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Formation of Modified Cytosine Residues in the Presence of Depurinated DNA

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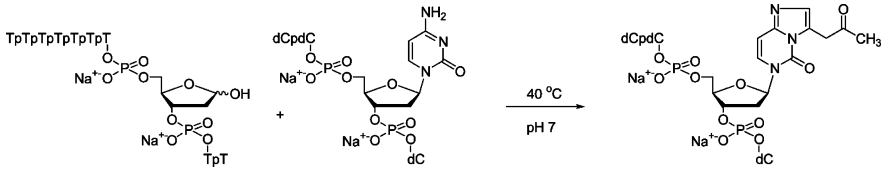
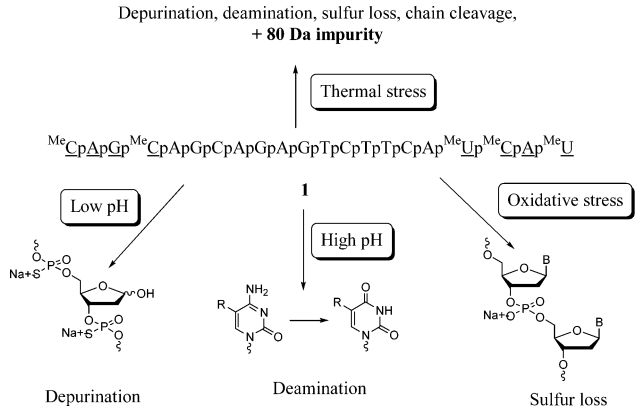


Depurination is an important degradation pathway for antisense phosphorothioate oligonucleotides under conditions of thermal stress. We present evidence showing that depurinated oligonucleotides react with cytosine-containing sequences giving products containing a 6-(2-deoxy-β-D-*erythro*-pentofuranosyl)-3-(2-oxopropyl)imidazo[1,2-*c*]pyrimidin-5(6*H*)-one residue. Further, we demonstrate that the same product is formed upon treatment of 2′-deoxycytidine with 4-oxo-2-pentenal, the latter being an expected byproduct of serial elimination reactions at apurinic sites. In addition to being important for synthetic oligonucleotides, apurinic site formation in cellular DNA is a common occurrence. Because repair of these sites can result in the production of 4-oxo-2-pentenal, it is interesting to speculate whether 6-(2-deoxy-β-D-*erythro*-pentofuranosyl)-3-(2-oxopropyl)imidazo[1,2-*c*]pyrimidin-5(6*H*)-one residues can form in vivo.

Introduction

The specific binding of synthetic oligonucleotides to mRNA or pre-mRNA sequences through Watson−Crick base pairing can result in highly selective inhibition of gene expression.[1−5] This is the principle of antisense drug therapy. Of the DNA analogues investigated to date, phosphorothioate diester oligonucleotides, wherein a nonbridging oxygen atom of the internucleotide linkage is replaced by a sulfur atom, are the most advanced clinical candidates. OGX-011 (**1**, Scheme 1) is a 21-residue phosphorothioate diester oligonucleotide containing both 2′-deoxy and 2′-*O*-(2-methoxyethyl) (MOE) nucleotides. The nucleotides are arranged such that four MOE nucleotides at the 5′ and 3′ ends of the sequence flank a gap of thirteen 2′-deoxynucleotides. Relative to their all-2′-deoxynucleotide counterparts, chimeric, second-generation antisense oligonucleotides such as **1** display increased binding affinities resulting in improved antisense potencies[6] and longer tissue half-lives in vivo, allowing for more relaxed dosing schedules and improved safety profiles.[7]

SCHEME 1. Base Sequence and Main Degradation Products of OGX-011 (1)[a]



[a] 2-(2-Methoxyethyl) ribose nucleotides are underlined; all other nucleotides are 2-deoxyribose and all internucleotide linkages are phosphorothioate diesters.

An important aspect in the development of any new drug is an assessment of its stability. In this regard, we have recently examined the effect of acids, bases, oxidiz-

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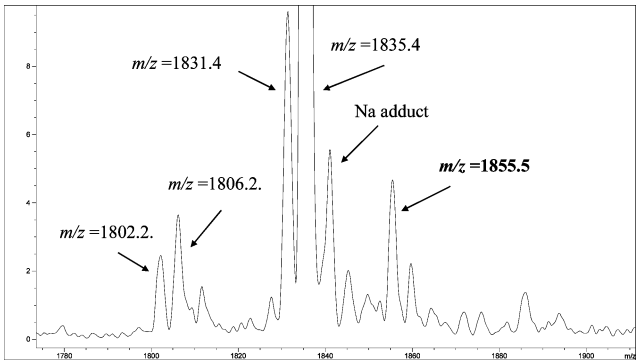


FIGURE 1. Average mass spectrum of the main UV peak obtained by HPLC analysis of **1** after thermal stress. In addition to the parent sequence (m/z = 1835.4), a number of other components are visible (see text).

ers, and heat on **1**. The results obtained under the first three conditions were largely in line with our expectations and with those reported in the literature. Under acidic conditions, the 2′-deoxyadenosine and 2′-deoxyguanosine residues in **1** underwent depurination to give molecules containing mainly a single abasic site.[8] It should be noted that the 2′-oxygen atom renders the MOE purine nucleotides of **1** much more resistant to depurination than their 2′-deoxy counterparts.[9] At high pH, the main degradation pathway was deamination of cytosine and 5-methylcytosine residues to uracil and thymine residues, respectively,[10] whereas treatment with a dilute solution of aqueous hydrogen peroxide resulted in increased levels of molecules containing mainly a single phosphate diester linkage (data not shown). In contrast to the limited number of products formed under each of these conditions, thermal stress produced a much more complicated pattern of degradation products, including depurinated, deaminated, desulfurized, and length-shortened oligonucleotides. In addition, we observed the formation of a product that had a molecular weight of 80 Da more than the parent sequence (+80 Da impurity). These observations are summarized in Scheme 1.

Here, we describe experiments using model compounds, the results from which allowed us to suggest a structure for the unexpected +80 Da degradation product. During the course of these studies, we obtained data regarding the reactivity of depurinated oligonucleotides that may be of some biological significance.

Results

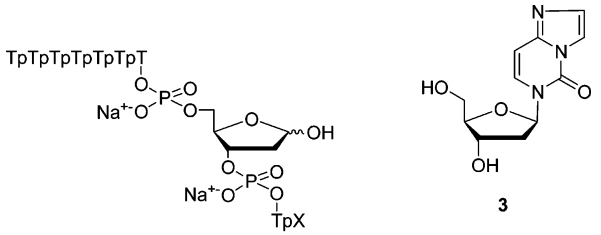
Figure 1 shows the average mass spectrum under the main UV peak obtained by HPLC analysis of a sample of **1** following four weeks of heating at 90 °C. The average mass spectrum of the main UV peak contained several identifiable components. The largest peak, m/z = 1835.4, corresponded to the −4 charge state of the full-length,

TABLE 1. Relative Amounts of Modified and Unmodified 4−10 Following Incubation with 2a or 2b[a]

sequence	unmodified (%)	+80 Da (%)	+160 Da (%)
4 d(ApApApA)	95	5	nd
5 d(CpCpCpC)	65	30	5
6 d(GpGpGpG)	100	nd	nd
7 d(TpTpTpT)	100	nd	nd
8 d(TpApT)	97	3	
9 d(TpCpT)	72	28	
10 d(TpGpT)	99.2	0.8	

[a] Calculated from the integrated ion currents (IC) due to each component assuming equivalent ionization efficiencies, e.g., relative amount + 80 Da = $IC_{80}/(IC_{unmodified} + IC_{80} + IC_{160}) \times 100\%$; nd = none detected (<0.5%).

CHART 1. Structures of Apurinic Oligonucleotides 2a and 2b and 3,N^4-Etheno-2′-deoxycytidine (3)



2a: X = 5-methyl-2'-MOE-uridine
2b: X = Thymidine

fully thioated oligonucleotide (calcd m/z = 1835.3). Also present were peaks corresponding to the −4 charge states of molecules containing a phosphate diester linkage in place of a phosphorothioate linkage (m/z = 1831.4, calcd 1831.3) and depurinated molecules that had lost either adenine or guanine and added water (m/z = 1806.2, calc 1806.0 and m/z = 1802.2, calc 1802.2, respectively). In addition to these known components, a signal at m/z = 1855.5, which was absent from a control sample stored at −20 °C, was observed. The mass difference between this unidentified component and the parent sequence was 80 Da. Extraction and integration of the ion currents due to this component and the parent molecule revealed the former was present at about 5% of the latter.

Consideration of the total product mixture formed by thermal stress of **1** suggested that depurination was an important route of degradation under these conditions. This suggestion was supported not only by the observed increase in apurinic oligonucleotides (Figure 1), but also by accumulation of the specific shorter oligonucleotides predicted to form[11] following cleavage at sites adjacent to the 2′-deoxyadenosine and 2′-deoxyguanosine residues in **1** (data not shown). With this in mind, we decided to investigate the hypothesis that the +80 Da impurity was also formed following an initial depurination reaction. To test this idea, we prepared a series of short, phosphate diester oligonucleotides (Table 1) and investigated their reactivity with oligonucleotides **2a** and **2b** that each contained an apurinic site (Chart 1).

Oligonucleotides **2a** and **2b** were accessed in a straightforward manner by acidic treatment of their 2′-deoxyadenosine-containing precursors. Completion of the depu-

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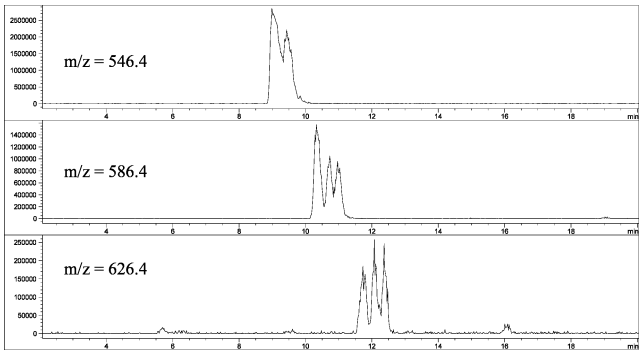


FIGURE 2. Ion chromatograms of reaction products of **5** with **2a**. The upper, middle, and lower panels show the ion currents due to **5**, **5** + **80 Da**, and **5** + **160 Da**, respectively.

rination reaction was confirmed by mass spectrometric analysis. Reaction mixtures containing **4**, **5**, **6**, or **7** (0.25 μmol) and **2a** (3.4 μmol) in water (50 μL) or **8**, **9**, or **10** (0.5 μmol) and **2b** (3.4 μmol) in water (100 μL) were incubated at 40 °C. The pH of the reaction mixtures was neutral. After 5 days, the reaction mixtures were analyzed by HPLC−MS. For each sample, we extracted the ions corresponding to the −2 charge state of the parent tetramers (or to the −1 charge of the parent trimers) and their +80 Da impurities. Where appropriate, we also extracted the ions corresponding to two additions of 80 Da. The ion chromatograms obtained for the $d(C)_4$ reaction mixture are shown in Figure 2.

Figure 2 shows that for the sample containing **5** and **2a** we were able to detect products that had molecular weights of 80 and 160 Da more than **5**, the latter being ascribed to doubly modified **5**. Integration of the ion chromatograms from each sample allowed us to estimate the relative amounts of the modified and unmodified components (Table 1).

Table 1 shows that only sequences containing 2′-deoxyadenosine and 2′-deoxycytidine reacted with **2a** or **2b** to give appreciable amounts of the +80 Da impurity. Under these conditions, **5** and **9** were about 7−10 times more reactive than **4** and **8**.[12] These results support the hypothesis that depurination and the formation of the +80 Da impurity are linked. The fact that reaction took place most readily with the 2′-deoxycytidine-containing oligonucleotides suggested that, in the case of **1**, reaction would occur mainly at one of its cytosines or, alternatively, 5-methylcytosines, rather than at the other aglycons or the internucleotide linkages or sugar residues.

Preparative HPLC of the $d(C)_4$ reaction mixture provided a sample enriched in the +80 Da impurity. Enzymatic digestion of this material using snake venom phosphodiesterase and alkaline phosphatase gave a mixture of nucleosides that were analyzed by reversed-phase HPLC (Figure 3).

In addition to 2′-deoxycytidine (t_R = 5 min) and thymidine (t_R = 15 min), the latter presumably present due to impurities in the donor sequence (**2a**) that were

(12) For **5**, assuming that unmodified and singly and doubly modified products have equivalent ionization efficiencies, the amounts of the three components were distributed approximately according to the binomial expansion $(x + y)^4$, where x and y are the decimal fractions of unmodified and modified cytosine residues, respectively. This suggests that reaction takes place equally at each of the four cytosine residues.

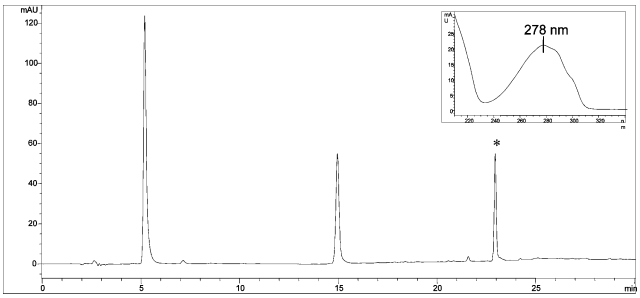
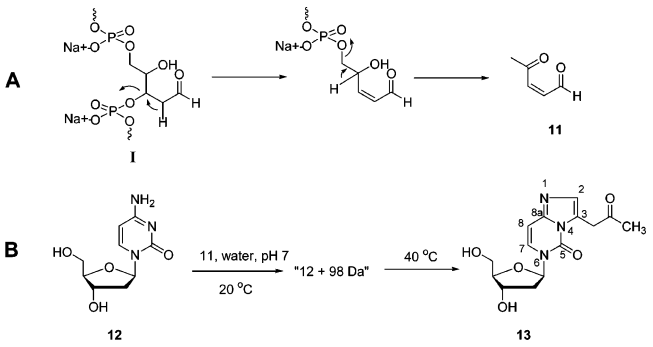


FIGURE 3. Reversed-phase HPLC chromatogram of isolated $d(C)_4$ +80 Da impurity after enzymatic digestion. Peaks at 5 and 15 min are 2′-deoxycytidine and thymidine, respectively. Modified nucleoside * had a molecular weight of 307 Da (80 Da more than 2′-deoxycytidine) and the UV spectrum shown inset.

SCHEME 2. (A) Predicted Formation of 4-Oxo-2-pentenal (11) from the Aldehyde Tautomer of an Apurinic Site. (B) Model Reaction of 2′-Deoxycytidine (12) with 11



isolated along with the modified tetramer, we observed a peak (labeled * in Figure 3) at 23 min. The molecular weight of this component was 307 Da, i.e., 80 Da more than 2′-deoxycytidine. The UV spectrum of this peak (inset in Figure 3) showed a maximum absorbance at 278 nm and appeared very similar to the UV spectrum of 3,N^4-ethenocytidine (**3**),[13] providing further evidence of a modified heterocyclic ring system. $(MS)^2$ analysis generated a 192 Da fragment ion that corresponded to the mass of cytosine plus an additional 80 Da (calcd $[M + H]^+$ = 192 Da) and confirmed the site of modification as the heterocycle.

To aid structural determination, we sought a simpler model system with which to generate larger quantities of the desired nucleoside. Consideration of the likely products of depurination led us to examine the reaction of 2′-deoxycytidine (**12**) with 4-oxo-2-pentenal (**11**), the latter being a possible product of sequential elimination reactions of the aldehyde tautomer (**I**) of an apurinic site (Scheme 2).

Treatment of an aqueous solution of **12** with about 12 equiv of **11** at 20 °C for 16 h resulted in complete consumption of the starting material and formation of two main product peaks (**12** + 98 Da), each with a mass 98 Da more than **12** (Figure 4, upper panel). Heating the reaction mixture at 40 °C for a further 13 h resulted in clean conversion of the **12** + 98 Da products into a

(13) Barrio, J. R.; Sattsangi, P. D.; Gruber, B. A.; Dammann, L. G.; Leonard, N. J. *J. Am. Chem. Soc.* **1976**, *98*, 7408−7413.

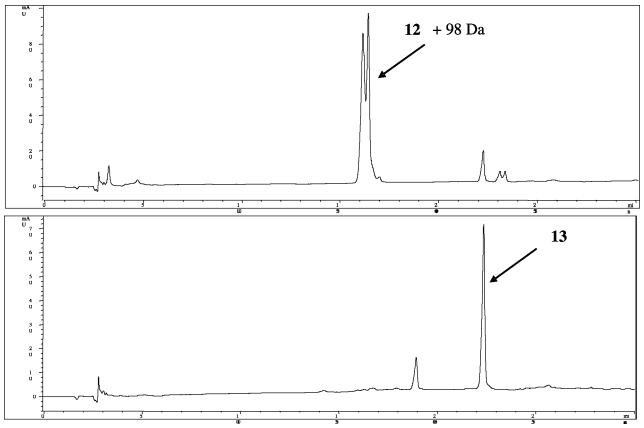


FIGURE 4. (Upper panel) HPLC trace of the reaction mixture formed by treatment of **12** with **11** for 16 h at 20 °C. The mass of the main product was 98 Da more than 2′-deoxycytidine. (Lower panel) The same reaction mixture after 13 h at 40 °C. The retention time, UV and mass spectra were identical to those of the modified nucleoside isolated from digestion of the +80 amu product formed by reaction of **5** and **2a**.

component (**13**) with a molecular weight of 80 Da more than **12** (Figure 4, lower panel).

It seems reasonable to suggest that the **12** + 98 Da product formed at room temperature lost water when heated to give a component with a molecular weight of 80 Da more than **12**. Silica gel chromatography of the crude reaction mixture produced after 13 h at 40 °C gave the main product in low yield (ca. 5%). We subsequently discovered that acetylation of the crude reaction mixture formed by reaction of **12** and **11** using an excess of acetic anhydride in pyridine, followed by silica gel purification and deacetylation (20% methylamine in water/MeOH, 1:1 v/v), gave the same product in a much improved, though still unoptimized, yield of 35%. Comparison of the HPLC retention times, mass spectrometry fragmentation patterns, and UVspectra[14] of this material and the modified nucleoside isolated by digestion of the +80 amu product formed from reaction of **5** with **2a** confirmed the two compounds were identical. This synthesis provided material in sufficient quantity and purity to allow us to determine the product as 6-(2-deoxy-β-D-*erythro*-pentofuranosyl)-3-(2-oxopropyl)imidazo[1,2-*c*]pyrimidin-5(6*H*)-one (**13**, Scheme 2). Salient features of the proton spectrum were one and two proton singlets at $\delta = 7.12$ and 4.15 ppm, assigned to imidazole hydrogen H_2 and the methylene hydrogens of the 2-oxopropyl side chain, respectively. All proton and carbon assignments were confirmed using two-dimensional proton (COSY) and C−H correlation (HMQC and HMBC) spectroscopy. The orientation of addition of 4-oxo-2-pentenal across the c-face of the pyrimidinone ring was confirmed by long-range C−H correlation spectroscopy (HMBC). An expanded view of the HMBC spectrum is shown in Figure 5 alongside **13** and its regioisomer **alt-13** anticipated from addition of 4-oxo-2-pentenal in the opposite orientation. The spectrum clearly shows the cross-peak from three bond coupling between H_2 and C_{8a} anticipated for regioisomer **13**. In contrast, the additional cross-peak between H_3 and C_5 that would be expected for structure **alt-13** was absent.

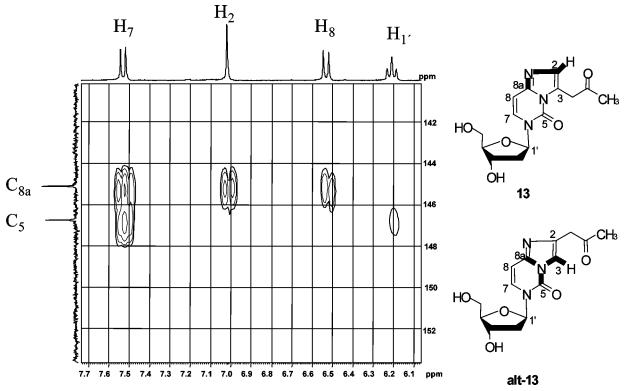
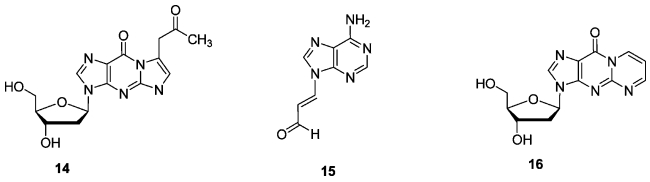


FIGURE 5. Expanded view of HMBC spectrum of **13**. Diagnostic, three-bond coupling relationships for **13** and regioisomer **alt-13** are shown in bold. The cross-peak between H_2 and C_{8a} expected for structure **13** was evident, while the additional cross-peak between H_3 and C_5 predicted for **alt-13** was not.

CHART 2. 3-(2-Deoxy-β-D-erythro-pentofuranosyl)-3, 4-dihydro-7-(2-oxopropyl)-9*H*-imidazo[1,2-*a*]purin-9-one (14), Adenine Propenal (15), and M_1DG (16)



Discussion

The results above indicate that in the presence of depurinated oligonucleotides, 2′-deoxycytidine-containing oligonucleotides react to give products that have molecular weights of 80 Da more than the parent sequence. 2′-Deoxyadenosine-containing oligonucleotides react to a lesser extent. In the case of 2′-deoxycytidine-containing oligonucleotides, reaction leads to formation of 6-(2-deoxy-β-D-*erythro*-pentofuranosyl)-3-(2-oxopropyl)imidazo[1,2-*c*]pyrimidin-5(6*H*)-one residues. We have demonstrated that this product is the same as that formed by reaction of 2′-deoxycytidine with 4-oxo-2-pentenal, the latter being a potential byproduct from sequential elimination reactions of depurinated oligonucleotides. It is known that 2′-deoxyguanosine also reacts with 4-oxo-2-pentenal, although the product, 3-(2-deoxy-β-D-*erythro*-pentofuranosyl)-3,4-dihydro-7-(2-oxopropyl)-9*H*-imidazo[1,2-*a*]purin-9-one (**14**, Chart 2), was reportedly formed to the extent of only about 4% after 7 h at 37 °C when about 3 equiv of purified **11** was used.[15] Although it is difficult to compare these results with our own directly, the fact that reaction of **12** with 12 equiv of crude **11** gave about 80% **13** suggests that reaction with 2′-deoxycytidine is

(14) The UV spectrum of this material recorded at pH 2 showed a λ_{max} at 296 nm, whereas at pH 7.0 λ_{max} equaled 278 nm. Changes in UV spectra as a function of pH are characteristic of 3,N^4-substituted cytidines: Singer, B. In *CRC Handbook of Biochemistry and Molecular Biology, Nucleic Acids*, 3rd ed.; Fasman, G. D., Ed.; CRC Press: Boca Raton, FL, 1975; Vol. 1, pp 409−447.

(15) Hecht, S. S.; Young-Sciame, R.; Chung, F.-L. *Chem. Res. Toxicol.* **1992**, *5*, 706−712.

more rapid than reaction with 2′-deoxyguanosine. The possibly reduced reactivity of 2′-deoxyguanosine, coupled with the reported instability of 4-oxo-2-pentenal,[16] may explain why we were unable to detect appreciable amounts of +80 Da product upon incubation of guanine-containing compounds **6** and **10** with depurinated oligonucleotides **2a** and **2b**. It should be noted, however, that we have no direct evidence for the generation of 4-oxo-2-pentenal from depurinated oligonucleotides under our conditions, and it may be that the observed lack of reactivity with guanine residues indicates the reaction proceeds via alternate pathways, e.g., reaction at apurinic sites prior to chain cleavage.[17]

Our efforts to date have focused on identifying those products formed by reaction of depurinated oligonucleotides, or their byproducts, with cytosine residues. We have yet to establish conclusively that this product and the +80 Da impurity formed upon thermal stress of **1** are identical. The two species do, however, have the same mass, and the latter is formed under conditions that give rise to depurination. For oligonucleotides such as **1**, which contain both methylated and unmethylated cytosine residues, the situation is likely complicated further as modification can presumably occur at either site. We are currently working to identify the products formed by reaction of 5-methylcytidine residues with depurinated oligonucleotides and to confirm that these and their unmethylated counterparts account for the +80 Da impurity formed by thermal stress of **1**.

Potential Biological Significance. In addition to DNA adduct formation due to exposure to exogenous chemicals, there is a growing body of evidence suggesting that certain endogenous chemicals, produced by normal metabolism, or under conditions of oxidative stress, can also react to form DNA adducts.[18] For example, it is known that conditions of oxidative stress lead to formation of base propenals e.g., **15**, that react with guanine residues to produce 3-(2-deoxy-β-D-*erythro*-pentofuranosyl)pyrimido[1,2-*a*]purin-10(3*H*)-one (M_1dG, **16**).[19] In the current context, it is probable that abasic sites, generated either by spontaneous hydrolysis[20] or by DNA glycosylases in the first step in the base excision repair (BER) pathway, are among the most common lesions in cellular DNA. Following base removal, the predominant BER pathway proceeds via AP endonuclease APE1-catalyzed hydrolysis of the phosphate diester bond 5′ to the abasic site and results in a nick that is subtended by a phosphorylated 2-deoxyribose moiety and a 3′-OH group. Polymerase β then introduces a nucleotide and excises the phosphorylated 2-deoxyribose moiety through a β-elimination mechanism, before the nick is finally sealed by DNA ligase III.[21] Although 4-oxo-2-pentenal is not produced by this mechanism, there is evidence to suggest that another DNA repair enzyme, the Fpg protein, catalyzes a different series of elimination reactions at abasic sites that liberate 4-oxo-2-pentenal as a byproduct.[16] It is interesting to speculate whether 4-oxo-2-pentenal produced in this way reacts with cellular DNA to generate 6-(2-deoxy-β-D-*erythro*-pentofuranosyl)-3-(2-oxopropyl)imidazo[1,2-*c*]pyrimidin-5(6*H*)-one residues in vivo.

Experimental Section

6-(2-Deoxy-β-D-*erythro*-pentofuranosyl)-3-(2-oxopropyl)imidazo[1,2-*c*]pyrimidin-5(6*H*)-one (13). 4-Oxo-2-pentenal (**11**) was prepared according to the method described by Hecht[15] as follows: a solution of 2-methylfuran (8.0 g, 98 mmol) in methylene chloride (40 mL) was added over 15 min to a stirred solution of *m*-CPBA (18.8 g, 82 mmol) in methylene chloride (120 mL) at 0 °C. The mixture was stirred a further 1 h at 0 °C, extracted with saturated aqueous sodium hydrogen carbonate solution (2 × 80 mL) and water (80 mL), dried (Na_2SO_4), and concentrated to give crude **11**.

Crude **11** was dissolved in water (40 mL). To 20 mL of this solution was added 2′-deoxycytidine hydrochloride (1.0 g, 3.8 mmol), and the products were stirred at 40 °C for 2 days. The reaction mixture was evaporated to dryness and azeotroped with dry pyridine (3 × 25 mL). The residue was redissolved in pyridine (50 mL) and acetic anhydride (5 mL, 53 mmol) added. The mixture was allowed to stir at room temperature overnight and then cooled on ice and water (10 mL) added. The products were concentrated to remove pyridine, and a solution of the residue in ethyl acetate (50 mL) was washed with saturated aqueous sodium hydrogen carbonate solution (2 × 50 mL). The organic layer was dried (Na_2SO_4) and concentrated under reduced pressure. The residue was purified by chromatography on silica gel. The fractions eluted with chloroform−MeOH (99:1 v/v) were combined and evaporated to give a yellow glass. To a solution of this material in methanol (10 mL) was added aqueous methylamine (40% w/v, 10 mL, 116 mmol). After 30 min, the products were concentrated to dryness, and the residue was purified by chromatography on silica gel. Combination and evaporation of the fractions eluted with chloroform−methanol (99:6 v/v) gave the title compound (0.41 g, 1.3 mmol, 35%) as a colorless solid. A portion of this material was crystallized from chloroform−methanol: R_f (CH_2Cl_2/MeOH, 9:1 v/v) 0.22; mp = 177−178 °C; UV λ_{max} (pH 7.0) 278 nm (ϵ = 11900), λ_{max} (pH 2.0) 296 nm (ϵ = 11700); ^{1}H NMR [$(CD_3)_2SO$] δ 7.63 (1H, d, J = 8.0 Hz), 7.12 (1H, s), 6.64 (1H, d, J = 7.9 Hz), 6.31 (1H, t, J = 6.7 Hz), 5.28 (1H, d, J = 4.2 Hz), 5.06 (1H, t, J = 5.2 Hz), 4.27 (1H, m), 4.15 (2H, s), 3.83 (1H, m), 3.60 (2H, m), 2.20−2.08 (5H, m); ^{13}C NMR [$(CD)_3]_2SO$ δ 204.2, 146.8, 145.1, 132.6, 127.8, 123.0, 98.8, 87.6, 84.7, 70.3, 61.2, 40.0, 39.7, 29.2; HRMS [M + Na]$^+$ calcd for $C_{14}H_{17}N_3O_5Na$ 330.106, found 330.1061. Anal. Calcd for $C_{14}H_{17}N_3O_5$: C, 54.72; H, 5.58; N, 13.67. Found: C, 54.72; H, 5.70; N, 13.55.

Supporting Information Available: General experimental procedures, HPLC−UV/MS conditions, HPLC chromatograms and mass spectra, and ^{1}H, ^{13}C, COSY, HMBC, and HMQC NMR spectra for **13**. This material is available free of charge via the Internet at http://pubs.acs.org.

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