa$ (M + Na$^+$) 386.0842, found 386.0843.

*NMR Characterization of **1***. One-dimensional NMR experiments were done on Varian Inova-400 and 500 MHz NMR spectrometers at 25 °C using a 5-mm pulsed-field-gradient ^{1}H indirect- detection probe. Acquisition parameters were similar on both spectrometers as follows: the 90 degree pulse width was 5.7 μs, the spectral window was 7000 Hz, the acquisition time was 2.2 s, and the delay between transients was 0.5 s. The residual HDO signal was eliminated with a 75 Hz CW presaturation pulse lasting 1.5 s prior to each of 32 signal averaging transients. All two-dimensional NMR spectra were recorded on a Varian Inova-400 MHz NMR spectrometer operating at 25 °C using a 5-mm pulsed-field-gradient ^{1}H indirect- detection probe. 2D-TOCSY was acquired using a z-filtered DIPSI mixing sequence, a 60 ms mixing time, 7 kHz spin-lock field, 4 repetitions, 256 (t_1) × 2048 (t_2) complex points, 7000 Hz sweep in each dimension. 2D-ROESY was acquired using a z-filtered mixing sequence with a 400 ms mixing time, 3 kHz spin-lock field, 8 repetitions, 256 (t_1) × 2048 (t_2) complex points, 7000 Hz sweep in each dimension. 2D-HSQC was acquired using 16 repetitions, 256 (t_1) × 1024 (t_2) complex points, 7000 Hz in

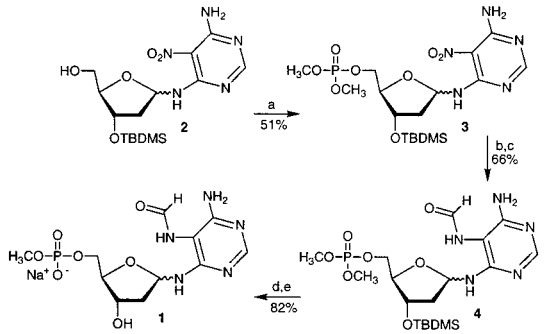
F2 and 15000 Hz in F1. The time between repetitions was 1.8 s for these experiments. Data were processed using Varian, Inc. VNMR software, version 6.1C. The final data size after forward linear-prediction in t_1 and zero-filling in both dimensions was 1024(F1) × 4096(F2) complex points for the homonuclear ROESY and TOCSY and 1024(F1) × 2048 (F2) complex points for the HSQC experiment.

*Epimerization Kinetics of **1**.* A freshly prepared solution of **1** (15 mM) in deuterated phosphate buffer (20 mM, pH 7.5) was placed in the probe of a 500 MHz NMR. Spectra were collected immediately ($t = 0$), at $t = 19.5$ min, and every 15 min thereafter. Prior to doing the kinetic measurements, a semiquantitative inversion−recovery (T_1) experiment was done at 400 MHz to estimate the T_1 relaxation times of the protons under the experimental conditions. Most of the ribose ring protons had T_1 values less than 1 s. However, the anomeric protons from both epimers had T_1 values of about 1.8 s. The kinetic experiment followed the course of the anomeric protons since these were judged by inspection to have the fewest interfering signals and were well resolved at 500 MHz. The parameters for this were the same as those described above for the presaturation experiment with the exception that a 30 degree excitation pulse (2 μs) was used. The acquisition computer took one spectral data point every 15 min, including allowance for the total acquisition time (143 s). To validate the integral intensities observed in the kinetics experiments, a single transient experiment using a 90 degree pulse was done on the sample after epimerization had arrived at the equilibrium ratio (24 h). The relative intensities resulting from the anomeric protons were compared with those found by doing a normal experiment consisting of 32 signal averaging transients. They were identical within experimental error (about 1%).

*Deglycoyslation Kinetics of **1**.* A solution containing phosphate diester **1** (1 mM) and internal standard (thymine, 0.5 mM) in phosphate buffer (10 mM, pH 7.5) were placed in an Eppendorf tube containing a screw cap with an O-ring. An aliquot (5 μL) was removed immediately, diluted with H_2O (25 μL), and stored in a freezer. The tube was placed in an oil bath whose temperature was controlled by a proportional digital controller, and verified using a standard thermometer. Aliquots (5 μL) were removed at the appropriate time and treated as above. Analysis was carried out using a C_{18}-reverse phase HPLC column (4.6 × 250 mm), UV detection (259 nm), and gradient elution. Solvent A: 50 mM ammonium formate (pH 6.2). Solvent B: 50 mM ammonium formate (pH 6.2), CH_3CN (1:1 by volume). Flow rate: 1.0 mL/min. Gradient: $t = 0 \rightarrow 10$ min, 0% B; $t = 10 \rightarrow 20$ min, 0−40% B linearly; $t = 20 \rightarrow 22$ min, 40−100% B linearly. Ret. times (min): FapyAde, 4.2; **1**, 5.8; thymine, 16.8.

*Alkaline Cleavage of an Abasic Site in **7**.* Oligonucleotide **7** (50 pmol) was labeled with ^{32}P at its 5′-terminus. Labeled DNA (0.5 mM) was treated with UDG (0.5 Units) at 37 °C for 30 min in DTT (0.45 mM), EDTA (0.45 mM), and HEPES (0.01 mM, pH 7.9). An aliquot (4 mL) was diluted to 1 nM ([DNA]) and treated with NaOH (0.1 M) at 55 °C for 20 min. The sample was cooled on ice and then neutralized with HCl (1 M). Calf-thymus DNA (0.12 mM) was added, and the DNA was precipitated, frozen, and centrifuged. The pellet was resuspended in formamide loading buffer and loaded on a 20% denaturing polyacrylamide gel. Following electrophoresis at 25 mA for 2.5 h, the gel was analyzed using a phosphorimager (Storm 820, Imagequant 3.3).

Scheme 2: Synthesis of Fapy·dA Nucleotide (**1**)[a]



[a] Key: (a) Dimethylchlorophosphate, (b) H_2, Pd/C, (c) formyl acetic anhydride, (d) $Et_3N·3HF$, (e) NaI.

Deglycosylation Kinetics of Fapy•dA and Fapy•dG in Oligonucleotides. A solution (50 μL for **6**, 100 μL for **5**) of 5′-^{32}P-labeled oligonucleotide was heated in phosphate buffer (10 mM, pH 7.5) at 55 or 90 °C. Aliquots (2 μL) were removed periodically and stored at −20 °C until all time points had been taken. The aliquots were warmed to room temperature, diluted with NaOH (final concentration, 0.1 M), and heated at 55 °C for 20 min. The samples were cooled on ice, neutralized with HCl (1.0 M), and diluted with formamide loading buffer. The samples were separated by gel electrophoresis and analyzed by using a phosphorimager as described above.

RESULTS

Substrate Synthesis and Characterization. The stability of Fapy•dA and Fapy•dG were measured in single-stranded DNA. The lability of the former was also examined at the monomeric level in phosphate diester **1**. Oligonucleotide substrates containing Fapy•dA and Fapy•dG were prepared as previously described (*21*, *22*). Experiments were not carried out on duplex DNA because we anticipated that the substrates would dehybridize over the lengthy period of time at the temperature required. Monomeric substrate (**1**) was prepared from **2** (Scheme 2). The nitro-substituted pyrimidine exists exclusively in its furanose (**2**) form. Following phosphorylation of the primary hydroxyl group (**3**), the nitro group was reduced, and the crude amine was formylated using formyl acetic anhydride. The formamidopyrimidine phosphate triester (**4**) was isolated as an inseparable mixture of anomers, which remained inseparable upon desilylation and subsequent monodemethylation to form **1**.

The stereochemistry and peak assignments of each anomer were assigned via 2-D ^{1}H NMR experiments (Table 1). A strong enhancement at the C4′ proton was observed when the isomer whose anomeric proton resonated at δ 5.9 was irradiated at this site (*23*). Irradiation of the C1′ proton of the other isomer showed enhancements of its respective C3′ and C5′ protons, but not C4′. A strong r.O.e. was also observed at the C2′ proton furthest downfield. Irradiation of the C2′ proton furthest downfield produced a very strong enhancement at the C2′ furthest upfield, as well as at the relatively more shielded respective C1′ and C3′ protons. These observations were consistent with those made in a TOCSY experiment of the epimeric mixture of **1**.

Table 1: ^{1}H NMR Chemical Shift Assignments of α-**1** and *β*-**1**[a]

proton	α-**1**	*β*-**1**
C1′	5.87	5.92
C2′ *pro-R*	1.84−1.87	2.04−2.10
C2′ *pro-S*	2.40−2.42	2.04−2.10
C3′	4.30	4.36
C4′	3.96	3.90
C5′ *pro-R, pro-S*	3.65−3.75	3.65−3.75
C2	7.83	7.83
H−C(O)-	8.19	8.18

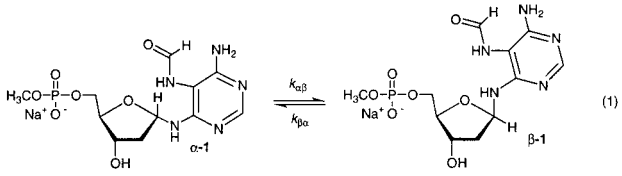
[a] Spectra were collected in deuterated phosphate buffer (pH 7.5, 10 mM).

Table 2: Rate Constants for Epimerization of **1** in Phosphate Buffer

anomer[a]	$k_{ab} \times 10^5$ (s^{-1})	$k_{ba} \times 10^5$ (s^{-1})
α	6.90	5.19
β	8.39	6.31

[a] Corresponds to the anomer whose rate of change is measured.

*Epimerization of **1** in Phosphate Buffer.* The ratio of anomers were measured by integrating the anomeric protons, which changed from 1.00:1.00 (*β*-:α-**1**) at time zero to 1.33:1.00 after 6 h. The first-order rate constants for equilibration (eq 1) between *β*-**1** and α-**1** were found to be within 18% of one another depending upon whether one measured the rate of change of the α- or *β*-anomer (Table 2).



(1)

*Deglycosylation of Monomeric Fapy•dA (**1**).* The rate of deglycosylation of **1** in phosphate buffer was measured by reverse phase HPLC at four temperatures from 37 to 90 °C (Table 3). Decomposition followed first-order kinetics for more than two half-lives. The rate constants for appearance of FapyAde at 37 and 55 °C were determined to be 2.30×10^{-6} and 1.05×10^{-5} s^{-1}, respectively. These data indicated that the half-life for decomposition of **1** was greater than 4 days at 37 °C, decreasing to less than 6 h at 90 °C. Using these data, the E_{act} for deglycosylation was determined to be 11.60 kcal/mol. The enthalpy ($\Delta H^{\ddagger}$) and entropy ($\Delta S^{\ddagger}$) of activation at 298 K were determined to be 11.01 kcal/mol and −48.6 e.u., respectively.


Fapy•Ade

Deglycosylation of Fapy•dA and Fapy•dG in Single-Stranded DNA. Formation of alkali-labile sites in DNA upon deglycosylation of formamidopyrimidines was exploited to determine the rate constants for this process by denaturing polyacrylamide gel electrophoresis in **5** and **6**. Alkaline cleavage conditions were chosen (0.1 M NaOH, 55 °C, 20 min) that produce 3.5 ± 0.7% damage of the Fapy•dA (**5**) and 1.3 ± 0.1% in Fapy•dG (**6**) containing oligonucleotides. Cleavage was only observed at the position of formamidopyrimidine lesions. Two sets of products corresponding to 3′-phosphate and *β*-elimination products were observed by gel electrophoresis, consistent with previous studies on abasic sites (*24*, *25*). The magnitude of background cleavage was determined for three separate samples and was subtracted from each time point when measuring the extent of deglycosylation in **5** and **6**. Upon the basis of previous studies concerning the lability of abasic sites, the cleavage conditions were expected to be sufficient for achieving 100% strand scission of deglycosylated substrate (*24*). This was verified using **7** as a precursor for DNA containing a single abasic site. Following treatment of **7** with UDG, the oligonucleotide (**8**) was subjected to the above alkaline cleavage conditions, resulting in 100% strand scission. The rate constant for the formation of alkali-labile lesions in **5** was measured in triplicate at 55 °C (Figure 1) and was found to be within experimental error of the rate of deglycosylation of **1** ($k = 8.89 \pm 0.39 \times 10^{-6}$ s^{-1}, $t_{1/2} = 21.6 \pm 0.9$ h). Analogous examination of Fapy•dG in **6** revealed that this lesion underwent deglycosylation at 55 °C with a first-order rate constant equal to $3.74 \pm 0.23 \times 10^{-7}$ s^{-1} ($t_{1/2} = 514 \pm 30$ h) that increased to $2.12 \pm 0.20 \times 10^{-6}$ s^{-1} ($t_{1/2} = 91 \pm 10$ h) at 90 °C. Given the slow rates of hydrolysis of Fapy•dG at even 55 °C, it was deemed impractical to measure the rate of deglycosylation at lower temperatures.

Table 3: Rate Constants for Deglycosylation of **1** in Phosphate Buffer

temp (°C)	$k_{Degly} \times 10^6$ (s^{-1})[a]	ave. $t_{1/2}$ (h)
37	1.87 ± 0.12	103
55	9.52 ± 1.47	20.5
72	17.1 ± 0.04	11.3
91	34.1 ± 0.04	5.7

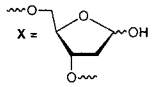
[a] Average rate constants are a minimum of two samples.

5′-d(CGT TCA ACG TGC ACT **X**AC AGC ACG TCC CAT)
5 (**X** = Fapy•dA)

5′-d(AGG CGT TCA ACG TGC AGT **X**AC AGC ACG TCC CAT GGT)
6 (**X** = Fapy•dG)

5′-d(GTC ACG TGC TGC AUA CGA CGT GCT GAG CCT)
7

5′-d(GTC ACG TGC TGC A**X**A CGA CGT GCT GAG CCT)
8



DISCUSSION

We provided the first determination of the rate constants for epimerization and deglycosylation of a formamidopyrimidine deoxyribonucleotide (Fapy•dA). Previously, such studies were limited by the lack of suitable synthetic substrates. By taking advantage of the ability of C5-substituents on the pyrimidine ring to prevent the furanose to pyranose rearrangement, we succeeded in synthesizing a 5′-phosphate diester of Fapy•dA (**1**) as a mixture of anomers. A similar synthetic strategy enabled us to chemically synthesize oligonucleotides containing Fapy•dA or Fapy•dG at defined sites and has been described elsewhere (*21*, *22*).

A combination of 2-D ^{1}H NMR experiments in deuterated phosphate buffer enabled us to assign the respective protons in the two molecules, as well as to determine which set of resonances belonged to each anomer. The various experi-

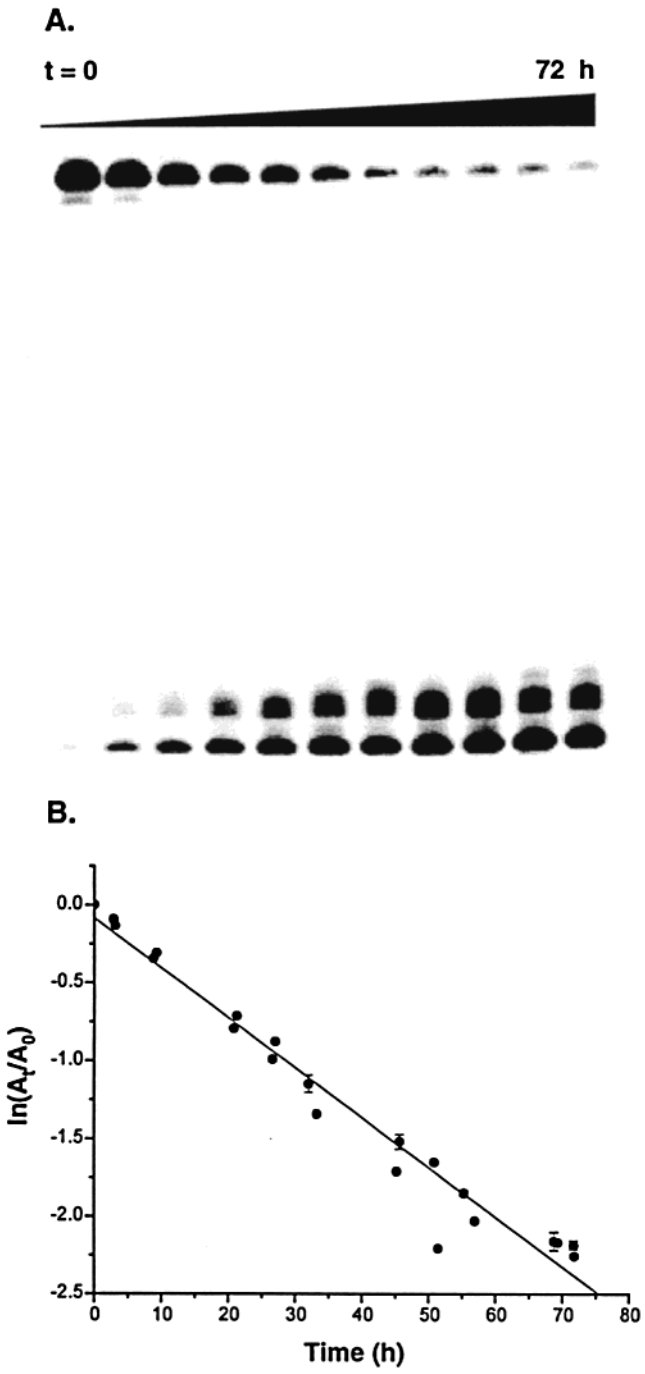
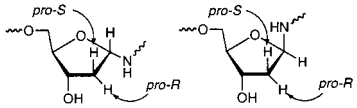


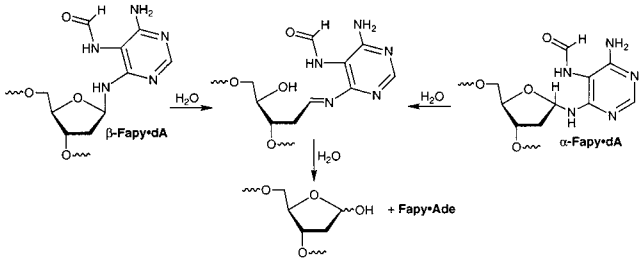
FIGURE 1: Deglycosylation of Fapy•dA in **5** at 55 °C as determined by the formation of alkali-labile lesions as a function of time. (A) Phosphorimage of 5′-^{32}P-**5** as a function of time following alkaline treatment. (B) Kinetic plot of the rate of disappearance of **5** (A_t) as a function of time.

ments were self-consistent and stereochemical assignments were unambiguous. r.O.e. experiments clearly distinguished between α- and *β*-anomers of **1** (*23*). Irradiation of the anomeric proton in *β*-**1** resulted in a strong enhancement of the appropriate C4′ proton. In contrast, enhancements were observed at C3′ and C5′ when the anomeric proton of α-**1** was irradiated. The α- and *β*-anomers of **1** were also distinguishable based upon the chemical shifts of their C2′ protons. The C2′ protons in α-**1** were separated by >0.5 ppm but were unresolved at 400 MHz in *β*-**1**. This effect is readily interpreted by considering that each prochiral C2′ proton in *β*-**1** is cis to a heteroatom substituent at either C1′ or C3′,

Scheme 3: Depiction of *pro-R* and *pro-S* Protons in α,*β*-1



Scheme 4: Epimerization and Deglycosylation of Fapy·dA



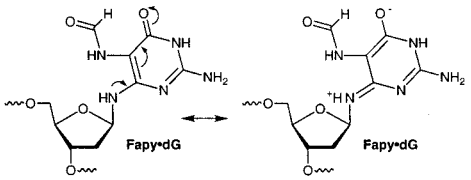
but in α-**1** the *pro-R* proton is shielded by the C3′ hydroxyl group and C1′ nitrogen (Scheme 3).

Resolution of the α- and *β*-anomers of **1** allowed us to measure the rate of epimerization of a formamidopyrimidine nucleotide at physiologically relevant pH for the first time. Previous studies had clearly shown that these molecules rearrange in water, but rate constants were unavailable (*19*, *20*). We showed that *β*-**1** is favored slightly over its anomer and that the two isomers are equilibrated within 6 h at 25 °C. The speed of the equilibration process is germane to the biological relevance of the process. The equilibration period for **1** is short as compared to the lifetime of lesions in DNA. For instance, equilibration of **1** occurs over a considerably shorter period of time than dehydration of the pyrimidine C6-hydrates (*26*−*28*). Hence, when studying the effects of formamidopyrimidine lesions on DNA repair and polymerase enzymes it will be important to consider the role of both anomers.

Decomposition of **1** was followed by HPLC. Examination of **1** enabled us to confirm that deglycosylation was the sole identifiable decomposition pathway by measuring the rate constant for appearance of FapyAde simultaneously. The half-life for deglycosylation in the monomeric substrate varied from more than 4 days to < 6 h over the temperature range between 37 and 90 °C. The measured activation parameters indicate that Fapy•dA will undergo first-order deglycosylation with a rate constant ∼1.2 × 10^{-6} s^{-1} ($t_{1/2}$ ∼1 week) at 25 °C. The rate constant of deglycosylation of Fapy•dA in an oligonucleotide measured at 55 °C was found to be within experimental error of that measured for **1**, indicating that the kinetics measured in the monomer are relevant to the longevity of Fapy•dA in DNA. The large negative entropy of activation is inconsistent with a mechanism for deglycosylation that involves formation of an oxonium ion, and we suggest that an "A-2" type process is operating under the weakly alkaline conditions employed (Scheme 4) (*29*−*31*). This mechanism is attractive in that it allows for epimerization and deglycosylation to proceed via a common intermediate, with epimerization being significantly more rapid than deglycosylation.

Fapy•dG was found to be considerably more stable, exhibiting a half-life of > 500 h at 55 °C. Deglycosylation was also measured at 90 °C (2.1 × 10^{-6} s^{-1}), where it was ∼16 times slower than the deglycosylation of **1** at the comparable temperature. The source of the greater stability

Scheme 5 : Resonance Stabilization of Fapy·dG



of Fapy•dG is unknown, but we suggest that the lone pair of electrons present on the anomeric nitrogen in Fapy•dG are less available for stabilizing the intermediate imine due to conjugation with the carbonyl group (Scheme 5). An analogous resonance structure cannot be drawn for Fapy•dA. Because of the modest rate for deglycosylation of Fapy•dA and Fapy•dG, it was impractical to measure the hydrolytic labilization in duplex DNA at a temperature at which we could be assured that the secondary structure was maintained.

The effects of Fapy•dA and Fapy•dG on polymerase enzyme activity in vitro and in vivo are uncertain. However, plasmid studies in which Fapy•dA is generated randomly and unselectively suggest that it gives rise to A → G transitions (*8*). *N*-Methyl Fapy•dG favors G → T and G → C transversions (*11*). The facile epimerization of these lesions augments the large number of possible hydrogen bond donor and acceptor patterns that can be presented to polymerase enzymes that encounter these lesions in DNA templates. This large number of structural possibilities provides a variety of possible biochemical consequences. The experiments described herein indicate that regardless of the mutagenic potential of these lesions, they are sufficiently stable in DNA to warrant full investigation of their genotoxicity.

SUPPORTING INFORMATION AVAILABLE

Table of r.O.e. enhancements measured for **1**. This material is available free of charge via the Internet at http://pubs.acs.org.

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