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5-Cyanoimino-4-oxomethylene-4,5-dihydroimidazole and 5-Cyanoamino-4-imidazolecarboxylic Acid Intermediates in Nitrosative Guanosine Deamination: Evidence from ^{18}O-Labeling Experiments

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Abstract: The nitrosative deaminations (37 °C, $NaNO_2$, NaAc buffer, pH 3.7) of guanosine **1r** in (^{18}O)-water (97.6%) and of [6-^{18}O]-**1r** in normal water were studied. [6-^{18}O]-**1r** was prepared from 2-amino-6-chloropurine riboside using adenosine deaminase. The reaction products xanthosine **3r** and oxanosine **4r** were separated by HPLC and characterized by LC/MS analysis and ^{13}C NMR spectroscopy. The ^{18}O-isotopic shifts on the ^{13}C NMR signals were measured and allowed the identification of all isotopomers formed. The results show that oxanosine is formed via 5-cyanoimino-4-oxomethylene-4,5-dihydroimidazole, **5**, and its 1,4-addition product 5-cyanoamino-4-imidazolecarboxylic acid, **6**. This hydration of **5** to **6** leads to aromatization and greatly dominates over water addition to the cyanoimino group of **5** to form 5-guanidinyliden-4-oxomethylene-4,5-dihydroimidazole, **7**. 5-Guanidinyl-4-imidazolecarboxylic acid, **8**, the product of water addition to **6**, is not involved.

Introduction

Deamination of DNA bases by nitrous acid was first reported in 1861,[1] and it was 100 years later that the toxicological effects of nitrous acid on nucleic acids were realized.[2] Subsequent studies showed that nitrosative deamination converts the nucleobase guanine to xanthine,[3] cytosine to uracil,[4,5] and adenine to hypoxanthine.[6] The possibility of endogenous DNA nitrosation was demonstrated in 1991 when Keefer reported that the bioregulatory agent nitric oxide[7] causes DNA base deaminations in vitro.[8] HNO_2[9] and NO[10] may also induce mutations through the formation of interstrand DNA cross-links. Shapiro et al. isolated and spectrometrically identified the cross-links dG-to-dG and dG-to-dA (Scheme 1) during the treatment of calf thymus DNA with nitrous acid.[11,12] Because of the numerous sources of dietary[13] and endogenous nitrosating agents, nitrosative DNA base deamination constitutes a major part of DNA damage[14] and contributes significantly to genomic instability and various diseases via mutagenesis and cytotoxicity. Several human DNA repair genes have been identified, and some of these may correct deamination damage.[15] Details of the detection of the DNA damage, the signaling to initiate repair, and the actual DNA repair are only now emerging.[16,17]

Dinitrogen trioxide (N_2O_3) is believed to be the active nitrosating agent in the deamination of DNA bases.[19] On the basis of the chemistry of primary amines, it is believed that the DNA damage by nitrogen oxides involves nitrosation of the exocyclic amino groups of the DNA bases leading to diazonium ions of the nucleobases as the *crucial reactive intermediates*. However, no diazonium ion of any nucleobase has ever been

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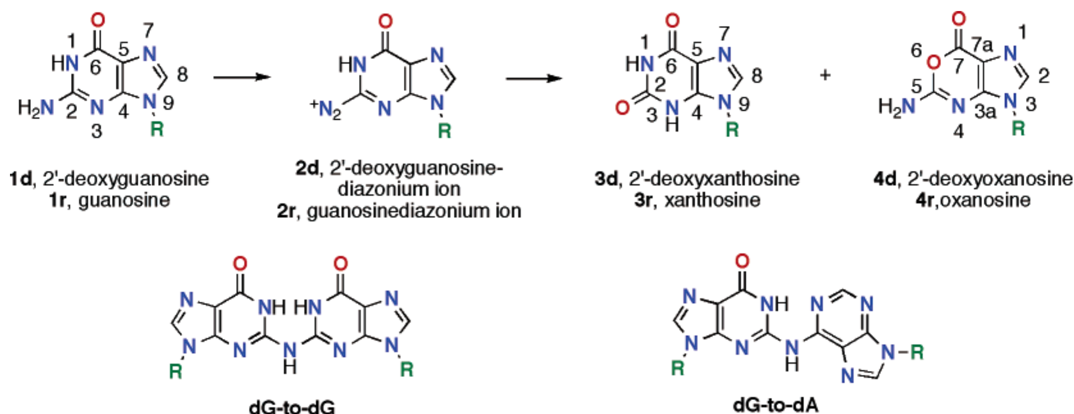
Scheme 1. Known Products of Nitrosative 2′-Deoxyguanosine Deamination[a]

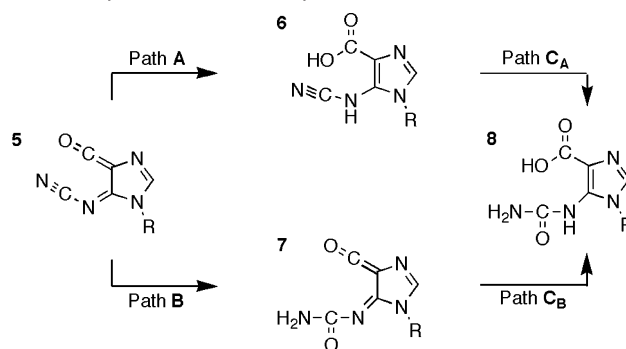


[a] The heterocycles are numbered, and the letter specifies R (**d**, R = 2′-deoxyribose; **r**, R = ribose; **h**, R = H).[18]

isolated or observed experimentally, and the properties and stabilities of these diazonium ions are not known, and their reaction chemistry is not well understood. The guaninediazonium ion **2** is assumed to undergo nucleophilic aromatic substitution of N_2 by water followed by tautomerization to **3**, whereas a nucleophilic aromatic substitution by an amino group of a neighboring nucleobase on the opposite strand would result in an interstrand DNA cross link. Suzuki et al. revisited the deamination of 2′-deoxyguanosine **1d** and isolated a novel product 2′-deoxyoxanosine **4d** in 21.5% yield in addition to 2′-deoxyxanthosine **3d** on treatment of 2′-deoxyguanosine, oligodeoxynucleotide, and calf thymus DNA with nitrous acid and nitric oxide (Scheme 1).[20] Several groups have since isolated **4d** during nitrosation of **1d**.[21] The formation of **4** cannot be explained by direct nucleophilic aromatic substitution on **2**.

Our theoretical studies of the unimolecular dediazoniations of the DNA base diazonium ions revealed that these ions are much more prone to lose dinitrogen[22,23] than the prototypical benzenediazonium ion.[24] For the isolated guaninediazonium ion **2h**, we found that its unimolecular dediazoniation is accompanied by concomitant amide bond cleavage and leads to the *N*-protonated derivative[25] of the pyrimidine ring-opened intermediate **5** shown in Scheme 2. In the presence of the complementary base cytosine, **2h** is not likely to exist at all, there is no barrier to proton transfer to cytosine, and **5** is the primary product of deprotonation and dediazoniation of **2**.[26] We have shown that deglycation of **5** is possible, but this reaction cannot compete with water addition to **5**.[27] Addition and recyclization chemistry of **5** could explain the formations of all known products of nitrosative guanine deamination—**3d**, **4d,** and the cross-links—and, hence, the results of our ab initio studies suggest the formation of all these products from a single common intermediate. Recently, Suzuki et al. subjected guanosine **1r** and its methyl derivatives 1-Me-**1r** to nitrosative deamination, and it was shown that the exocyclic amino group of **4r** arises from the imino nitrogen of **1r**.[28] This finding corroborates our mechanistic hypothesis.

Scheme 2. Postulated Intermediates 5-Cyanoimino-4-oxomethylene-4,5-dihydroimidazole **5** and Hydration Products 5-Cyanoamino-4-imidazolecarboxylic Acid **6**, 5-Guanidinyliden-4-oxomethylene-4,5-dihydroimidazole **7**, and 5-Guanidinyl-4-imidazolecarboxylic Acid **8**



It is our goal to test the mechanistic hypothesis that pyrimidine ring opening occurs along the reaction channels leading to the formation of **3r** and **4r** by nitrosative deamination of **1r** and, if so, to determine the paths to product formation. The key intermediate **5** contains two highly electrophilic functional groups. The addition of one water to the ketene[29] moiety (path A) or to the cyanoimine functionality[30] (path B) would result in **6** or **7**, respectively. The addition of a second water molecule

(18) International Union of Pure and Applied Chemistry, Recommendations on Organic & Biochemical Nomenclature, Symbols & Terminology etc. World Wide Web material by G. P. Moss at URL http://www.chem.qmul.ac.uk/iupac. (a) Glossary of Class Names of Organic Compounds and Reactive Intermediates Based on Structure, Section Nucleic Acids and Component Units. (b) Nomenclature for Isotopically Substituted Compounds and Specifically Labeled Compounds: Section H-2, Names for Isotopically Modified Compounds.
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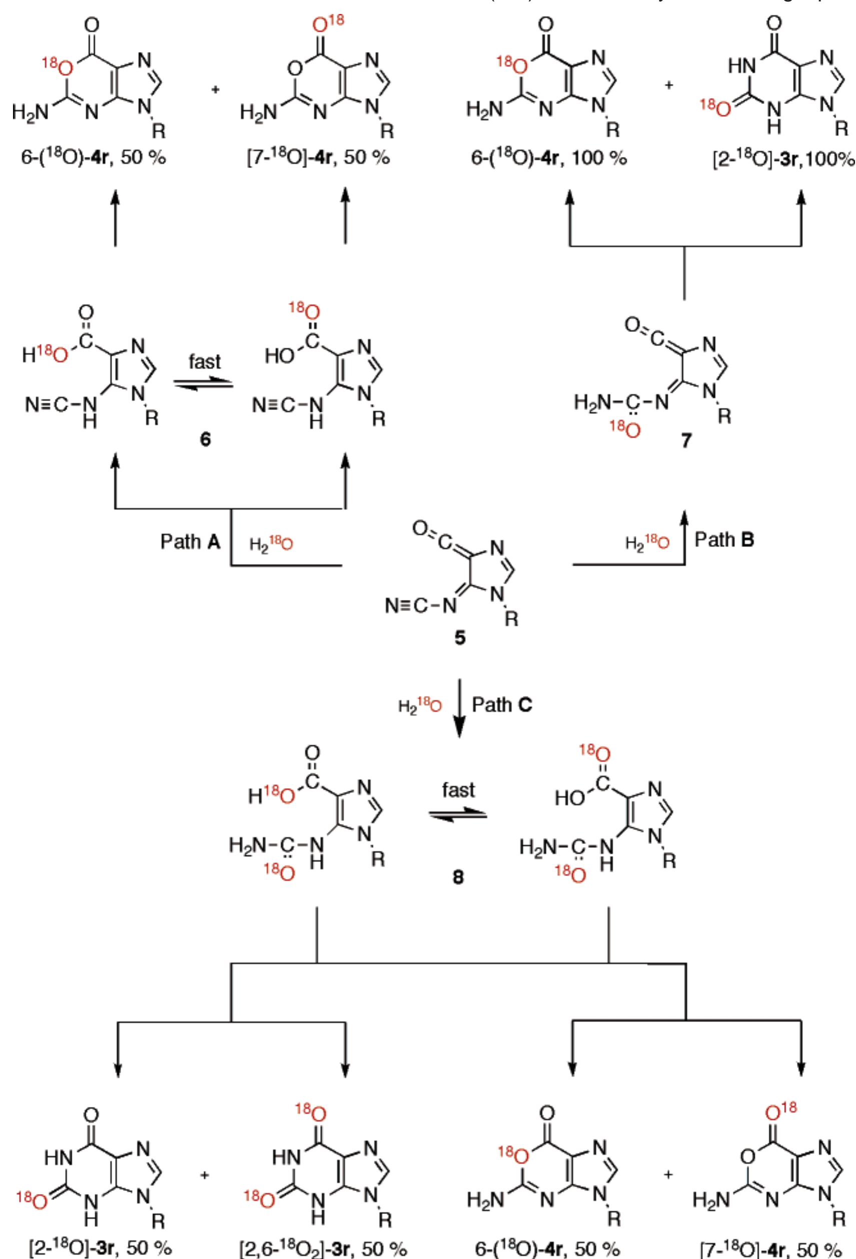
Scheme 3. Possibilities for Product Formations in the Deamination of **1r** in (^{18}O)Water with Pyrimidine Ring Opening[a]



[a] Percentages refer to *relative* yields of isotopomers.

to either **6** or **7** would give **8**, and we refer to its formation as path C. **3r** could be formed by direct nucleophilic aromatic substitution of the guaninediazonium ion or via pyrimidine ring opening and recyclization of **7** or **8** (vide infra) but not **6**. Recent theoretical studies of the bimolecular reaction of guaninediazonium ion with water suggest that a direct replacement is possible for some reaction trajectories.[31] **4r** could be formed from any of the compounds **6**−**8**.

Experimental Design

To discriminate between the reaction options of Scheme 2, we studied the nitrosative deaminations of **1r** in (^{18}O)water and of [6-^{18}O]-**1r** in normal water. We discuss possible outcomes with the help of Schemes 3 and 4 and present some precedent in Scheme 5. The predicted outcomes of the two labeling experiments are summarized in Table 1.

We analyzed the ^{18}O-labeling by two methods. First, we determined the *number of labels* in the products in a straightforward manner by electrospray ionization mass spectrometry.[32] Second, we determined the *positions of the labels* by analysis of the ^{18}O-isotopic shifts in the ^{13}C NMR spectra of the products. Such isotope effects on the chemical shifts of attached NMR nuclei have been observed for ^{13}C as well as several other nuclei.[33] The effect primarily is a consequence of a small difference in the distances between the isotopic atoms and the

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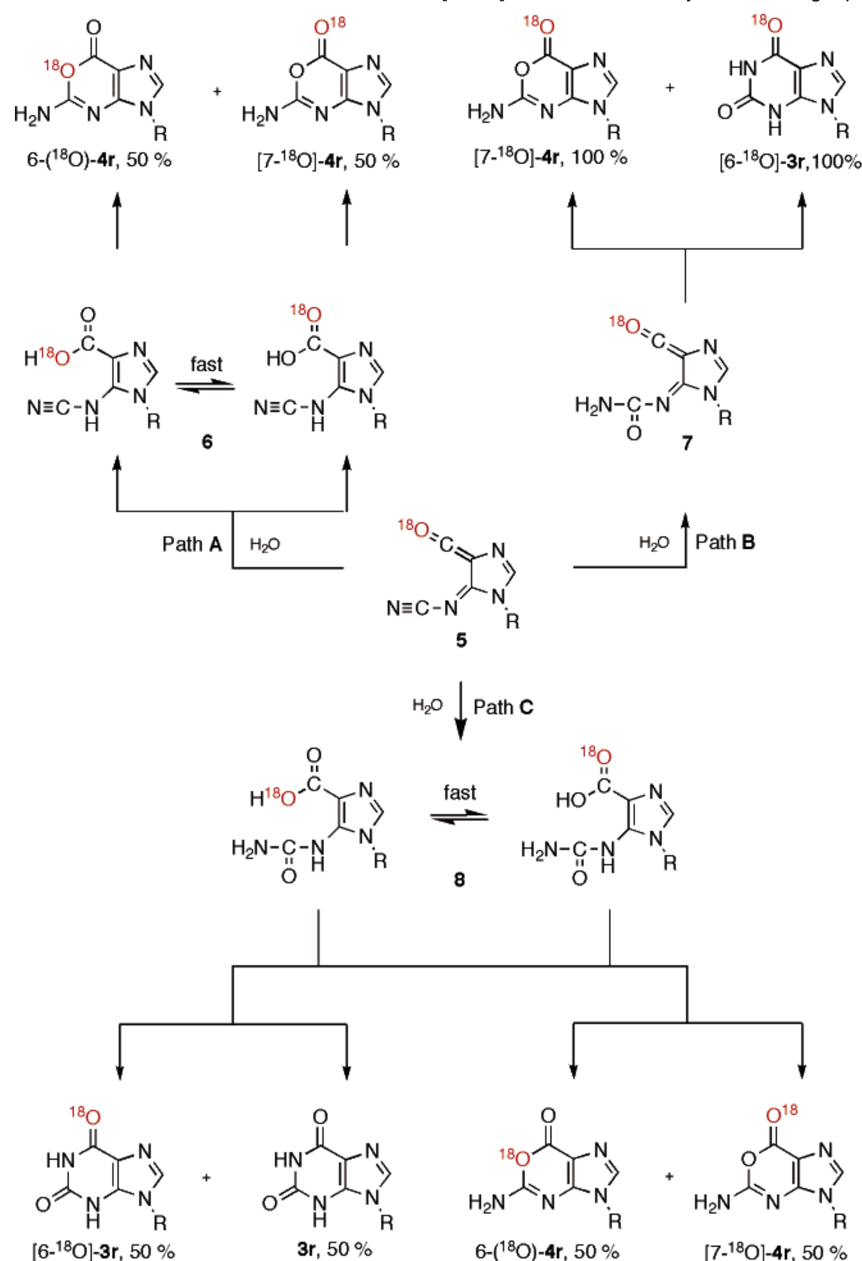
Scheme 4. Possibilities for Product Formations in the Deamination of [6-^{18}O]-**1r** in Water with Pyrimidine Ring Opening via **5**[a]



[a] Percentages refer to *relative* yields of isotopomers.

NMR nucleus that affects the chemical shielding.[34] The small distance differences result from the anharmonicity of the potential-energy surfaces of the systems in combination with the difference in the vibrational energy levels of the isotopic systems. ^{18}O-isotopic shifts of ^{13}C NMR signals have been studied for several functional groups, and their magnitudes vary in ways that are not easily predictable.[35,36]

A previously reported enzymatic synthesis of [6-^{18}O]-**1r** involves the hydrolysis of 2-amino-6-chloropurine riboside in the presence of enzyme 5′-adenylic acid deaminase (AMPDA).[37] In our laboratory, this reaction required 7 days to run to completion and resulted in low yields (<70%). Hence, we developed a fast and quantitative enzymatic synthesis of [6-^{18}O]-**1r** from 2-amino-6-chloropurine riboside using adenosine deaminase.[38b]

Deamination of 1r in (^{18}O)Water with Pyrimidine Ring Opening. The possibilities for the hydration of **5** in the presence of $^{18}OH_2$ are discussed in Scheme 3. 1,4-Addition of water to **5** would lead to the imidazolecarboxylic acid **6** (path A). An equal distribution of the ^{18}O-label between the carbonyl-O and the hydroxyl-O of **6** is expected because rotations about

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Table 1. Predicted Outcome of the Two Labeling Experiments

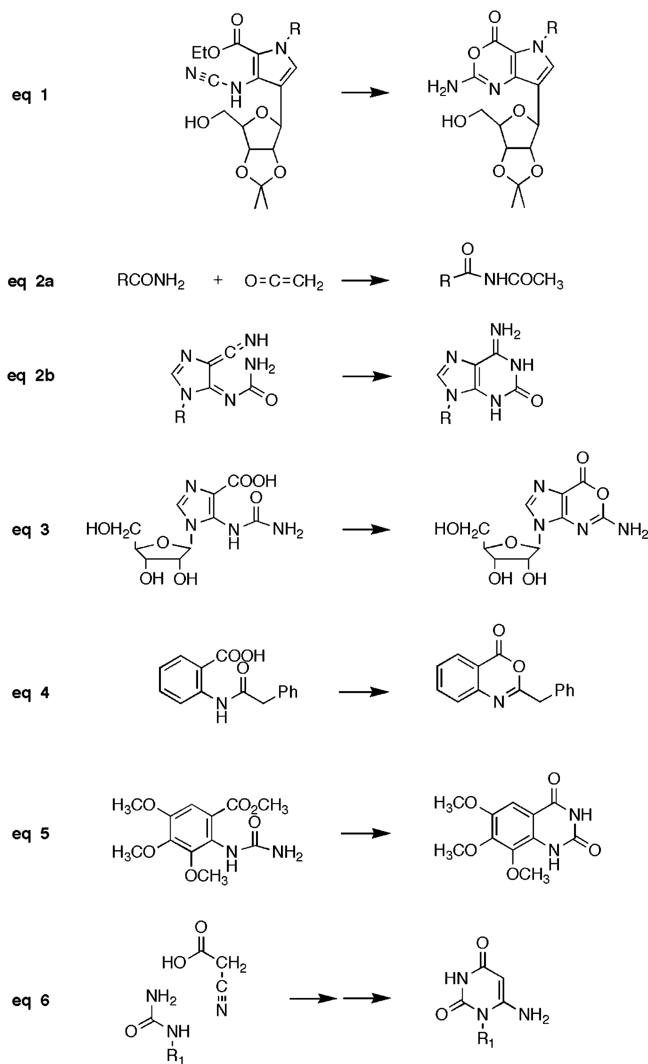
reaction path	3r isotopomers	4r isotopomers
	deamination of **1r** in ^{18}O-labeled water	
A		50% 6-(^{18}O)-**4r**, 50% [7-^{18}O]-**4r**
B	100% [2-^{18}O]-**3r**	100% 6-(^{18}O)-**4r**
C	50% [2-^{18}O]-**3r**, 50% [2,6-$^{18}O_2$]-**3r**	50% 6-(^{18}O)-**4r**, 50% [7-^{18}O]-**4r**
	deamination of [6-^{18}O]-**1r** in normal water	
A		50% 6-(^{18}O)-**4r**, 50% [7-^{18}O]-**4r**
B	100% [6-^{18}O]-**3r**	100% [7-^{18}O]-**4r**
C	50% [6-^{18}O]-**3r**, 50% **3r**	50% 6-(^{18}O)-**4r**, 50% [7-^{18}O]-**4r**

C−COOH bonds are fast.[39] Cyclization by addition of the carboxylic group to the cyanamide then results in the formation of equal amounts of 6-(^{18}O)-**4r** (label in the ring) and [7-^{18}O]-**4r** (exocyclic label). The synthesis of 3-deazaoxanosines by nucleophilic addition of esters to cyanamides has precedent (Scheme 5, eq 1).[40]

Addition of water to the cyanoimine leads to **7** (path B). Cyclization by addition of the urea amino group to the ketene could give [2-^{18}O]-**3r**, whereas the addition of the carbonyl oxygen would form 6-(^{18}O)-**4r**. The acetylation of amides by ketenes studied by Dunbar and White[41] provides a precedent for the ring closure of **7** to **3** (Scheme 5, eq 2a), and we provided evidence for a closely related system (Scheme 5, eq 2b).[42] There is no precedent for the ring closure of **7** to **4**.

The formation of **8** in $^{18}OH_2$ (path C in Scheme 3) would result in some doubly ^{18}O-labeled products. As with **6**, one would expect an equal distribution of the ^{18}O-label between the carbonyl-O and the hydroxyl-O in the carboxylic acid group of **8**. Nucleophilic attack of the carboxylic acid group at the urea's carbonyl carbon would form equal amounts of 6-(^{18}O)-**4r** and [7-^{18}O]-**4r**. The ^{18}O-label of the urea moiety is invariably lost if **4** is formed from **8**. The work by Luk and co-workers[43] provides precedent for the formation of **4** via mild cyclodehydration of carboxylic acids and ureas (Scheme 5, eq 3). Also, phenylacetylated anthranilic acid is known to cyclize to the oxanosine-type ring system benzooxazinone (Scheme 5, eq 4).[44] On the other hand, amide bond formation between the urea and the carboxylic acid group of **8** would form **3**. Such a ring closure would involve a tetrahedral intermediate with one ^{18}OH and one ^{16}OH group, and either one would be eliminated with (almost) equal probability to form (almost) equal amounts of [2-^{18}O]-**3r** (one ^{18}O-label) and [2,6-$^{18}O_2$]-**3r** (two ^{18}O-labels). This type of ring closure also has precedent. Hammen and Allen[45] synthesized quinazolinediones by way of the addition of the amino group of urea to esters (Scheme 5, eq 5), and a compound containing the oxanine-type ring system also was isolated. Pyrimidine ring formation by addition of ureas to carboxylic acids is well known (Scheme 5, eq 6).[46]

Scheme 5. Literature Precedent for Cyclizations Discussed in Schemes 3 and 4



Deamination of [6-^{18}O]-1r in Normal Water with Pyrimidine Ring Opening. The deamination of [6-^{18}O]-**1r** with pyrimidine ring opening would lead to **5**, and the ^{18}O-label would be at the ketene oxygen. The hydration of the ^{18}O-labeled intermediate **5** is discussed in Scheme 4. In analogy to the previous discussion, an equal distribution is expected for the ^{18}O-label in **6,** and ring closure would form equal amounts of 6-(^{18}O)-**4r** and [7-^{18}O]-**4r**. Again, **3r** cannot be produced via path A. Hydration of the cyanoimine would lead to **7** and on to [7-^{18}O]-**4r** and [6-^{18}O]-**3r**, respectively. The reaction of **4** would lead to equal amounts of 6-(^{18}O)-**4r** and [7-^{18}O]-**4r** and equal amounts of [6-^{18}O]-**3r** and **3r**.

Experimental Details

General Procedures. Most chemicals were purchased from Aldrich. 2-Amino-6-chloropurine riboside was purchased from Sigma (purity > 95%) and used without further purification. Adenosine deaminase (calf intestine) was purchased from Boehringer Mannheim in solution containing 50% glycerol (v/v) and 10 mM potassium phosphate. ^{18}O-

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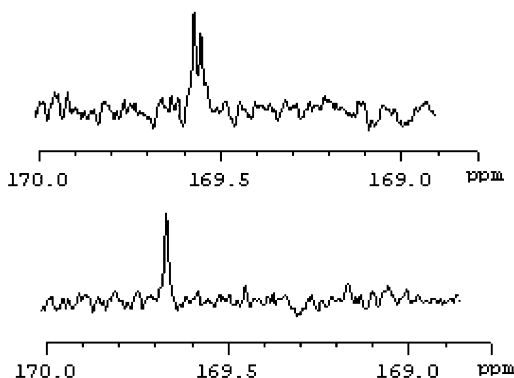


Figure 1. ^{13}C NMR spectra of pure [6-^{18}O]-**1r** (bottom) and a mixture of [6-^{18}O]-labeled and unlabeled **1r** (top). The upfield ^{18}O-isotopic shift of the C6 signal is 18.3 ppb (2.3 Hz). The chemical shift variations are due to pH variations; see Experimental Details.

Labeled water was obtained from Isotech Isotopes Inc. in 97.6% enrichment. HPLC-grade acetonitrile was obtained from Fisher. Triethylammonium acetate buffer (0.1 M) was prepared from glacial acetic acid and triethylamine, filtered through 0.45 μm filter paper under reduced pressure, and sonicated for 15 min before use. Triethylamine was purified by distillation over calcium hydride. Distilled water was filtered through 0.45 μm filter paper and sonicated before use.

Enzymatic Synthesis of [6-^{18}O]Guanosine with Adenosine Deaminase. A sodium phosphate buffer solution with pH = 7.0 was prepared in ^{18}O-labeled water. 2-Amino-6-chloropurine riboside (33 mM) was incubated at 37 °C with adenosine deaminase (50.0 μg, 10 unit) in 2.85 mL of phosphate buffer (0.1M, pH = 7.0) and 0.15 mL of dimethyl sulfoxide. DMSO was used to increase the solubility of **1r** at pH = 7.0. The reaction was monitored by TLC. **1r** began to precipitate after 20 min, and all starting material was consumed after 20 h. The reaction mixture was centrifuged to separate the precipitated **1r**. The supernatant liquid was discarded. **1r** was washed 3 times with 1 mL aliquots of water. The product was dried by lyophilization, and the final yield was 90%. The ^{13}C NMR spectrum of [6-^{18}O]-**1r** is shown in Figure 1.

Deamination of Guanosine. **1r** (12.49 mM) was incubated at 37 °C with $NaNO_2$ (124.9 mM) in 4 mL of sodium acetate buffer (3N, pH 3.7). The reaction was continued for 20−24 h to ensure that all of the **1r** had reacted. HPLC analysis of the reaction mixture indicated the formation of **3r** (t_R = 9.8) and **4r** (t_R = 14.2) and the absence of **1r**, and these observations were confirmed by LC/MS analysis. The reaction mixture was then separated by HPLC, the fractions were lyophilized to prevent any product decomposition, and ^{13}C NMR spectra were recorded.

HPLC Analyses and Preparation. The HPLC analyses were performed on a Shimadzu LC system that consisted of a LC-10ATvp pumping system, CTO-10Avp column oven, and a SPD-M10Avp photodiode array detector. The temperature of the column oven was set at 25 °C. The samples were injected with a SIL 10A autosampler, the initial mobile phase was 100% 0.1 M triethylammonium acetate buffer, and acetonitrile was then mixed into the mobile phase in such a way as to increase the acetonitrile concentration linearly from 0% to 10% over 20 min. For analytical purposes, a Supelcosil octadecylsilane column (25 × 4.6 mm i.d., 5 μm particle size) was used and the flow rate was 1 mL/min. The volume of sample injected was 10 μL. For semipreparative work, a Supelcosil octadecylsilane column (25 × 10 mm i.d., 5 μm particle size) was used and the flow rate was maintained at 4.73 mL/min. The volume of the sample injected was 142 μL. The fractions with the desired products were collected in multiple runs with a FRC-10A fraction collector. The samples were kept cool (−5 °C) by a sample cooler during the HPLC analysis to prevent decomposition.

LC/MS Analyses. The LC component consisted of a Finnigan P4000 pump, AS3000 autosampler, and UV6000 LP detector. The mobile phase initially was 100% 0.1 M triethylammonium acetate buffer, and an acetonitrile concentration was then built up linearly from 0% to 10% over 20 min. The flow rate was 1 mL/min, and the injection volume was 20 μL. The separations were carried out on a Supelcosil octadecylsilane column (25 × 4.6 mm i.d., 5 μm particle size). The LC was coupled to a TSQ 7000 triple-quadrupole mass spectrometer (Thermoquest, San Jose, CA), which was operated in the negative-ion electrospray ionization mode for analysis of the **1r** deamination products. The temperature of the heated capillary was 350 °C.

NMR Spectroscopy. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker Avance DRX500 spectrometer equipped with a 5 mm broadband probe. The probe temperatures were 298 and 288 K for **3r** and **4r** samples, respectively. A 30° excitation pulse was applied to record both ^{1}H and ^{13}C spectra. All ^{13}C NMR spectra were acquired with broadband ^{1}H decoupling. The carrier frequencies were 500.1330885 (^{1}H) and 125.7713108 MHz (^{13}C). Sweep widths were 10330 (^{1}H) and 39682 Hz (^{13}C). Repetition times were 3.78 (^{1}H) and 1.82 s (^{13}C). ^{1}H NMR spectra were recorded after 16 scans, while the number of scans for the ^{13}C NMR spectra ranged from 20k to 40k depending on sample concentration. In general, a line broadening of 0.20 (^{1}H) and 1 Hz (^{13}C) was applied. Data size after zero-fill ranged from 32K to 128K depending on the digital resolution required for each spectrum. The CD_3OD solvent signals at $\delta(^{1}H) = 3.30$ and $\delta(^{13}C) = 49.0$ were used as internal chemical shift standards. Chemical shifts are reported in parts per million (ppm). The isotopic shift is reported in parts per billion (ppb) and in Hertz. The peaks were assigned based on literature data.

Choice of the NMR Solvent. The isotope effect of ^{18}O vs ^{16}O on the ^{13}C NMR chemical shift is very small, only 2−5 Hz, and the choice of solvent was critical for their measurement. The carbonyl peaks in the ^{13}C NMR spectrum of **3r** were difficult to obtain in DMSO because of the solvent's high viscosity and the difficult relaxation of the carbonyl carbons. These carbonyl peaks could only be recorded allowing for longer relaxation time (6.65 s) but unfortunately were then too broad 30−40 Hz. The carbonyl peaks also appear upon addition of 50 mM relaxation reagent $Cr(acac)_3$ in DMSO, but these conditions resulted in extreme signal broadening (60 Hz). Methanol-d_4 was found to be the ideal solvent to measure the isotopic shifts; in this solvent very sharp peaks occurred with widths of only 2−4 Hz. While **4r** easily dissolved in methanol, **1r** and **3r** are insoluble in methanol and their solubility was increased by addition of ca. 50−200 μL of 1 N NaOH in D_2O. These pH variations cause the significant chemical shift differences in the ^{13}C NMR spectra of **1r** and **3r** (Figures 1, 4, and 7).

The ^{18}O-isotope shifts were measured as follows. The ^{13}C NMR spectrum of the ^{18}O-labeled compound was recorded first. Then a small quantity of unlabeled compound (ca. 1 mg) was added to the NMR tube, and a second spectrum was recorded. In the case of **3r**, NaOH was used as needed to make the sample clear for the NMR measurements.

Results

Deamination of 1r in (^{18}O)Water. The HPLC chromatogram of the reaction mixture is shown in Figure 2. The peaks with retention times of 9.8 and 14.2 min are **3r** and **4r**, respectively, and the small peak with a retention time of 10.7 min corresponds to unreacted **1r**. **3r** and **4r** have a molecular weight of 284. Negative-ion ESI−LC/MS gave a base peak with m/z = 285 for **3r** and **4r** (Figure 3) and shows the incorporation of one ^{18}O-label in both **3r** and **4r**. The small $[M-H]^-$ peak at m/z = 283 indicated trace amounts of unlabeled **3r** and **4r** in the reaction mixture (the enrichment of (^{18}O)water was 97.6%). There are no peaks in the mass spectrum of **3r** that would indicate the presence of two labels in the same ion.

The ^{13}C NMR spectrum of unlabeled **3r** was recorded and assigned as follows: δ 63.67 (C5′), 73.31 (C3′), 76.57 (C2′), 87.78 (C4′), 91.49 (C1′), 116.82 (C5), 138.02 (C8), 154.57 (C4),

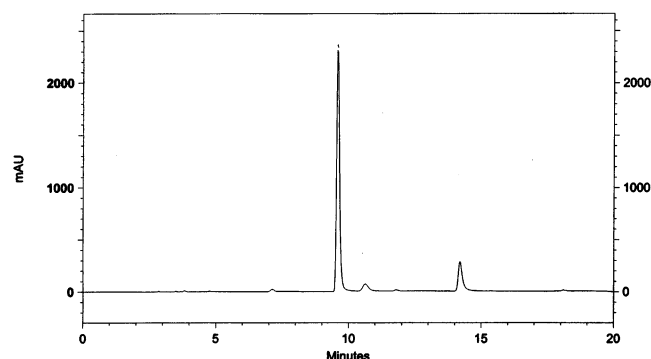


Figure 2. HPLC chromatogram of the deamination of **1r** in $^{18}OH_2$ shows the formations of **3r** and **4r**.

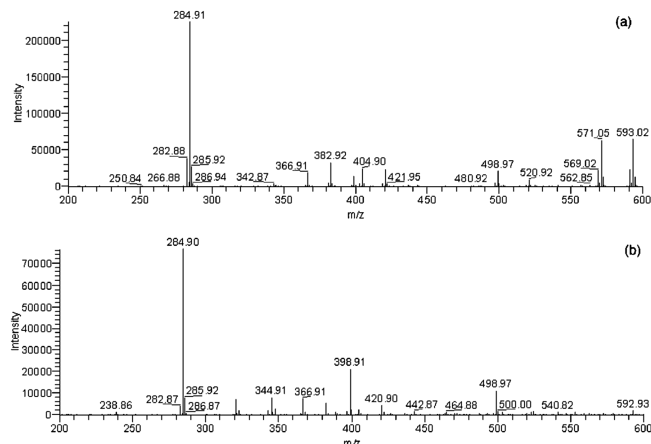


Figure 3. Mass spectra (ESI, negative-ion mode) of (a) **3r** and (b) **4r** obtained by deamination of **1r** in $^{18}OH_2$.

161.10 (C2), 162.35 (C6). The ^{13}C NMR spectrum of [2-^{18}O]-**3r** shows an ^{18}O-isotopic upfield shift of 22 ppb (2.75 Hz) for the C2 signal (Figure 4, bottom), [2-^{18}O]-**3r**. The small peak at the base of the C2 signal is due to the presence of small amounts of (unlabeled) **3r,** and addition of more **3r** increased this peak and confirmed the upfield shift (Figure 4, top).

The ^{13}C NMR spectrum of unlabeled **4r** was recorded and assigned as follows: δ 62.90 (C5′), 72.03 (C3′), 75.89 (C2′), 87.21 (C4′), 89.92 (C1′), 112.78 (C7a), 138.87 (C2), 154.23 (C3a), 155.80 (C5), 161.79 (C7). The ^{13}C NMR spectrum of ^{18}O-labeled **4r** is shown in the bottom in Figure 5 and features splittings of the C5 and C7 peaks at 155.80 and 161.79 ppm, respectively. Addition of unlabeled **4r** to the sample of labeled **4r** increased the downfield parts of both signals (top, Figure 5; Table 2).

One would expect four C signals for the C5 and C7 atoms for a mixture of 6-(^{18}O)-**4r** and [7-^{18}O]-**4r**. The in-ring label of 6-(^{18}O)-**4r** should cause upfield shifts for the C5 and C7 signals. The exocyclic label in [7-^{18}O]-**4r** should cause an upfield shift of C7 and less, if any, for C5. The interpretation of the C5 signal is thus rather straightforward in that the upfield signal is that of 6-(^{18}O)-**4r** and the downfield signal is that of [7-^{18}O]-**4r**. The intensity of the downfield signal increases on addition of **4r** and shows that the C5 isotopic shift in [7-^{18}O]-**4r** is negligible as expected. Interpretation of the C7 signal is slightly more involved. Upon addition of unlabeled **4r**, one might have expected three signals: one for C7 of unlabeled **4r** and two signals due to the isotopic shifts of the C7 atoms of 6-(^{18}O)-**4r** and [7-^{18}O]-**4r**. Yet, only two signals are observed, and the addition of **4r** merely increases (without much broadening) the downfield signal. Hence, the upfield resonance of the C7 signal is due to either 6-(^{18}O)-**4r** or [7-^{18}O]-**4r**, and the ^{18}O-label in the other isotopomer happens to leave the C7 shielding unaffected. We assign the C7 upfield-shifted signal to [7-^{18}O]-**4r** because the combination of the shorter C=O bond length with the larger electron density in the C=O bonding region make this bond more susceptible to a distance effect on shielding. Hence, the C5 upfield shift is 23 ppb (2.93 Hz), and it is due to the in-ring label in 6-(^{18}O)-**4r**. The C7 upfield shift is 18 ppb (2.25 Hz), and it is due to the exocyclic-O in [7-^{18}O]-**4r**. Finally, note that the upfield and downfield components of the C5 and C7 signals show about equal intensity, and we can conclude that 6-(^{18}O)-**4r** and [7-^{18}O]-**4r** are formed in about equal amounts.

Deamination of [6-^{18}O]-1r in Normal Water. The deamination of [6-^{18}O]-**1r** in normal water was carried out, and the reaction mixture was analyzed by HPLC, LC/MS, and ^{13}C NMR spectroscopy.

The HPLC chromatogram of the reaction mixture was similar to the one shown in Figure 2. Negative-ion ESI−LC/MS gave a base peak with m/z = 285 for **3r** and **4r** (Figure 6) and showed

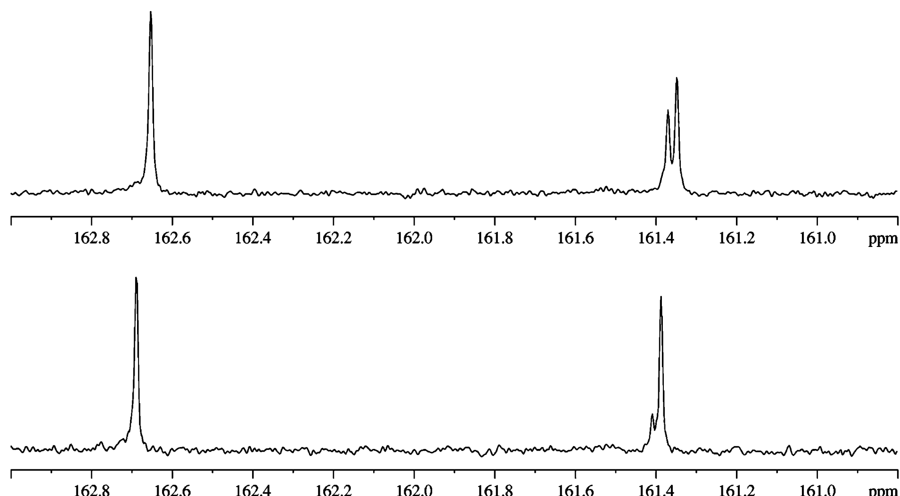


Figure 4. Deamination of **1r** in $H_2{}^{18}O$. The ^{13}C NMR spectrum of (almost fully) ^{18}O-labeled **3r** is shown on the bottom. The spectrum on top was recorded after addition of unlabeled **3r** to the sample of ^{18}O-labeled **3r**. The isotopic shift in the C2 signal is clearly observed. The chemical shift variations are due to pH variations; see Experimental Details.

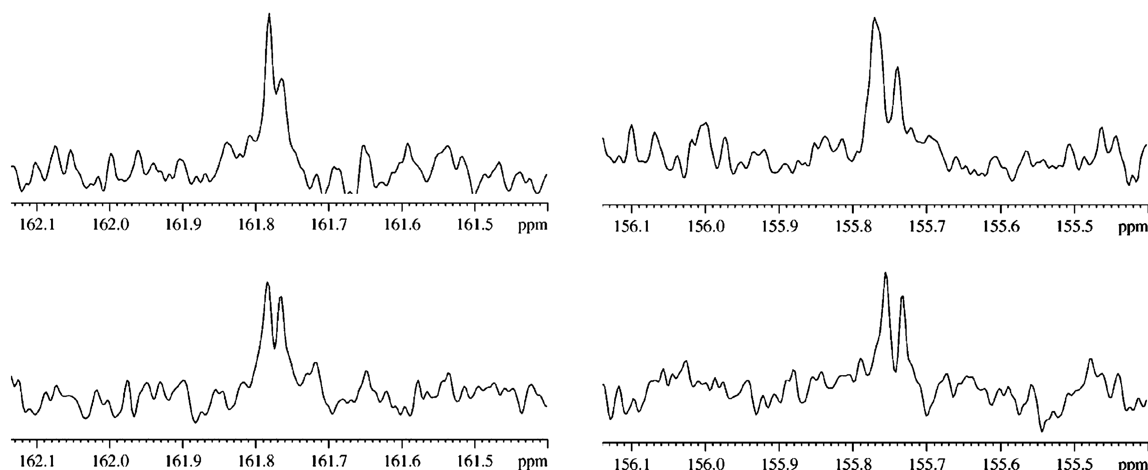


Figure 5. Deamination of **1r** in $H_2{}^{18}O$. The ^{13}C NMR spectrum of (almost fully) labeled **4r** shows isotopic shift in the resonance position of C5 (right) and C7 (left). The spectrum on top was recorded after the addition of unlabeled **4r** to the sample of labeled **4r**.

Table 2. Measured ^{18}O-Isotopic Shifts of ^{13}C Resonance

^{13}C-nucleus	experiment 1 **1r** and $^{18}OH_2$	experiment 2 [6-^{18}O]-**1r** and OH_2	average
C6 in [6-^{18}O]-**1r**		18.3 ppb 2.3 Hz	
C2 in [2-^{18}O]-**3r**	22 ppb 2.75 Hz		
C6 in [6-^{18}O]-**3r**		29 ppb 3.66 Hz	
C5 in 6-(^{18}O)-**4r**	23 ppb 2.93 Hz	22 ppb 2.75 Hz	22.5 ± 0.5 ppm 2.85 ± 0.10 Hz
C7 in 6-(^{18}O)-**4r**	ca. 0, not expected		
C5 in [7-^{18}O]-**4r**	ca. 0, as expected		
C7 in [7-^{18}O]-**4r**	18 ppb 2.25 Hz	13 ppb 1.70 Hz	15.5 ± 2.5 ppb 1.95 ± 0.25 Hz

the incorporation of one ^{18}O-label in both **3r** and **4r** as expected. The small $[M-H]^-$ peak at $m/z = 283$ is due to trace amounts of unlabeled **3r** and **4r**. The peak at $m/z = 321$ in the mass spectrum of **4r** corresponds to the adduct of $[M - H]^-$ with two water molecules.

The ^{13}C NMR spectrum of the labeled **3r** was recorded, and it featured an ^{18}O-isotopic upfield shift of 29 ppb (3.66 Hz) of the C6 resonance (Figure 7, bottom), [6-^{18}O]-**3r**. The small peak at the base of the C6 peak is due to small amounts of unlabeled **3r**, and this peak was shown to increase upon addition of unlabeled **3r** to the sample (Figure 7, top).

In the ^{13}C NMR spectrum of the labeled **4r** (Figure 8), an ^{18}O-isotope splitting occurs for the C5 and C7 peaks at 155.80 and 161.79 ppm, respectively. The addition of unlabeled **4r** tothe almost fully labeled sample increased the peak on the left. The spectra show all the same features as in the respective spectra shown in Figure 5, and their interpretation is the same. Hence, the C5 upfield shift is 22 ppb (2.75 Hz) due to the in-ring label, and the C7 upfield shift is 13 ppb (1.70 Hz) due to the exocyclic-O. As with the spectra in Figure 5, the upfield and downfield components of the C5 and C7 peaks show about equal intensity, corroborating that 6-(^{18}O)-**4r** and [7-^{18}O]-**4r** are formed in about equal amounts.

Discussion

The deamination of **1r** in $H_2{}^{18}O$ produced xanthosine molecules with only one label, [2-^{18}O]-**3r** (Figures 3 and 4). The formation of [2-^{18}O]-**3r** via **8** can be excluded because isotopomer [2,6-$^{18}O_2$]-**3r** is not formed. This finding is not surprising as one would expect the rates for the intramolecular cyclizations of **6** or **7** to be faster than the bimolecular addition of water to **6** or **7** to form **8**. If **8** is not observed and **3r** is not formed via **8**, it is in principle still possible that 6-(^{18}O)-**4r** and [7-^{18}O]-**4r** could be formed via **8**. Yet, the latter is not likely for kinetic reasons. While compounds of type **8** do cyclize (vide supra), these compounds do not play a role during deamination because **6** and **7** cyclize fast and path C is not important.

The deamination of [6-^{18}O]-**1r** in normal water gives [6-^{18}O]-**3r** as expected (Figures 6 and 7). **3r** (which is unlabeled) is not observed, and the implications are exactly the same as were drawn from the absence of [2,6-$^{18}O_2$]-**3r** in the other experiment.

It is clear that **3r** is not formed from **8**, and the question is now whether xanthosine is formed from **5** or by direct nucleophilic substitution without pyrimidine ring opening. **3r** could be formed via pyrimidine ring opening only if the hydration of **5** followed path B. The question as to whether path B plays a significant role can be addressed via the relative yields of 6-(^{18}O)-**4r** and [7-^{18}O]-**4r**. This ratio is unity (a) if only path A is operative or (b) if paths A *and* B are traveled and path B produced exclusively **3r** (Table 1). It is reasonable to expect a preference for the reaction **7** → **3r** (N-nucleophile) over the reaction **7** → **4r** (O-nucleophile). It is also reasonable to expect a kinetic advantage for the reaction **5** → **6** (diffusion controlled[27]) over the reaction **5** → **7**, and this must be true since the overall yield of xanthosine exceeds the yield of oxanosine. The ^{13}C NMR spectra of **4r** (Figures 5 and 8) produced by deamination of **1r** in $H_2{}^{18}O$ and of [6-^{18}O]-**1r** in normal water both show that almost equal amounts of 6-(^{18}O)-**4r** and [7-^{18}O]-**4r** are formed. Hence, the deduction can be made with certainty that path A is the dominant path for the formation of **4r**, while the NMR data do not allow small contributions from path B to be excluded. The dominance of path A is entirely consistent with the results of our recent studies of the electronic structures of **5**.[25,27] Electronic structure analysis of **5** shows that the zwitterionic resonance form **5−2** dominates over the fulvene-type structure **5−1** (Scheme 6). The anionic character of the imino-*N* in **5** leads to preferred imino-*N* protonation and converts what is already a latent acylium moiety into an extremely electrophilic actual acylium group. Thus, **5** is perfectly set up for protonation at the imino-*N* and nucleophile addition at the ketene C atom,

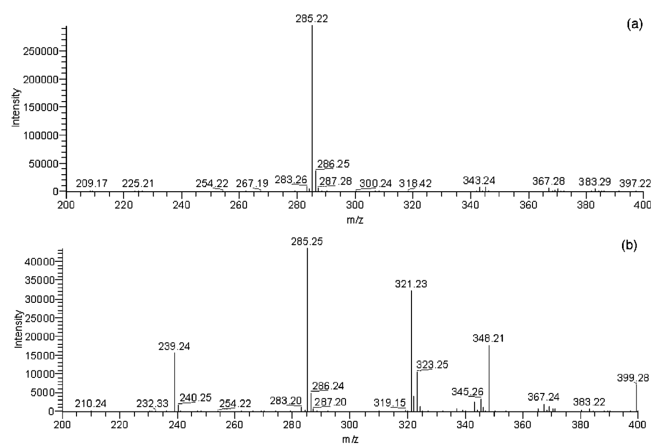


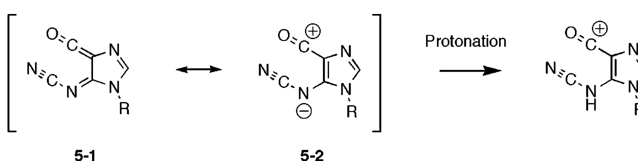
Figure 6. Mass spectra (ESI, negative-ion mode) of (a) **3r** and (b) **4r** after deamination of [6−^{18}O]-**1r** in water.

and the product of the 1,4-addition contains an aromatic imidazole ring.

Conclusion

The labeling experiments demonstrate that the oxanosine formation in nitrosative guanosine deamination proceeds via the

Scheme 6. Resonance Structures of **5** and *N*-Protonation



5-cyanoimino-4-oxomethylene-4,5-dihydroimidazole **5** and 5-cyanoamino-4-imidazolecarboxylic acid **6**. The carbodiimide tautomer **6′** of the cyanoamine **6** might be involved in the cyclization. This hydration of **5** by way of 1,4-addition is much faster than guanidine formation by water addition to the cyanoimine group. These experimental findings fully corroborate theoretical studies of the electronic structure of 5-cyano-imino-4-oxomethylene-4,5-dihydroimidazoles and of their *N*-protonation.[25] The cyclization of **6** is fast compared to a second water addition, and 5-guanidinyl-4-imidazolecarboxylic acid, **8**, does not play a role in nitrosative guanosine deamination. The studies demonstrate for the first time the intermediacy of 5-cyanoamino-4-imidazolecarboxylic acid **6** in the nitrosative guanosine deamination on the path to oxanosine. The significance of this discovery increases with the realization that oxanosine formation is not the only reaction option for **6** inside of DNA. Hence, we

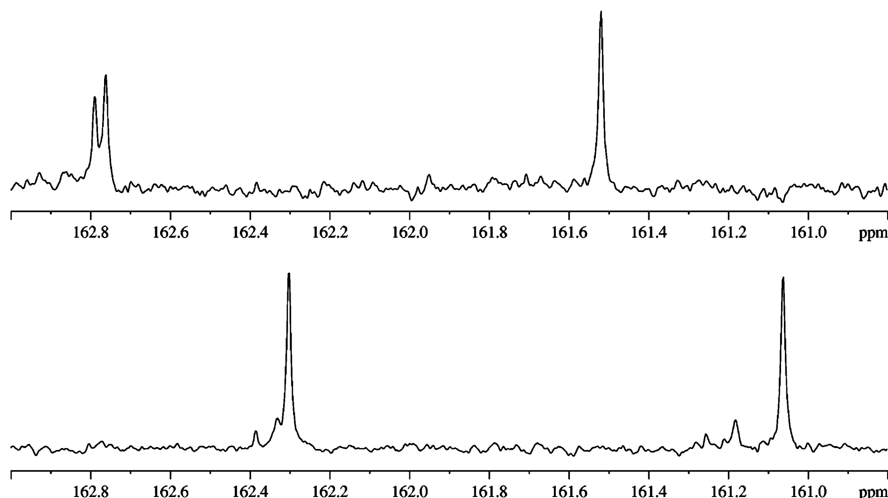


Figure 7. Deamination of [6-^{18}O]-**1r** in normal water. The ^{13}C NMR spectrum of (almost fully) labeled [6-^{18}O]-**3r** shows isotopic shift for the C-6 signal (bottom). The spectrum shown on top was recorded after addition of some unlabeled **3r** to the sample of [6-^{18}O]-**3r**. The chemical shift variations are due to pH variations; see Experimental Details.

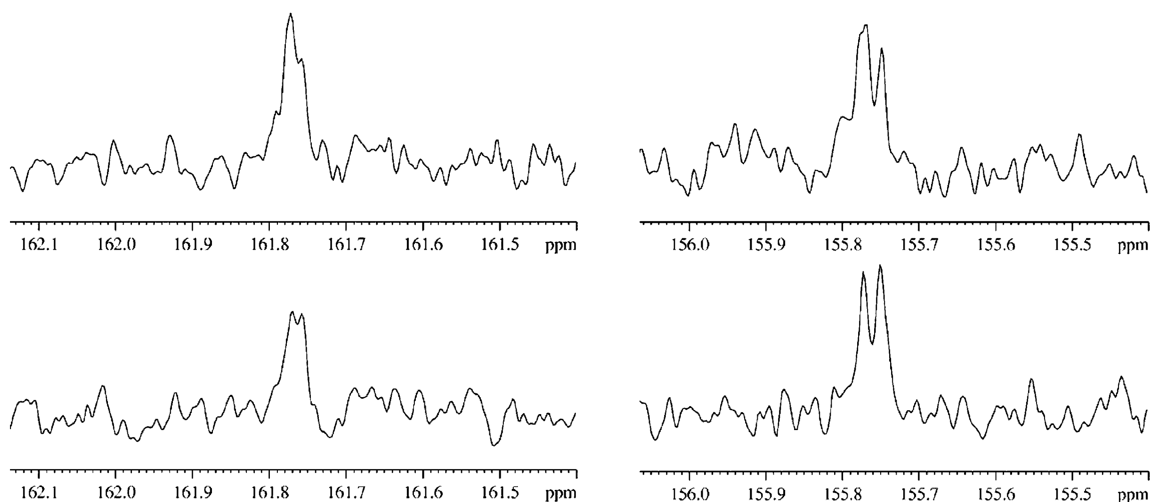
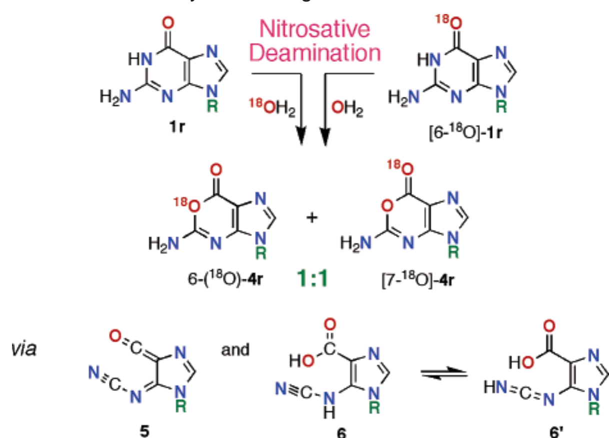


Figure 8. Deamination of [6−^{18}O]-**1r** in normal water. The ^{13}C NMR spectrum of (almost fully) labeled **4r** (bottom) shows isotopic shifts of the C5 (right) and C7 (left) signals. The spectrum on top was recorded after addition of unlabeled **4r** to the sample of labeled **4r**.

Scheme 7. Summary of Labeling Studies



are now exploring the chemistry of **6** and some pertinent derivatives (Scheme 7).

The results presented are the results of mechanistic studies in *homogeneous solution,* and one needs to keep in mind that the chemistry in the *anisotropic environment of DNA* may differ in a variety of ways, and these include the availability of water and the molecularity of the water addition, the possibility of changes in mechanism within DNA, as well as issues of rotational flexibility. (1) The present results show that **5** is not the one and only common intermediate for the formation of xanthosine and oxanosine in homogeneous solution, and the dediazoniation of the guanosinediazonium ion in solution therefore cannot be strictly unimolecular (at least for the xanthosine-forming reaction). For the heteroaromatic diazonium ion, the rates for the two reaction channels are dependent on the water concentration, and this dependence might affect product ratios. In particular, any such bimolecular process in DNA would be influenced by solvent accessibility. (2) While the present results show that most of the xanthosine is formed by nucleophilic aromatic substitution in homogeneous solution, the extrapolation that the same chemistry occurs in DNA is not justified based on current knowledge. In contrast, the currently available theoretical evidence[26] suggests that deprotonation occurs before dediazoniation in DNA, and this would require xanthosine formation via intermediates **5** and **7**. (3) Another important way in which the DNA environment would affect the labeling experiments concerns the isotopomer ratios because the assumption of free rotation about the C−COOH bonds would certainly no longer hold. With the new enzymatic synthesis of [6-^{18}O]-**1r**, we are now studying deamination in oligonucleotides with labeled guanosine to explore such effects of the anisotropic environment.

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