Mechanism of Action of (2-Haloethyl)nitrosoureas on DNA: Discrimination between Alternative Pathways of DNA Base Modification by 1,3-Bis(2-fluoroethyl)-1-nitrosourea by Using Specific Deuterium Labeling and Identification of Reaction Products by HPLC/Tandem Mass Spectrometry

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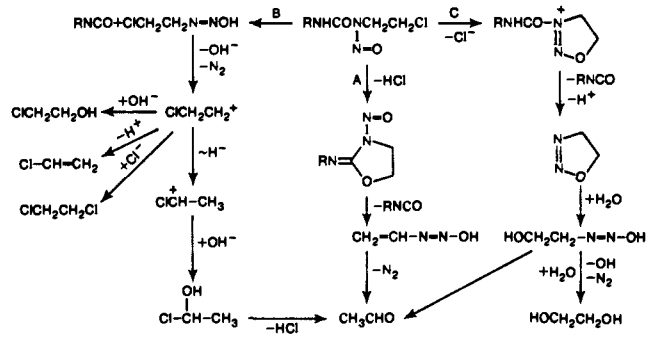
Abstract: Reaction of deoxyguanosine with 1,3-bis(2-fluoro-1,1-dideuterioethyl)-1-nitrosourea in Tris or cacodylate buffer, pH 7.1, at 37 °C affords, among the products, 7-(2-fluoro-2,2-dideuterioethyl)deoxyguanosine and 7-(2-hydroxy-2,2-dideuterioethyl)deoxyguanosine as shown by HPLC/MS/MS analysis. A similar reaction in the presence of 10 molar equiv of KBr resulted in the formation, in addition to the two modified nucleosides, of 7-(2-bromo-2-monodeuterioethyl)deoxyguanosine, in accord with the formation of an intermediate species in which the fluorine is rendered labile and exchangeable. Formation of the monodeuterated bromo derivative and comparable monodeuterated hydroxy and fluoro derivatives is consistent with generation of a monodeuterated diazoalkane deoxyguanosine species, promoted by the high ionic strength conditions, and trapping of the latter by the counterion. In contrast, BFNU is inert to attempted halide exchange in the absence of deoxyguanosine. These and other data are consistent with the initial formation of a tetrahedral intermediate from reaction of O^6-dG with BFNU or its iminourea. The results rule out a suggested mechanistic pathway via nucleophilic displacement directly on the tetrahedral intermediate. However, the results are in accord with an alternative pathway in which the tetrahedral intermediate cyclizes to an *N*-nitrosooxazolidine, followed by nucleophilic opening at the 5-position by N7-guanine. In principle, the results of the deuterium-labeling experiments are also consistent with an "anchimeric" pathway in which the initially formed O^6-dG tetrahedral intermediate cyclizes to a 1,2,3-oxadiazolinium species that undergoes subsequent nucleophilic opening at the 5-position. However, the contribution of the anchimeric pathway is likely to be minimal in view of estimates of ca. 20% contribution on the basis of experiments with BCNU-*2,2,2′,2′-d_4-N*-^{18}O in conjunction with in situ reduction of the released carbonyl compounds by liver alcohol dehydrogenase.

(2-Haloethyl)nitrosoureas (HENUs),[1] including 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU), 1,3-bis(2-fluoroethyl)-1-nitrosourea (BFNU), 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU), 1-(2-chloroethyl)-3-(4-*trans*-methylcyclohexyl)-1-nitrosourea (MeCCNU), and chlorozotocin, have an established place in the clinical treatment of a range of human malignancies, including Hodgkin's disease, Burkitt's lymphoma, and cerebral neoplasms.[2-4] Unlike other anticancer agents, HENUs and aryltriazenes such as DTIC decompose spontaneously under physiological conditions to give rise to electrophiles that react with sensitive cellular macromolecules.[2,3,5,6] Several lines of evidence indicate that DNA is one of the principal cell targets,[2,3] and specific lesions have been identified.[6-10] These include alkylation of both base and phosphate residues,[10] interstrand cross-linking,[7,8] and single-strand breaks[10] of two types corresponding to the two types of alkylation. Studies on the biochemical pharmacology of the HENUs support the view that, of these, the critical and lethal lesions are associated with alkylation of the DNA bases, including certain interstrand cross-links,[7,8,11] in part because they are difficult

Scheme I. Suggested Competing Pathways of Spontaneous Aqueous Decomposition of (2-Haloethyl)nitrosoureas



(1) The abbreviations used are as follows: HENU, 1-(2-haloethyl)-1-nitrosourea; BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; BFNU, 1,3-bis(2-fluoroethyl)-1-nitrosourea; CCNU, 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea; MeCCNU, 1-(2-chloroethyl)-3-(4-*trans*-methylcyclohexyl)-1-nitrosourea; dG, deoxyguanosine; ClEt, 2-chloroethyl group; HOEt, 2-hydroxyethyl group; FEt, 2-fluoroethyl group; MPE, (methidiumpropyl-EDTA)iron(II); SoSo, 2-chloroethyl (methylsulfonyl)methanesulfonate; DTIC, 5-(3,3-dimethyl-1-triazenyl)-1*H*-imidazole-4-carboxamide; API, atmospheric pressure ionization; CAD, collision-activated decomposition.

(2) Ludlum, D. B. In *Cancer, a Comprehensive Treatise*; Becker, F. F., Ed.; Plenum Press: New York, 1975; Vol. 5, p 285.

(3) Wheeler, G. P. *A Review of Studies on the Mechanism of Action of Nitrosoureas*; ACS Symposium Seres 30; American Chemical Society: Washington, DC, 1976; p 87.

(4) (a) Weinkam, S. J.; Lu, H.-S. *Adv. Pharmacol. Chemother.* **1982**, *19*, 1-33. (b) Hemminki, K.; Ludlum, D. B. *JNCI, J. Natl. Cancer Inst.* **1984**, *73*, 1021. (c) Proceedings of the 7th New Drug Symposium on Nitrosoureas. In *Cancer Treat. Rep.* **1976**, *60*, 651.

(5) Brundrett, R. B.; Cowens, J. W.; Colvin, M. *J. Med. Chem.* **1976**, *19*, 958.

(6) Lown, J. W. In *New Approaches to the Design of Antineoplastic Agents*; Bardos, T. J., Kalman, T. I., Eds.; Elsevier: Amsterdam, The Netherlands, 1982; p 61.

(7) Kohn, K. W. *Cancer Res.* **1977**, *37*, 1450.

(8) Lown, J. W.; McLaughlin, L. W.; Chang, Y.-M. *Bioorg. Chem.* **1978**, *7*, 97.

(9) (a) Tong, W. P.; Ludlum, D. B. *Biochem. Pharmacol.* **1979**, *28*, 1135. (b) Tong, W. P.; Kirk, M. C.; Ludlum, D. B. *Cancer Res.* **1982**, *42*, 3102. (c) Gombar, C. T.; Tong, W. P.; Ludlum, D. B. *Biochem. Biophys. Res. Commun.* **1979**, *90*, 878. (d) Parker, S.; Kirk, M. C.; Ludlum, D. B.; Koganty, P. R.; Lown, J. W. *Biochem. Biophys. Res. Commun.* **1986**, *139*, 31. (e) Nibbering, N. M. M. In *High Performance Mass Spectrometry: Chemical Applications*; Gross, M. L., Ed.; ACS Symposium Series 70; American Chemical Society: Washington, DC, 1978; p 80. Theissling, C. B.; Nibbering, N. M. M.; de Boer, T. J. *Adv. Mass Spectrom.* **1971**, *5*, 642. Venema, A.; Nibbering, N. M. M.; de Boer, T. J. *Org. Mass Spectrom.* **1970**, *3*, 583.

(10) Lown, J. W.; McLaughlin, L. W. *Biochem. Pharmacol.* **1979**, *28*, 1631.

(11) Erickson, L. C.; Bradley, M. O.; Ducore, J. M.; Ewig, R. A. G.; Kohn, K. W. *Proc. Natl. Acad. Sci. U.S.A.* **1980**, *77*, 467.

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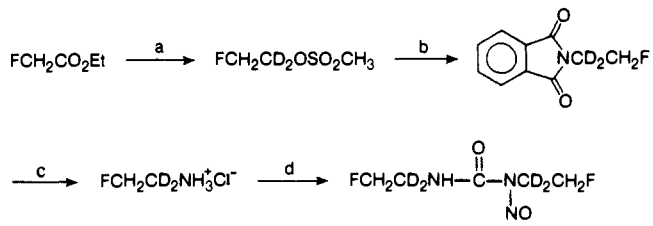
Scheme II[a]



[a] Reaction conditions: (a) $LiAlD_4$, Et_2O, then CH_3SO_2Cl, Et_3N, CH_2Cl_2; (b) potassium phthalimide, DMF, 130–140 °C; (c) NH_2NH_2 in EtOH, then HCl, (d) $COCl_2$, Et_3N, then $NaNO_2$, HCOOH, 0 °C.

to repair.[11] It is therefore essential to determine the mechanism of reaction of HENUs with DNA bases and to attempt to account for the observed sequence and site selectivity of the reaction.[12]

Progress in understanding the details of the mechanism of action of these valuable clinical agents has been hampered by the extraordinary chemical complexity of HENUs in solution.[5,6,13-19] Consequently, the pathways leading to the formation of the critical lesions, e.g., the interstrand cross-link between cytosine and guanine,[9a] have been by no means evident. It has become clear, however, that the events ultimately responsible for the biological activity of HENUs may well occur before or during the spontaneous aqueous decomposition; these include conformational changes,[18] NH proton abstraction by base from the HENU, and the formation of transient tetrahedral intermediates by reversible hydration of the carbonyl group.[19] These events are governed by the type and nature of the HENU, pH, temperature, and the nature and polarity of the microenvironment.

At least three pathways of decomposition have been suggested[19b] for which supporting experimental evidence has been provided (Scheme I). Invoking stereoelectronic control in the decomposition of HENUs, which requires that available lone pairs on two heteroatoms in the tetrahedral intermediates be aligned antiperiplanar to the bond to be broken,[20] accounts satisfactorily for the plethora of products identified.[19b] Despite these advances in our understanding, several questions remain unanswered, especially with regard to the key steps leading to the modification of the individual DNA bases.

A controversy has arisen with regard to the interpretation of the marked sequence selectivity of DNA modification by anticancer alkylators, with advocates presenting arguments for alternative explanations on the basis of (a) regioselective alkylation controlled by intramolecular events on the DNA[21] and (b) chemoselection on the basis of global DNA electrostatics[22a] directing an incoming positively charged species.[12a-f,22b] Some of the suggested mechanisms[21] are amenable to testing by appropriate physical–organic techniques. Accordingly, we report a study of the reaction of BFNU-*1,1,1',1'*-d_4 with deoxyguanosine under various experimental conditions, which permits discrimination between some alternative mechanisms of DNA base modification that have been proposed. Evidence based on HPLC/MS/MS analysis of reaction products[23] for a clean molecular rearrangement during formation of the critical lesions 7-(2-fluoroethyl)deoxyguanosine and 7-(2-hydroxyethyl)deoxyguanosine was obtained, which has a direct bearing on the above-mentioned controversy. The placement of the deuterium labels also permitted the detection of an elusive diazoalkane intermediate under certain experimental conditions.

Synthesis of Deuterium-Labeled BFNUs. Reduction of ethyl fluoroacetate with lithium aluminum deuteride in ether followed by treatment with methanesulfonyl chloride gave the deuterated fluoroethyl methanesulfonate. Successive treatment with potassium phthalimide, hydrazine, and hydrochloric acid afforded the labeled fluoroethylamine hydrochloride. Treatment of the latter with phosgene and nitrosation in formic acid afforded BFNU-*1,1,1',1'*-d_4 as outlined in Scheme II.

Brief Description of LC/MS/MS Methodology Used. The proofs of structure given under Results are based on LC/MS/MS apparatus and methodology. The LC/MS interface utilized in this instrument is relatively novel and needs to be described. The LC effluent is vaporized in a heated nebulizer[23a,b] by passing the liquid through a heated capillary and entraining the vapor and liquid droplets escaping the capillary tip with heated N_2 gas at near atmospheric pressures. The resulting gas mixture is exposed to atmospheric pressure ionization (API).[23c] In LC/API, the gasified analytes, all of which are nitrogen bases M, are ionized by protonation with reagent ions NH_4^+ produced by the electric gas discharge in the presence of NH_3 originating from the LC buffer. The protonation reaction

$$NH_4^+ + M \rightleftharpoons MH^+ + NH_3$$

is of relatively low exothermicity and therefore fragmentation of MH^+ is generally not expected. The API-generated ions contained in this atmospheric gas mixture are transferred to vacuum, and mass analysis is obtained with a quadrupole, Q_1.

When tandem mass spectrometry is employed (MS/MS), structural information about the ion MH^+ (or other ions) can be obtained with collision-activated decomposition (CAD) of MH^+.[23d] In this technique, the ion mass selected with the first quadrupole Q_1 and accelerated by a given potential V is exposed to collisions with a gas (Ar) in a second quadrupole, Q_2, which is operated so as to transmit all ions. The fragment ions produced by the collisional breakup of MH^+ (fragment ions) and undecomposed MH^+ are mass-analyzed in a third quadrupole, Q_3. The resulting

(12) (a) Hartley, J. A.; Gibson, N. W.; Kohn, K. W.; Mattes, W. B. *Cancer Res.* **1986**, *46*, 1943. (b) Mattes, W. B.; Hartley, J. A.; Kohn, K. W. *Nucleic Acids Res.* **1986**, *14*, 2971. (c) Hartley, J. A.; Lown, J. W.; Mattes, W. B.; Kohn, K. W. *Acta Oncol.* **1988**, *27*, 503. (d) Kohn, K. W.; Hartley, J. A.; Mattes, W. B. *Biochem. Pharmacol.* **1988**, *37*, 1799. (e) Briscoe, W. T.; Duarte, S. P. *Biochem. Pharmacol.* **1988**, *37*, 1061. (f) Wurdeman, R. L.; Gold, B. *Chem. Res. Toxicol.* **1988**, *1*, 146. (g) Richardson, K. K.; Richardson, R. C.; Crosby, R. M.; Swenberg, J. A.; Skopek, T. R. *Proc. Natl. Acad. Sci. U.S.A.* **1987**, *84*, 344. (h) Zarbl, H.; Sukumar, S.; Arthur, A. V.; Martin-Zanca, D.; Barbacid, M. *Nature (London)* **1985**, *315*, 382.

(13) Lown, J. W.; Chauhan, S. M. S. *J. Med. Chem.* **1981**, *24*, 270.

(14) Weinkam, R. J.; Lin, H. S. *J. Med. Chem.* **1979**, *22*, 1193.

(15) Lown, J. W.; Chauhan, S. M. S. *J. Org. Chem.* **1981**, *46*, 2479.

(16) (a) Montgomery, J. A.; James, R.; McCaleb, G. S.; Johnston, T. P. *J. Med. Chem.* **1963**, *6*, 669. (b) Johnston, T. P.; McCaleb, G. S.; Opliger, P. S.; Montgomery, J. A. *J. Med. Chem.* **1966**, *9*, 892. (c) Montgomery, J. A.; James, R.; McCaleb, G. S.; Johnston, T. P. *J. Med. Chem.* **1967**, *10*, 668. (d) Brookes, P.; Lawley, P. D. *J. Chem. Soc.* **1961**, 3923.

(17) Chatterjii, D. C.; Greene, R. F.; Galleti, J. F. *J. Pharm. Sci.* **1978**, *67*, 1527.

(18) (a) Lown, J. W.; Chauhan, S. M. S.; Koganty, R. R.; Sapse, A.-M. *J. Am. Chem. Soc.* **1984**, *106*, 6401. (b) Sapse, A.-M.; Allen, E. B.; Lown, J. W. *J. Am. Chem. Soc.* **1988**, *110*, 5671. (c) Lown, J. W.; Chauhan, S. M. S. *Tetrahedron Lett.* **1981**, *22*, 401. (d) Lown, J. W.; Chauhan, S. M. S. *J. Org. Chem.* **1983**, *48*, 3901.

(19) (a) Snyder, J. K.; Stock, L. M. *J. Org. Chem.* **1980**, *45*, 1990. (b) Lown, J. W.; Chauhan, S. M. S. *J. Org. Chem.* **1981**, *46*, 5309.

(20) Deslongchamps, P. *Tetrahedron* **1975**, *31*, 2463. Deslongchamps, P. *Stereoelectronic Effects in Organic Chemistry*; Pergamon: New York, 1983.

(21) (a) Buckley, N. *J. Am. Chem. Soc.* **1987**, *109*, 7918. (b) Buckley, N. *J. Org. Chem.* **1987**, *52*, 484. (c) Buckley, N.; Brent, T. P. *J. Am. Chem. Soc.* **1988**, *110*, 7520. (d) A reviewer has pointed out that this mechanism was proposed[21a-c] for regioselective reactions at DNA and that the displacement may occur in duplex B-DNA because of the more favorable entropy for the DNA reaction. Figures 6–8 are modified versions of published figures[21b,c] representing events on B-DNA.

(22) (a) Pullman, A.; Pullman, B. *Q. Rev. Biophys.* **1981**, *14*, 289. (b) Strictly speaking, the paper of Wurdemann and Gold (ref 12f) refers to the sequence-specific alkylation of DNA by *N*-alkyl-*N*-nitrosoureas.

(23) (a) Henion, J. D.; Thomson, B. A.; Dawson, P. H. *Anal. Chem.* **1982**, *54*, 451. Covey, T. R.; Lee, E. D.; Henion, J. D. *Anal. Chem.* **1986**, *58*, 2453. (b) Alexander, J. A.; Kebarle, P.; Fuciarelly, A. F.; Raleigh, J. A. *Anal. Chem.* **1987**, *59*, 2484. (c) Sunner, J.; Nicol, G.; Kebarle, P. *Anal. Chem.* **1988**, *60*, 1300. Sunner, J.; Ikonomou, M. G.; Kebarle, P. *Anal. Chem.* **1988**, *60*, 1308. (d) Johnson, J. V.; Yost, R. A. *Anal. Chem.* **1985**, *57*, 758A–768A. (e) Whitehouse, C. M.; Dreyer, R. N.; Yamashita, M.; Fenn, J. B. *Anal. Chem.* **1985**, *57*, 675. Bruins, A. P.; Covey, T. R.; Henion, J. D. *Anal. Chem.* **1987**, *59*, 2642. Ikonomou, M. G.; Blades, A. T.; Kebarle, P. *Anal. Chem.*, submitted for publication. (f) A reaction time of 0.5 s is evaluated for 5% decomposition of 7-(2-fluoroethyl)guanine with the assumption that the preexponential factor is $A = 10^{15}$ s^{-1} (see Table X1.5 of: Benson, S. W. *The Foundations of Chemical Kinetics*; McGraw-Hill: New York, 1960) and the activation energy $E_A = 60$ kcal/mol.

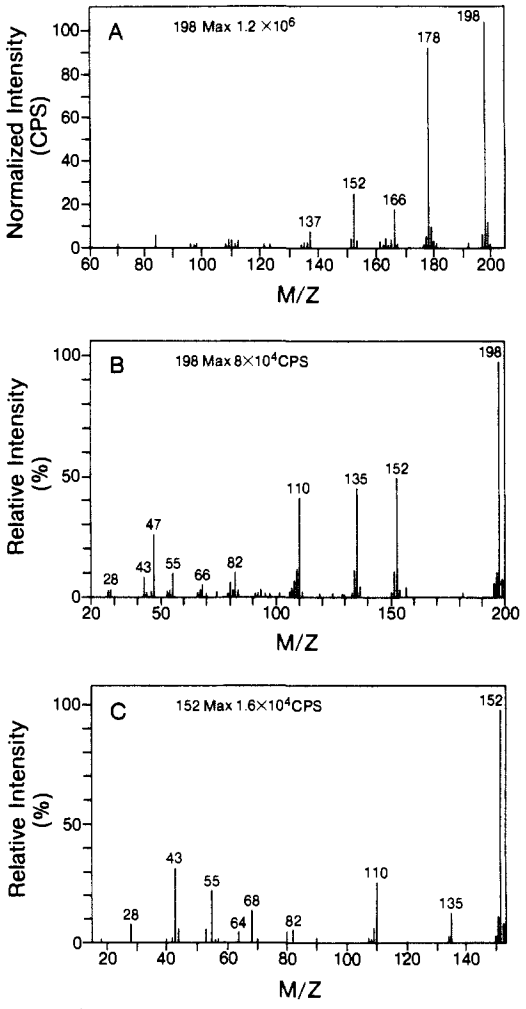


Figure 1. (A) Positive-ion API mass spectrum of authentic 7-(2-fluoroethyl)guanine after background subtraction obtained under LC/MS conditions [1 nmol of 7-(2-fluoroethyl)guanine dissolved in 10% MeOH and 0.1 M NH_4Ac was injected via the column under the conditions described in the Experimental Section]. (B) LC/MS/MS CAD fragment ion mass spectrum of protonated 7-(2-fluoroethyl)guanine (MH^+, m/z 198). (C) LC/MS/MS CAD fragment ion spectrum of protonated guanine (BH_2^+), m/z 152, obtained from flow-injection analysis of an authentic sample of 10 nmol of guanine dissolved in H_2O (LC/MS conditions as described in the Experimental Section).

CAD spectrum can be used for the deduction of structural features of MH^+ in a manner similar to that used for the interpretation of electron-impact mass spectra.[23d]

In the present study, a triple-quadrupole instrument employing the type of interface described above was used to characterize products of the reaction of deoxyguanosine with BFNU or BFNU-*1,1,1',1'-d*$_4$. Products of all reactions were hydrolyzed prior to HPLC/MS/MS analysis in order to release the modified bases from the deoxyguanosine nucleosides.

Results

API and CAD Mass Spectra of Authentic 7-(2-Fluoroethyl)-guanine Samples. The positive-ion API spectrum of authentic 7-(2-fluoroethyl)guanine obtained under LC/MS conditions is shown in Figure 1A. Four significant ions that are crucial in this work are clearly shown in the spectrum. These are the protonated molecular ion MH^+ at m/z 198 and the fragment ions at m/z 178 corresponding to loss of HF from MH^+, m/z 166 corresponding to loss of CHF from MH^+, and m/z 152, which corresponds to the mass of the protonated guanine base and corresponds to loss of C_2H_3F from MH^+.

The mechanisms by which the ions m/z 178, 166, and 152 originate need to be discussed. In a separate experiment, the MH^+ ion was obtained from solutions of 7-(2-fluoroethyl)guanine by using not a heated nebulizer but electrospray as the interface to the mass spectrometer. Electrospray is a very mild form of ionization by which ions present in solution are transferred to the gas phase at ambient temperature.[23e] With this technique, only the one ion, MH^+, was observed. This experiment shows that the fragments m/z 178, 166, and 152 are probably due to the high temperature associated with the nebulizer.

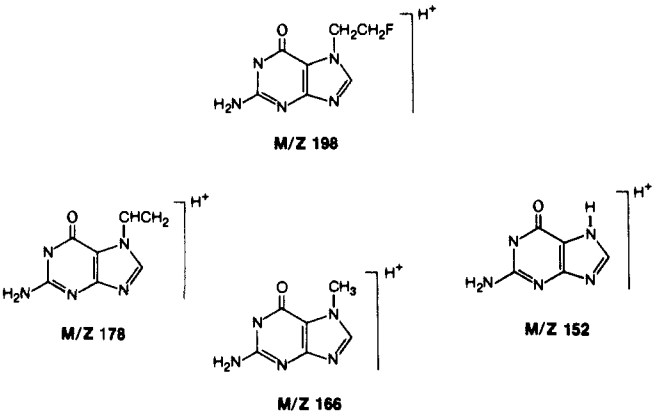


Figure 2. Structures of MH^+ and other characteristic ions resulting from the HPLC/API-MS analysis of 7-(2-fluoroethyl)guanine.

Two alternatives can be considered: pyrolysis of MH^+ to yield the ions m/z 178, 166, and 152 or pyrolysis of neutral M in the heated nebulizer followed by API protonation of neutral pyrolysis products. As a guide to decomposition of vibrationally excited MH^+, one can use the MS/MS collision-activated decomposition (CAD) fragment spectrum of MH^+; see Figure 1B. This spectrum was obtained with MH^+ produced with the nebulizer, but essentially identical spectra were obtained with electrospray-generated MH^+. The m/z 152 ion is seen to be the only important high-mass fragment, and m/z 178 and 166 are completely absent. The formation of m/z 152 from MH^+ corresponds to production of the stable product fluoroethylene, and this decomposition can be considered likely. On the other hand, the m/z 166 corresponds to formation of CHF, a high-energy product, and this decomposition pathway is very unlikely for vibrationally excited MH^+, an expectation that agrees with the absence of m/z 166 from the MH^+ CAD spectrum.

The above results suggest that the ion 166 and probably also 178 and 152 in the LC/MS spectrum (Figure 1A) are not due to pyrolytic decomposition of MH^+ but are due to pyrolysis of neutral M in the hot zones of the nebulizer. The ion at m/z 178 could be due to loss of HF from the neutral M, followed by protonation of the neutral reaction product in the API source. Similarly, the m/z 166 could be due to cleavage of the ethyl C–C bond and formation of CH_2F and an $(M - CH_2F)$ free radical that subsequently abstracts an H atom from gas molecules like CH_3OH. The product of this reaction will be 7-methylguanine. Protonation of this product in the API source would then lead to m/z 166, which can be designated as $(M - CH_2F + H)H^+$. Possible structures of these ions at m/z 198, 178, 166, and 152 are given in Figure 2. The pyrolytic cleavage of the CH_2–CH_2F bond in 7-(2-fluoroethyl)guanine can be justified. The two radicals CH_2F and $(M - CH_2F)$ that result are both stabilized by resonance, particularly so the $(M - CH_2F)$, which could be considered benzyl radical like. Thus a bond dissociation energy of ~60 kcal/mol could be expected. The temperature of the gas in the nebulizer is below 500 °C, but wall temperatures close to 550 °C could be present. With these estimates, one can show that the bond cleavage could occur within the short (~0.1 s) contact time in the nebulizer.[23f] Some if not all of the m/z 152 could be due to pyrolytic loss of the CH_2CH_2F radical from M, followed by H atom abstraction leading to guanine and API protonation of the guanine. The resulting ion m/z 152 can be designated $(M - C_2H_4F + H)H^+$ or BH_2^+.

Although the pyrolysis products available from the heated nebulizer are sufficient to provide structural information on the protonated molecular ion, CAD fragment ion spectra of MH^+ (m/z 198), Figure 1B, obtained under LC/MS/MS conditions

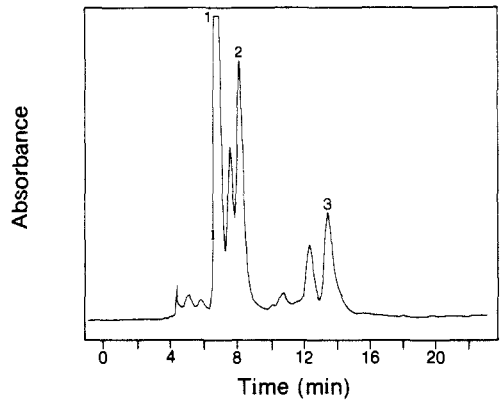


Figure 3. HPLC/UV chromatogram of the products resulting from the reaction of BFNU with guanosine in Tris buffer at pH 7.1, followed by hydrolysis. Numbered peaks correspond to compounds that were characterized from their mass spectra: peak 1, guanine; peak 2, 7-(2-hydroxyethyl)guanine; peak 3, 7-(2-fluoroethyl)guanine.

also yielded several ions for structural confirmation of MH^+. The ions of interest in this spectrum are m/z 152, corresponding to $(MH^+ - C_2H_4F + H)$, and m/z 47, representing $[CH_2CH_2F]^+$. Most of the remaining ions originate from the decomposition of the base moiety (protonated guanine, m/z 152). This was confirmed by obtaining CAD fragment mass spectra of m/z 152 from an authentic sample of guanine under standard LC/MS/MS conditions; see Figure 1C. Thus, the lower intensity ions from the CAD of MH^+, Figure 1B, were assigned to sequential loss of H_2NCN from m/z 152 (m/z 110, 68) and successive losses of NH_3 and C_3NH_5 from m/z 152 (m/z 135, 80). These same fragment ions are present in the CAD spectrum of the authentic guanine sample, except for the ion at m/z 47. Its absence from the spectrum in Figure 1C indicates that m/z 47 represents $[CH_2CH_2F]^+$, which must arise from MH^+. Apart from this, and minor variations in ion intensities, the spectra of Figure 1, part B (m/z 20–152 range) and part C, are identical.

Characterization of 7-(2-Fluoroethyl)guanine and 7-(2-Hydroxyethyl)guanine Resulting from the Reaction of Deoxyguanosine with BFNU in Tris. The reaction of deoxyguanosine with BFNU under the conditions reported in the Experimental Section led to a number of products that were separated by using high-performance liquid chromatography (Figure 3) and characterized from LC/API-MS and LC/MS/MS data. Peaks corresponding to the presence of guanine (peak 1), 7-(2-hydroxyethyl)guanine (peak 2), and 7-(2-fluoroethyl)guanine (peak 3) were identified (a) by comparing the chromatographic data with those of authentic samples, (b) by examining the reconstructed ion chromatograms and API mass spectra for specific ions expected for each of the species, and (c) by obtaining CAD fragment mass spectra of MH^+ for each of the components of interest in the products of this reaction. More details are available in Table I.

Characterization of Dideuterated 7-(2-Fluoroethyl)guanine Resulting from the Reaction of BFNU-*1,1,1′,1′-d*$_4$ with Deoxyguanosine. The products of the reaction were separated by HPLC and analyzed by UV, LC/MS, and LC/MS/MS by a procedure similar to that outlined in the preceding sections. In the present case, since the m/z of the protonated molecular ion was not known, a search for ion maxima at the retention time 13.5 min (established from authentic samples) corresponding to 7-(2-fluoroethyl)guanine was carried out.

From the selected set of reconstructed ion chromatograms shown in Figure 4A, it is evident that the ions at m/z 200, 180, and 166 have characteristic maxima at the correct retention time. The m/z 200 is assigned to the protonated molecular ion MH^+, indicating the presence of two deuterium atoms in this derivative. The 7-(2-fluoroethyl)guanine is thus dideuterated.

The retention time of this dideuterated fluoroguanine derivative being the same as that of the authentic 7-(2-fluoroethyl)guanine sample indicates that the fluorine atom must be located on the

Table I. Deoxyguanosine Modification by BFNU or BFNU-*1,1,1′,1′-d*$_4$[a]

reaction and dG-N7 conditions	$HOCH_2CH_2$- N7-dG m/z 196	$HOCHDCH_2$- N7-dG m/z 197	$HOCD_2CH_2$- N7-dG m/z 198	FCH_2CH_2- N7-dG m/z 198	FCHDCH$_2$- N7-dG m/z 199	FCD_2CH_2- N7-dG m/z 200	$BrCH_2CH_2$- N7-dG m/z 258	BrCHDCH$_2$- N7-dG m/z 259
1. BFNU with dG in Tris	10[b,c]			9[b,c]				
2. BFNU-*1,1,1′,1′-d*$_4$ with dG in Tris	1[c,d]	10[c,d]	1.6[d,e]	trace[d,e,i]	0.35[d,e]	2.5[b,c]		
3. BFNU with dG and excess kBr in Tris	4[b,c]			3.3[b,c]			10[b,c]	
4. BFNU-*1,1,1′,1′-d*$_4$ with dG and excess KBr in Tris	3.3[d,e]	10[c,d]	3.3[d,e]	2[d,e,i]	10[d,e]	2[b,c]		0.2[e,h]
5. BFNU with dG in cacodylate	10[b,c]			9[b,c]				
6. BFNU-*1,1,1′,1′-d*$_4$ with dG in cacodylate	1[d,e]	10[c,d,f]	10[c,d,g]		0.8[d,e]	5[b,c]		
7. BFNU with dG and excess KBr in cacodylate	10[b,c]			6.7[b,c]			6.7[b,c]	
8. BFNU-*1,1,1′,1′-d*$_4$ with dG and excess KBr in cacodylate	10[c,d]	3.3[c,d]	10[d,e]	trace[d,e,i]	trace[d,e,i]	1.3[b,c]		0.3[e,h]

[a] Hydroxyethyl-N7-dG and haloethyl-N7-dG products analyzed by HPLC/MS and HPLC/MS/MS after hydrolysis with 1N HCl—approximate relative yields from peak heights (MH^+ peak and major fragments) in the reconstructed ion chromatograms. [b] Presence confirmed from reconstructed single-ion chromatograms of MH^+ and specific fragments $(M - HX)H^+$, $(M = CH_2X + H)H^+$, and protonated guanine $(BH + H^+)$. [c] Additional structural information obtained by characterizing MS/MS CAD fragment ion spectra of MH^+. [d] Presence deduced from reconstructed ion chromatograms of MH^+ at expected elution time. Supporting fragments cannot be assigned due to high degree of isotopic overlap. [e] MH^+ signal intensity is not sufficiently strong to obtain reliable MS/MS CAD fragment ion spectra. [f] MS/MS CAD fragment ion spectra indicate that only one deuterium atom is present and that this atom is located on the ethyl group. [g] MS/MS CAD fragment ion spectra indicate that one deuterium is located on the ethyl chain and the other on the base. [h] Presence confirmed by the isotopic pattern of MH^+ and the strong presence of m/z 179 indicating loss of HBr from MH^+. [i] Reconstructed ion chromatograms of MH^+ peaks are very weak in intensity; therefore the identification of this species is tentative.

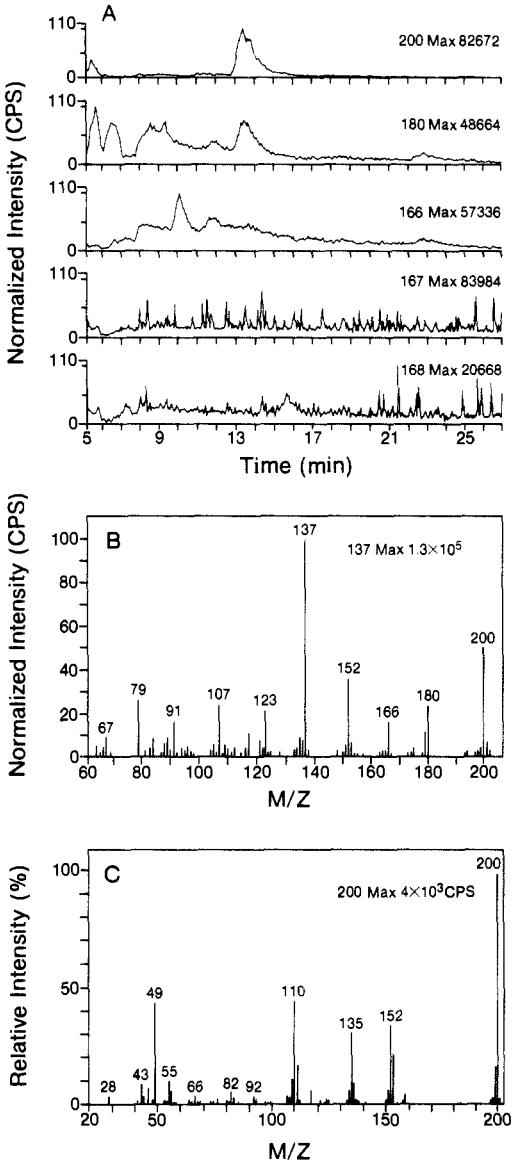


Figure 4. (A) Reconstructed ion chromatograms for peaks corresponding to MH^+ (m/z 200) and characteristic fragments m/z 180 and 166 of dideuterated 7-(2-fluoroethyl)guanine, all indicating ion maxima at the correct retention time of 13.5 min. Reconstructed ion chromatograms for m/z 167 and 168 are also plotted in this figure, demonstrating the backgroundlike behavior of these ions. No distinct maxima are indicated at the retention time of interest. (B) Background-subtracted LC/API mass spectrum deduced from the peak maxima of 13.5 min shown in (A). (C) LC/MS/MS CAD fragment ion mass spectrum of m/z 200 [MH^+ of dideuterated 7-(2-fluoroethyl)guanine] obtained at 13.5 min.

ethyl chain and also in the C_2-position. A different retention time would have been expected otherwise.

The mass spectral analysis of nebulized authentic 7-(2-fluoroethyl)guanine (see preceding sections) showed that the m/z 152 corresponds to protonated guanine. This ion is also present in Figure 4B, while m/z 153 and 154 are negligible. This shows that there is no deuterium on the protonated guanine and confirms that both deuterium atoms are on the fluoroethyl group. Furthermore, the absence of m/z 153 and 154 shows that no deuterium is transferred from the fluoroethyl group to the guanine moiety in the nebulizer-produced LC/API mass spectrum.

Further confirmation that the fluoroethyl group is dideuterated is obtained from the LC/MS/MS CAD fragment ion spectrum of m/z 200 taken at the correct (13.5 min) retention time, shown in Figure 4C. This spectrum contains a prominent peak at m/z 49. In the CAD fragment ion spectrum of MH^+ m/z 198 obtained from authentic 7-(2-fluoroethyl)guanine, the prominent peak was m/z 47 (seen in Figure 1B), and this peak was identified as $[CH_2CH_2F]^+$. The shift of that peak to m/z 49 in Figure 4C shows that the fluoroethyl group is now dideuterated.

The ion at m/z 180, see Figure 4B, corresponds to loss of HF from m/z 200. The major loss being HF rather than DF indicates that the two deuteriums are on the same carbon atom as the fluorine, since one expects that the elimination will involve H or D atoms on the carbon β to the fluorine.

The ion at m/z 166 in Figure 4B is obviously due to an analogous process as the ion of the same mass obtained from authentic 7-(2-fluoroethyl)guanine; see Figure 1A. As pointed out in the preceding discussion, it is unlikely that this ion is due to a fragmentation of MH^+ m/z 198, since that would lead to a neutral product, CHF, which is of high energy. The observation of the same mass, m/z 166, from the dideuterated compound confirms this conclusion, since the ion resulting from a cleavage reaction from the dideuterated MH^+ should have contained a miminum of one deuterium atom when the neutral loss was CDF or two deuterium atoms for the neutral loss of CHF. Since no deuterium atoms are present in m/z 166, some of the hydrogen in m/z 166 is of foreign origin, i.e., due to a bimolecular reaction. The neutral group lost then is not CHF but CH_2F (CDF and CD_2F, respectively, from the dideuterated compound). The clean loss of CD_2F from the dideuterated compound provides very strong evidence that both deuterium atoms are on the α-carbon, i.e., that the fluoroethyl group is CH_2CD_2F. In the section dealing with mass spectral data for authentic 7-(2-fluoroethyl)guanine = M, evidence was presented that losses of CH_2F occur from M due to pyrolysis of neutral M in the nebulizer and that the resulting neutral ($M - CH_2F$) radical picks up a hydrogen atom by a bimolecular hydrogen abstraction reaction. The resulting ($M - CH_2F + H$) becomes protonated in the API region and is detected as $(M - CH_2F + H)H^+$, m/z 166. The presence of a lone m/z 166 peak without deuterium satellites in the nebulizer spectrum of the dideuterated compound, shown in Figure 4B, is consistent with that mechanism [where m/z 166 is now $(M - CD_2F + H)H^+$] and provides further confirmation for its validity. However, it should be noted that the proof of structure of the 7-(2-fluoro-2,2-dideuterioethyl)guanine is not dependent on the exact mechanism by which the ions are formed. The pyrolysis of M just represents the most probable such mechanism. Finally, the background-like behavior of ions m/z 167 and 168 at 13.5 min (see Figure 4A) further verifies the assignment of the deuterium atoms α to the fluorine. One deuterium β to the fluorine and one α would have resulted in a maximum of m/z 167 at 13.5 min, representing the $(M - CHDF + H)H^+$ ion, formed by a mechanism identical with that discussed for m/z 166. Similarly, the presence of two deuterium atoms β to the fluorine would have indicated a maximum of m/z 168 at 13.5 min, since the $(M - CH_2F + H)H^+$ ion would have been expected to form.

In order to ensure that the data presented in this section were reproducible, two reactions of deoxyguanosine with BFNU-*1,1,1',1'-d*$_4$ were performed and five aliquots of each reaction product were analyzed by HPLC/MS. The presence of 7-(2-fluoro-2,2-dideuterioethyl)guanine was confirmed every time. This was necessary, since significant variations in the relative intensities of the ions m/z 179 and 166 were observed from one experiment to the other.

In addition to dideuterated 7-(2-fluoroethyl)guanine, five other distinct species were detected and identified in the products of this reaction by employing the same experimental procedures and criteria outlined above. The results of these experiments are summarized in Table I. A tentative quantitative comparison considering all the problems involved (see Experimental Section) is also given in Table I.

Earlier mass spectra[9d] of dideuterio(2-chloroethyl)guanine obtained from BCNU-*2,2,2',2'-d*$_4$ and deoxyguanosine were interpreted to indicate that the deuteration of the ethyl group was CD_2CH_2Cl, i.e., corresponding to rearrangement as found in the present work by using BFNU-*1,1,1',1'-d*$_4$. The deuterium assignment[9d] was based on a comparison of the electron-impact (EI) mass spectrum of the deuterated derivative with that of authentic

undeuterated (2-chloroethyl)guanine. In general, EI spectra are not too well suited to provide a definite assignment of the position of deuterium atoms unless an EI spectrum of the authentic deuterated compound is available. Hydrogen or even carbon scrambling can occur in the excited molecular radical ion, which leads to loss of positional information for labeled atoms. These rearrangement processes have unfavorable entropy transition states but are of lower energy than, and can compete with, the more direct dissociation and cleavage processes that retain positional information for the label. For examples involving studies of systems with some relevance to the present case, see Nibbering.[9e] In the EI study,[9d] neither of the two major fragmentations provides unambiguous proof of the position of the deuterium. Only a lower abundance loss of CH_2Cl in the undeuterated isomer ion is seen to remain as a loss of CH_2Cl in the deuterated isomer. This peak, if one assumes absence of prior scrambling, indicates the CD_2C-H_2Cl structure. In the present study, as was discussed earlier, the fragments of the (2-haloethyl)guanines are produced by thermal decomposition of the neutral molecule and then ionized by protonation with a weak protonating agent. No deuterium scrambling is expected to occur for these low-energy processes, and the data provide multiple confirmations for the equivalent rearrangement corresponding to BFNU-*1,1,1',1'-d*$_4$ and leading to CH_2CD_2X.

Characterization of 7-(2-Hydroxyethyl)guanine and 7-(2-Haloethyl)guanines and Their Deuterium-Labeled Counterparts from the Reactions of Deoxyguanosine with BFNU or BFNU-*1,1,1',1'-d*$_4$ in Tris and in the Presence of KBr. These were experiments similar to those of the two previous sections, but here the reactions were carried out in the presence of KBr (see Experimental Section). HPLC/MS and HPLC/MS/MS analysis of the products of reactions 3 and 4 (see Table I) led to the detection and identification of a number of important species in each case; these have been summarized in Table I. Similarly, the experimental procedures, conditions, and criteria used to identify these products were identical with those discussed in the previous sections. Extensive documentation and discussion regarding the identification of these products have been presented elsewhere.[24]

Characterization of 7-(2-Hydroxyethyl)guanine and 7-(2-Haloethyl)guanines and Their Deuterium-Labeled Counterparts from the Reactions of Deoxyguanosine with BFNU or BFNU-*1,1,1',1'-d*$_4$ in Different Buffers. Having determined that the key individual products of the base modification could be separated and identified and the deuterium labels located within them, a systematic study of the reactions was undertaken. The conditions varied according to the presence or absence of deuterium labeling in the substrate, or of competing external halide ion, and the nature of the buffer, i.e., nucleophilic or nonnucleophilic. Eight distinct species were detected and identified according to the reaction conditions (Table I). Quantifying these products poses severe problems, including differences in solubility of the deglycosidated modified bases and the difficulty of HPLC/MS detection of low concentration components eluting next to very high concentration components, as well as the coelution of isotopic components. However, several approaches to these problems were investigated, and techniques were devised to give reproducible approximate relative yields of products; the results are summarized in Table I.

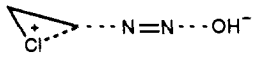
The first general conclusion is that identical products are obtained when either Tris or sodium cacodylate buffer is used. The major products, in the absence of bromide ion, are HOEt-N7-dG and FEt-N7-dG. When BFNU-*1,1,1',1'-d*$_4$ is used, the isotopic labeling confirms clean rearrangement, so that the deuteriums are now α to the OH group or F atom. The detection of monodeuterated and undeuterated counterparts of these species indicates the operation of an exchange mechanism, plausibly via the diazoalkane (see Figure 8 and Discussion). The extent of the latter in situ deuterium exchange for FCD_2CH_2-N7-dG is severely suppressed in the nonnucleophilic sodium cacodylate buffer in both experiments 6 and 8, suggesting the possible involvement of Tris in this process in experiments 2 and 4.

When the reactions are carried out in the presence of a 10-fold molar excess of bromide ion, halide exchange occurs (experiments 3 and 7) to give BrEt-N7-dG. No such halide exchange takes place on BFNU in the absence of dG, even in the presence of 18-crown-6 and bromide ion in appropriate control reactions. The position of the deuterium label confirms complete molecular rearrangement of the side chain. Only the monodeuterated bromo species is detected and none of the dideuterated component, suggesting an unusually facile isotopic exchange under these high ionic strength conditions (see Figure 8 and Discussion) even though the extent of halide exchange is relatively low. However, the extent of halide exchange is evidently sensitive to precise reaction conditions (compare reaction 7).

Discussion

Several candidates have been considered for the key electrophile involved in the reaction of HENUs with DNA bases. These include (*E*)- and (*Z*)-(2-haloethyl)diazoates, the diazonium ion, the diazoalkane, and the (2-haloethyl)carbenium ion. Evidence in the case of DNA base alkylation by BCNU[9,33] requires primarily S_N2 processes, which rules out a free carbenium ion. Results from specifically deuterated HENUs[5,15] also apparently eliminate the diazoalkane as a candidate electrophile (however, see evidence below for its intermediacy under special conditions). Specific *N*-^{18}O labeling of BCNU in conjunction with in situ reduction of the products of spontaneous decomposition under physiological conditions by alcohol dehydrogenase indicates the intermediacy of 1,2,3-oxadiazoline.[25] The latter plausibly arises from (*Z*)-(2-chloroethyl)diazohydroxide, a species in which the N–O bond is still intact and therefore disfavors the alkyldiazonium ion, at least for this pathway.

The intermediacy of (*E*)- or (*Z*)-(2-chloroethyl)diazonium ions implies incipient chlorine participation in the following way and has been invoked by advocates of the "global electrostatic" interpretation of HENU sequence selectivity.[12a-d] Since no com-



parable stabilization by participation is possible for the analogous fluorine species,[19] we therefore considered the BFNU would be valuable and informative as a mechanistic probe when compared with BCNU.[26,27]

The results of the isotopic-labeling experiments with BFNU-*1,1,1',1'-d*$_4$ reacting with deoxyguanosine, analyzed by HPLC/MS/MS, confirm transfer of the 2-fluoroethyl group to the N7-position of guanine, with complete rearrangement of the two deuterium atoms from their initial position β to the fluorine in BFNU-d_4 to positions α to the fluorine in the nucleoside adduct. This rules out direct S_N2 displacement on (*E*)- or (*Z*)-(2-fluoro-1,1-dideuterioethyl)diazohydroxide (or the corresponding diazonium ion) by the deoxyguanosine nucleophile. Similar direct S_N2 displacement of fluoride from BFNU may also be ruled out on the basis of extensive literature on the behavior of alkyl fluorides toward nucleophiles[27] and of the control experiments wherein BFNU is inert to bromide ion, contrasted with the facile halide exchange that takes place with BFNU in the presence of deoxyguanosine. The latter key observation suggests formation of a

(24) Ikonomou, M. G.; Naghipur, A.; Lown, J. W.; Kebarle, P. *Biomed. Environ. Mass Spectrom.* In press.

(25) Lown, J. W.; Chauhan, S. M. S. *J. Org. Chem.* **1982**, *47*, 851.

(26) (a) BFNU and BCNU have many similar properties to support this selection. The decomposition rates of BFNU and BCNU in aqueous phosphate buffer, pH 7.2, at 37 °C are identical ($k = 12.2 \times 10^{-3}$ min^{-1})[3,16] and in aqueous solution are closely comparable ($k = 2.84$ and 2.27×10^{-3} min^{-1}, respectively).[3,16] The alkylating activities of BFNU and BCNU, measured with 4-(*p*-nitrobenzyl)pyridine in EtOAc are comparable.[3] BFNU and BCNU are also equally effective against L1210 leukemia; however, there are differences in their toxicities that may be attributable to the fact that BFNU generates fluoroacetic acid, a known toxin.[2-4]

(27) An important distinction between BCNU and BFNU is the anticipated lack of reactivity of the latter toward possible direct S_N2 displacement of F by nucleophiles in the absence of exceptional activation (vide infra). Parker, R. E. Mechanisms of Fluorine Displacement. *Adv. Fluorine Chem.* **1963**, *3*, 63.

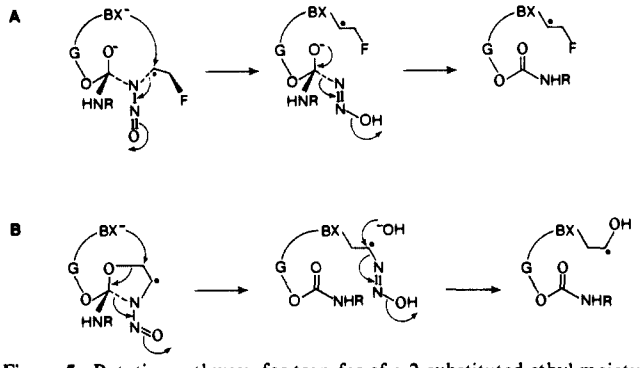


Figure 5. Putative pathways for transfer of a 2-substituted ethyl moiety to a neighboring DNA base site from an initially formed tetrahedral intermediate derived from an HENU.

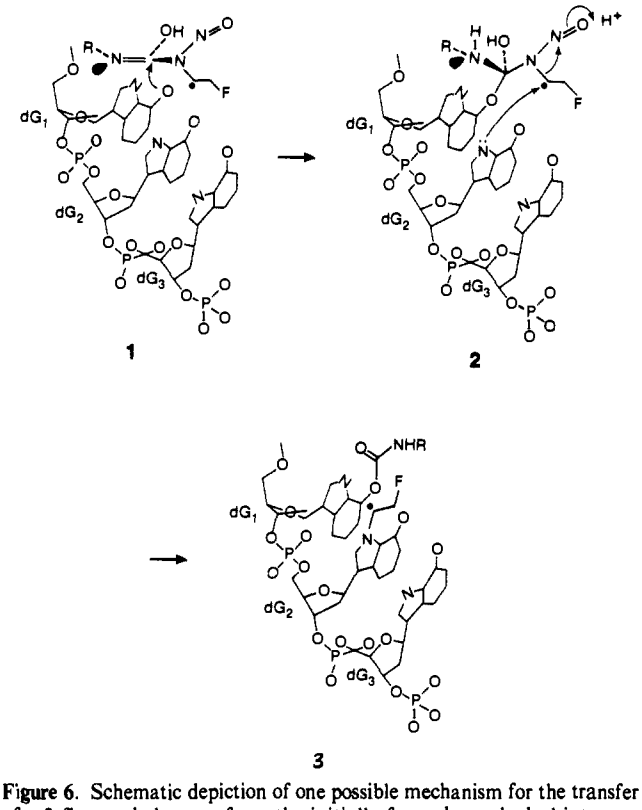


Figure 6. Schematic depiction of one possible mechanism for the transfer of a 2-fluoroethyl group from the initially formed tetrahedral intermediate to a 3′-adjacent N7-dG position showing incorrect prediction of deuterium isotope label placement, designated ●.

reactive intermediate capable of accommodating both the molecular rearrangement and halide exchange, depending on the reaction conditions.

Several lines of evidence implicate formation of tetrahedral intermediates from HENUs resulting from reversible addition of water or a DNA base nucleophile to the nitrosourea carbonyl group or the corresponding iminourea.[18a,c,19,21] One such species was considered recently in an attempt to interpret the striking sequence-selective alkylation and interstrand cross-linking of runs of G residues in DNA. In this view, the tetrahedral intermediate formed by the reaction of the O^6-guanine is subject to S_N2 displacement of the 2-haloethyl group by the N7 atom of a second deoxyguanosine.[21] As may be seen from Figure 5A, the results of the deuterium-labeling experiments are not in accord with this proposal. Therefore, the suggestion[21] that the tetrahedral intermediate **2** could react with a 3′-neighboring guanine moiety to yield the FEt-N7-dG **3** by displacement on the methylene α to the nitrosated N_1, as shown in Figure 6, must be rejected.[21d]

An alternative pathway was proposed[21c] involving rearrangement of the initially formed tetrahedral intermediate **2**. This involves formation of a tetrahedral intermediate by addition of

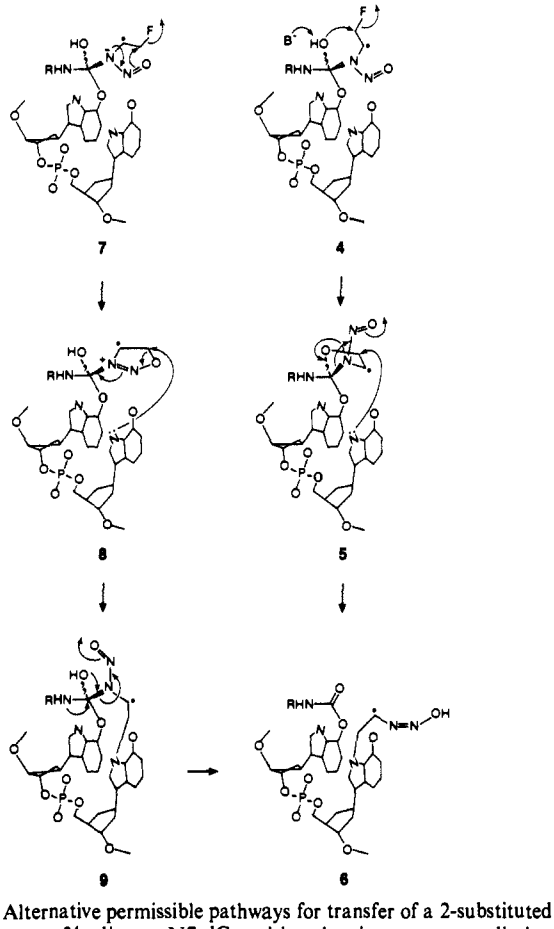
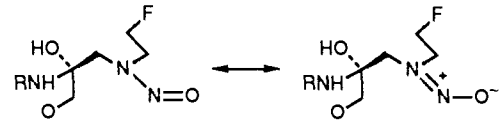


Figure 7. Alternative permissible pathways for transfer of a 2-substituted ethyl group to a 3′-adjacent N7-dG position showing correct prediction of isotope label placement, designated ●.

the O^6-guanine to the carbonyl group of the iminourea of the HENU to give **4** (Figures 5B, 7), followed by ring closure to form the nitrosooxazolidine **5** (Figures 5B, 7). Considering this proposal in the light of the present results, it is clear that the deuterium-labeling studies prove conclusively that the C–F bond is broken and subsequently reformed in the reaction of BFNU and deoxyguanosine. Given the fact that C–F bonds are normally inert to nucleophilic displacement,[19,27] then clearly some unusual circumstances must obtain, and the results point to a favored intramolecular displacement.[28] Among the factors contributing to the polarization and weakening of the C–F bond, rendering it more susceptible to nucleophilic displacement, is the resonance of the adjacent *N*-nitroso group with concomitant inductive



electron withdrawal from the neighboring carbon atoms.[29] Certainly fluoride ion is released during the hydrolysis of BFNU at pH 7 and 37 °C, indicating the lability of the C–F bond (by certain cyclization pathways) under these mild conditions.[19]

The alternative proposed mechanism thus involves formation of the *N*-nitrosooxazolidine **5** and attack at position 5 of the

(28) The cyclic fluoronium ion, which would correspond to putative comparable participation by fluorine, has attracted considerable theoretical interest (Lischka, H.; Kohler, H. J. *J. Am. Chem. Soc.* **1978**, *100*, 5297. Clark, D. T.; Lilley, D. M. J. *J. Chem. Soc. D* **1970**, 1042. Hopkinson, A. C.; Lien, M. H.; Yates, K.; Czismadia, I. G. *Theor. Chim. Acta* **1975**, *38*, 21). Unlike the other halo analogues, all attempts to detect the cyclic fluoronium ion experimentally have failed (Olah, G. A.; Bollinger, J. M. *J. Am. Chem. Soc.* **1968**, *90*, 947. Olah, G. A.; Bollinger, J. M.; Brinich, J. M. *J. Am. Chem. Soc.* **1968**, *90*, 2587. Olah, G. A.; Beal, D. A.; Westerman, P. W. *J. Am. Chem. Soc.* **1973**, *95*, 3387. Olah, G. A.; Schilling, P.; Westerman, P. W.; Lin, H. C. *J. Am. Chem. Soc.* **1974**, *96*, 3581.

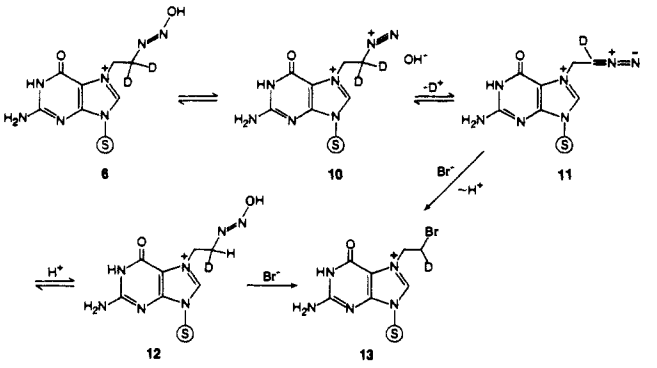


Figure 8. Suggested mechanism for formation of $BrCHDCH_2$-N7-dG from the diazoic acid via a diazoalkane and promoted by high ionic strength conditions.

heterocycle by nucleophiles on the neighboring bases, e.g., by N7-guanine, leading to the rearranged diazoic acid **6** (X = OH) (Figure 7).[21d,29] In the absence of an adjacent base in a complementary strand of DNA, the intermediate can hydrolyze to HOEt–N7-dG or react with fluoride ion within the solvent cage to form FEt–N7-dG.[30] It has been demonstrated independently that the halogen group in 7-(2-haloEt)dG derivatives is not subject to hydrolysis to HOEt-N7-dG,[9a,31] so the above competing mechanisms provide a plausible explanation for the observed formation of $HOCD_2CH_2$-N7-dG together with FCD_2CH_2-N7-dG.[32]

Moreover, the halide-exchange experiments, in which a 10-fold molar excess of KBr is present during the reaction of BFNU with deoxyguanosine, result in formation of BrEt-N7-dG. By contrast, no such halide exchange occurs on FEt-N7-dG directly or even in the presence of 18-crown-6 to enhance the nucleophilicity of the bromide ion.[32d] This supports the suggestion of a labile species (the diazoic acid) subject to effective competitive nucleophilic substitution by Br^- (nucleophilicity value 3.5 on Wells scale[32a]) combined with the mass law effect. Supporting evidence for the intermediacy of the $HON{=}NCH_2CH_2$-N7-dG moiety **6** is obtained by the detection of $BrCHDCH_2$-N7-dG (i.e., bearing only one deuterium adjacent to bromine)[32b] in the KBr-exchange reaction with BFNU-*1,1,1′,1′-d*$_4$. This is most plausibly explained by enhanced reversible formation of diazoalkane **11** from the diazonium ion **10** under the high ionic strength conditions[32c] (Figure 8). Formation of $BrCHDCH_2$-N7-dG conceivably takes place in the presence of high Br^- concentration either by nucleophilic displacement on the partially exchanged diazoic acid **12** or by addition of Br^- to the diazoalkane group followed by extrusion of N_2 and collapse of ions in the solvent cage to give **13**. The detection of only **13** and none of the corresponding dideuterium or protium counterparts argues for a preferred pathway of **6** → **10** → **11** → **13** under these conditions.

The mechanism shown in Figure 7 (**4**, **5**, **6**) has the merit that it correctly predicts the positions of the deuterium labels observed in the present study. Another appealing feature of this mechanism for the formation of ClEt-N7-dG or FEt-N7-dG, which cannot form interstrand cross-links,[21c] is that it also accounts for the observed preferential N7-alkylation of individual G residues in runs of Gs, i.e., with transfer of the 2-haloethyl group to the neighboring base site.[21]

An alternative mechanism, the so-called "anchimeric" pathway,[21c] must also be considered in the light of the deuterium isotope labeling results. This involves formation, from the initially generated tetrahedral intemediate **7** (Figure 7), of a 1,2,3-oxadiazolinium species **8**. Subsequent ring opening via an adjacent N7-dG nucleophile would, in principle, lead to FEt-N7-dG and HOEt-N7-dG via **9** to **6** with the correct placement of the deuterium labels. Experimental evidence for the contribution of this pathway in HENU hydrolysis under neutral conditions has been obtained by using specific ^{18}O labeling in conjunction with in situ reduction of the released [^{18}O]acetaldehyde by alcohol dehydrogenase.[25] It was concluded that this anchimeric pathway contributed in the case of BCNU only to the extent of 21% of the overall reaction.[25] It is conceivable that the relative contributions of the two pathways involving cyclic intermediates (at least one of which is demanded in order to accommodate the deuterium-labeling results) may be altered on the surface of the DNA.[35] However, the lower energy of the nitrosooxazolidine intermediate compared with the positively charged 1,2,3-oxadiazolinium species suggests the former pathway may be more significant.

There remains the question of the possible contribution of the chemoselection on the basis of global DNA electrostatic effects. Advocates of this position argue that, because HENUs of different structures give essentially the same pattern of selective alkylation of DNA, this points to a common intermediate, e.g., the (2-haloethyl)diazohydroxide, whereas an alkylator such as SoSo, which is expected to react by a different electrophile, does not exhibit a similar sequence selectivity.[12a-d] This argument has some appeal except that the structurally quite different alkylating saframycin antitumor antibiotics A and S[36a-c] display a DNA

(29) There are precedents for this reaction in the cyclization of (β-haloethyl)ureas to oxazolidines (Kreling, M. E.; McKay, A. F. *Can. J. Chem.* **1959**, *37*, 504) and in the isolation of 2-(alkylimino)-3-nitrosooxazolidines from HENUs,[13] as well as 3-(2-chloroethyl)urea adducts formed by treating lysine or polylysine with BCNU (Wheeler, G. P.; Bowden, B. J.; Struck, R. F. *Cancer Res.* **1975**, 2974). In addition, the 2-(alkylimino)-3-nitrosooxazolidines, which had been previously postulated[3,16] as intermediates in the hydrolysis of HENUs, were synthesized and found to hydrolyze to products found for HENUs.[13]

(30) Chatterjii et al. have remarked that "simple nucleophilic displacement of fluorine is extremely difficult and liberation of the fluoride ion almost certainly points to an intramolecular substitution by a negatively charged atom...".[17]

(31) Another factor favoring the ring closure to the *N*-nitrosooxazolidine is that such *exo-tet* ring closures are particularly favorable for five-membered ring formation, since this ring size represents the best balance between enthalpy and entropy terms; i.e., the ring is strain free and the transition state is accessible (Baldwin, J. E. *J. Chem. Soc., Chem. Commun.* **1976**, 734).

(32) (a) The fluoride ion, although present in low concentration compared with H_2O, evidently can compete successfully owing to its close proximity to the newly generated *N*-nitrosooxazolidine and to the higher nucleophilicity on the Wells scale of F^- = 2.0, compared with H_2O = 0 (*Chem. Rev.* **1963**, *63*, 171). (b) The predominant elimination of only one deuterium atom by the intermediacy of a diazoalkane is similar to the analogous elimination of one deuterium in the base treatment of α,α-dideuterated alkylsulfonyl chlorides that proceeds via a sulfone intermediate (Opitz, G. *Angew. Chem., Int. Ed. Engl.* **1967**, *6*, 107–123. King, J. *Acc. Chem. Res.* **1975**, *8*, 10–17). (c) Increased ionic strength of the medium is known to accelerate reactions that result in an increase in charge separation, either in the production of ion pairs as in the case of **6** → **10** or in polarization within a molecule as in **10** → **11** (Bronsted, A. *Z. Phys. Chem., Stoichiom. Verwandtschaftsl.* **1922**, *102*, 169; **1925**, *115*, 337). A simplified treatment of the effect of ionic strength on a reaction that produces ions or that results in an increase in intramolecular polarization has been given by Bateman, L. C.; Church, M. G.; Hughes, E. D.; Ingold, C. K.; Taber, W. A. *J. Chem. Soc.* **1940**, 979; it yields the relationship

$$\ln k_r = \ln k_r^0 + \alpha Z^2 \mu d \qquad (1)$$

where k_r^0 is the rate constant at zero ionic strength, α a constant characteristic of the solvent and temperature, μ the ionic strength, Z the fractional charge on each "end" of the dipole, and d the distance between the "ends". The application of this equation to the quantitative prediction of salt effects is limited largely by the difficulty in estimating, a priori, the fractional charge Z and the separation of charge d associated with an activated complex whose existence is momentary and which therefore may be studied only by indirect means (Gould, E. S. *Mechanism and Structure in Organic Chemistry*, Holt, Rinehart, and Winston: New York, 1980). Qualitatively, one may predict from eq 1 that a 10-fold increase in molality of KBr is expected to accelerate the steps **6** → **10** and **10** → **11** compared with normal reaction conditions and thereby permit the generation and trapping of the diazoalkane **11**. (d) A reviewer has suggested that, in the Br^- exchange experiments, the crown ether may dissociate from the Br ion and complex with the diazoic acid, which would thereby facilitate the exchange reaction.

(33) Ludlum, D. B.; Tong, W. P. *Biochem. Pharmacol.* **1978**, *27*, 2391.

(34) It is noteworthy that 2-haloethylation and 2-hydroxyethylation of N7-dG are similarly enhanced in runs of Gs in the reaction of HENUs with DNA.[12a-d]

(35) A similar conclusion has been reached on the basis of other criteria.[21c] Buckley has suggested that, while HENU hydrolysis is a kinetically controlled process, reactions occurring on DNA are more likely to be thermodynamically controlled.[21c]

sequence selectivity of GGG[36d] very similar to that of HENUs, and they certainly do not react via an alkanediazoic acid but rather by an iminium species.[36a]

On the other hand, opponents of the global DNA electrostatics view have argued that species such as (2-haloethyl)diazohydroxides, or the kinetically equivalent diazonium ions, would react indiscriminately with DNA.[21] This is not necessarily the case, as may be shown, for example, by a hard and soft acids and bases (HSAB) treatment, taking into account HOMO–LUMO interactions between electrophilic (2-haloethyl)diazohydroxides and alternative nucleophilic sites on the DNA.[18a,b] Nevertheless, the free (2-haloethyl)diazohydroxide must now be rejected in the light of the deuterium isotope labeling experiments. The results with the saframycin antibiotics, which both bind and bond to DNA,[34] suggest a subtle interplay of conformational adaption and molecular recognition overlaid on the global electrostatic effect of the GGG sequence over the incoming positively charged iminium species. It may transpire that the sequence selectivity of the HENUs involves a similar interplay of stereochemical factors, of the type discussed herein, as well as electrostatic influences. Experiments to investigate these factors are continuing and will be reported in due course.

Experimental Section

Melting points were determined on a Fisher-Johns apparatus and are uncorrected. The IR spectra were recorded on a Nicolet 7199 FT spectrophotometer, and only the principal bands are reported. The ^1H and ^{19}F NMR spectra were recorded on Bruker WH-200 or WH-400 spectrometers. EI mass spectra were determined on an Associated Electrical Industries (AEI) MS-9 double-focusing high-resolution mass spectrometer with ionization energies at 70 eV. Peak measurements were made by comparison with perfluorotributylamine at a resolving power of 15 000 on an AEI-MS-50 mass spectrometer. Kieselgel 60 (230–400 mesh) of E. Merck was used for flash chromatography, and precoated silica gel 60F-254 sheets of E. Merck were used for TLC, with the solvent system indicated in the procedure. TLC plates were visualized by using UV light or 2.5% phosphomolybdic acid in methanol with heating. Ethanol was freshly distilled from magnesium turnings, ether was dried over $LiAlH_4$ and stored over sodium wire, dimethylformamide was distilled from calcium hydride and stored over molecular sieves (3A), and triethylamine was treated with potassium hydroxide and then distilled from barium oxide and stored over molecular sieves (3A).

Materials. Crystalline 1,3-bis(2-fluoroethyl)-1-nitrosourea (BFNU), 7-(2-hydroxyethyl)guanine, and 7-(2-fluoroethyl)guanine were synthesized by published methods.[9,16] Deoxyguanosine hydrate, potassium phthalimide, lithium aluminum deuteride, and 18-crown-6 ether were obtained from Aldrich Chemical Co. Sodium cacodylate was obtained from Pfaltz and Bauer, Inc., Chemical Co. Tris(hydroxymethyl)-aminomethane, enzyme grade, was obtained from Bethesda Research Laboratories. Phosgene (20% in toluene) was obtained from Fluka Chemical and Biochemical Co.

2-Fluoro-1,1-dideuterioethyl Methanesulfonate. A solution of ethyl fluoroacetate (12.6 g, 119 mmol) in dry ether (100 mL) was added dropwise over a period of 30–40 min to a cold (5–10 °C) and stirred suspension of lithium aluminum deuteride (5 g, 119 mmol) in dry ether (200 mL). The reaction mixture was refluxed under argon for 7–8 h, at which time TLC analysis showed complete disappearance of starting material. The product was treated with water (5 mL), 15% NaOH solution (5 mL), and water (15 mL). The precipitate was removed by filtration; drying of the filtrate (K_2CO_3) followed by solvent removal under vacuum afforded 2-(fluoro-1,1-dideuterio)ethanol as a pale yellow liquid. The residual yellow liquid was distilled [30 °C (35 mmHg)] to obtain the pure product: 10.6 g (68% yield); ^1H NMR ($CDCl_3$) δ 3.5 (br s, 1 H, exch OH), 4.5 (d, 2 H, CH_2F, $J_{^{19}F-^1H}$ = 47 Hz).

To the product (10.6 g, 160 mmol) in dry dichloromethane (100 mL), containing a 50% molar excess of triethylamine (30.3 g, 300 mmol) at −10 °C, was added a 10% excess of methanesulfonyl chloride (25 g, 220 mmol) over a period of 20–30 min. The reaction mixture was stirred at −10 °C for 30 min and washed successively with cold water, 10% HCl solution, a saturated aqueous solution of sodium bicarbonate, and sodium chloride solution. Drying of the dichloromethane solution ($MgSO_4$) followed by solvent removal under vacuum afforded 2-fluoro-1,1-dideuterioethyl methanesulfonate as a pale yellow oil. The residual yellow oil was distilled [75 °C (0.5 mmHg)] to obtain the pure product: 20 g (86.9% yield); ^1H NMR ($CDCl_3$) δ 2.90 (s, 3 H, OSO_2CH_3), 4.5 (d, 2 H, CH_2F, $J_{^{19}F-^1H}$ = 47 Hz).

***N*-(2-Fluoro-1,1-dideuterioethyl)phthalimide.** A mixture of 2-fluoro-1,1-dideuterioethyl methanesulfonate (11.9 g, 82.6 mmol), potassium phthalimide (15.28 g, 82.6 mmol), and DMF (400 mL) was stirred at 130–140 °C for 3 h. The mixture was diluted with cold water (1 L), filtered, washed with cold water, and dried in vacuo over P_2O_5. The crude product was purified by column chromatography (silica gel, hexane with 25% ethyl acetate). Recrystallization from absolute ethanol afforded *N*-(2-fluoro-1,1-dideuterioethyl)phthalimide: 11 g (69% yield); mp 100 °C as white needles; ^1H NMR ($CDCl_3$) δ 4.6 (d, 2 H, CH_2F, $J_{^{19}F-^1H}$ = 47 Hz), 7.5–7.90 (m, 4 H, Ph); ^{19}F NMR ($CDCl_3$) δ F = −225.62 (3 q, $^2J_{^{19}F-^1H}$ = 47.06 Hz, $^3J_{^{19}F-D}$ = 3.76 Hz); EI-MS, *m/z* (rel intensity) 195 (M^+, 4.70), 162 ($M^+ - CH_2F$, 100), 134 (6.47), 104 (7.95), 76 (13.79), 66 (7.68), 51 (2.16).

2-Fluoro-1,1-dideuterioethylamine Hydrochloride. To a stirred solution of *N*-(2-fluoro-1,1-dideuterioethyl)phthalimide (2.63 g, 13.5 mmol) in absolute ethanol (50 mL) was added a solution of hydrazine hydrate (0.9 g, 18.5 mmol). The reaction mixture was stirred under reflux for 1.5 h. The resulting semisolid mixture was acidified to about pH 2–3 with dry HCl gas, stirred, refluxed for 1–2 h more, cooled to 5–10 °C, and filtered to remove phthalohydrazide. The filtrate was evaporated to dryness in vacuo, and the residue was dissolved in water (15–20 mL) and filtered through Celite. Removal of the water by lyophilization gave the product, which was further dried in vacuo over P_2O_5 to afford 2-fluoro-1,1-dideuterioethylamine hydrochloride: 1.37 g (100% yield); mp 92–93 °C as a white crystalline solid; ^1H NMR (Me_2SO-d_6) δ 4.65 (d, 2 H, CH_2F, $J_{^{19}F-^1H}$ = 47 Hz), 8.5 (br s, 3 H, exch $NH_3^+Cl^-$); EI-MS, *m/z* (rel intensity) 65 (M^+ − HCl, 100).

1,3-Bis(2-fluoro-1,1-dideuterioethyl)urea. To a stirred suspension of 2-fluoro-1,1-dideuterioethylamine hydrochloride (2.5 g, 25 mmol) in dry dichloromethane (25 mL) was added slowly a solution of triethylamine (5.05 g, 50 mmol) in dry dichloromethane (25 mL) at −5 °C. The reaction mixture was stirred at −5 °C for 25–30 min, and then a solution of 20% phosgene in toluene (7.5 mL) was added. The solution was diluted with dry ether (200 mL), and again a solution of triethylamine (5.05 g, 50 mmol) in dry ether (25 mL) was added. The resulting reaction mixture was stirred at −5 °C for 5–6 h and then allowed to warm to ambient temperature and stirred for a further 12 h. The reaction mixture was concentrated to give a solid residue and then water was added; the mixture was extracted with ethyl acetate and dried over $MgSO_4$, and the solvent was removed under vacuum. The residual solid was recrystallized from $CHCl_3$ to give the urea: 3 g (79% yield); mp 145–146 °C as a white crystalline solid; ^1H NMR ($CDCl_3$) δ 4.45 (d, 4 H, $2CH_2F$, $J_{^{19}F-^1H}$ = 47 Hz), 5.15 (br s, 2 H, exch 2 NH); IR (film) ν_{max} 3328, 1670 cm^{-1}; EI-MS, *m/z* (rel intensity) 156 (M^+, 100), 123 (56.25), 108 (14.77), 92 (21.97), 64 (15.26).

1,3-Bis(2-fluoro-1,1-dideuterioethyl)-1-nitrosourea. A solution of the urea (0.46 g, 3 mmol) in 97% formic acid (8 mL) was cooled to 0 °C, and sodium nitrate (0.82 g, 12 mmol) was gradually added with stirring. Stirring was continued for another 2.5 h. Then the mixture was diluted with 50 mL of water and extracted with $CHCl_3$ (3 × 60 mL). The combined extracts were dried ($MgSO_4$) and the solvent was removed. The residual yellow oil was chromatographed (silica gel, $CHCl_3$) to afford 1,3-bis(2-fluoro-1,1-dideuterioethyl)-1-nitrosourea as a yellow crystalline solid: 0.28 g (57% yield); mp 38–39 °C; ^1H NMR ($CDCl_3$) δ 4.4 (d, 2 H, $J_{^{19}F-^1H}$ = 47 Hz), 4.6 (d, 2 H, $J_{^{19}F-^1H}$ = 47 Hz), 7.25 (br s, 1 H, exch NH); ^{19}F NMR ($CDCl_3$) δ F_1 = −225.125 (3 q, $^2J_{^{19}F-^1H}$ = 47.06 Hz, $^3J_{^{19}F-D}$ = 3.76 Hz), F_2 = −225.29 (3 q, $^2J_{^{19}F-^1H}$ = 47.06 Hz, $^3J_{^{19}F-D}$ = 3.765 Hz); IR (film) ν_{max} 3421, 2960, 2845, 1721, 1526, 1490, 1034 cm^{-1}; EI-MS, *m/z* (rel intensity) 185 (M^+, 8.88), 94 (100), 92 (56.41), 72 (86.48), 61 (59.26).

Reaction of Deoxyguanosine with BFNU. Equimolar amounts of normal or deuterated BFNU and deoxyguanosine were dissolved in dimethyl sulfoxide (0.2 mL), and tris(hydroxymethyl)aminomethane (Tris) buffer [50 mM, pH 7.1 (0.8 mL)] or sodium cacodylate buffer [50 mM, pH 7.1 (0.8 mL)] was added to produce a final concentration of 0.12 M BFNU and 0.12 M deoxyguanosine. Reactions were also performed in the presence of 1.2 M KBr. Reaction mixtures were incubated at 37 °C for 24 h and then diluted with H_2O; the DMSO was removed by lyophilization.

Isolation of Nucleoside Derivatives. Deoxyguanosine derivatives were separated by HPLC (see below). Derivatives were identified by their HPLC retention times and ultraviolet spectra by comparison, whenever possible, with known standards as described previously. Modified bases were released from the deoxyguanosine nucleosides by hydrolysis with

(36) (a) Lown, L. W.; Joshua, A. V.; Lee, J. S. *Biochemistry* **1982**, *21*, 419. (b) Lown, J. W.; Hanstock, C. C.; Joshua, A. V.; Arai, T.; Takahashi, K. *J. Antibiot.* **1983**, *36*, 1184. (c) Lown, J. W.; Joshua, A. V.; Chen, H.-H. *Can. J. Chem.* **1981**, *59*, 2945. (d) Rad, K. E.; Lown, J. W. *Chem. Res. Toxicol.* In press. Saframycins A and S show strict GGG sequence binding preference as shown by MPE footprinting on *HindIII*/*EcoRI* restriction fragments of pBr322.

1.0 N HCl for 60 min at 100 °C. Hydrolysates were neutralized with 1 N KOH to pH 7.0 and the solutions lyophilized. The residues, without further purification, were dissolved in water and 100-μL aliquots were injected for HPLC/MS and/or HPLC/MS/MS analysis. The amount of water added to the various residues investigated was not the same. In each individual case enough water was added to the residue until a satisfactory concentration for the components of interest was detected by the mass spectrometer.

Exchange Control Experiments. (a) 7-(2-Fluoroethyl)deoxyguanosine (0.5 g, 1.5 mmol) and potassium bromide (0.178 g, 15 mmol) were added to 3 mL of 50 mM Tris buffer (pH 7.1) in 5-mL airtight Reactivials equipped with Teflon septa, thermostated at 37 °C for 24 h. The clear solution was evaporated under vacuum at room temperature and the residue heated in 1 N hydrochloric acid (2 mL) at 100 °C for 1 h. The solution was made neutral with 20% potassium hydroxide and the resulting precipitate washed with cold water and dried. Recrystallization from methanol gave only unreacted 7-(2-fluoroethyl)guanine. Reactions were also performed in a 50 mM sodium cacodylate buffer (pH 7.1) with all other conditions being the same, and no halide exchange was observed.

(b) A solution of 7-(2-fluoroethyl)deoxyguanosine (0.5 g, 1.5 mmol) and 3 mL of 50 mM Tris buffer in D_2O/DCl (pH 7.1) was thermostated at 37 °C for 24 h. The solution was evaporated to dryness under vacuum, and the residue was heated in 1 N deuterium chloride (2 mL) at 100 °C for 1 h. The solution was made neutral with 20% potassium hydroxide and the precipitated filtered and washed with water and dried. Recrystallization from methanol gave trideuterated 7-(2-fluoroethyl)guanine with no deuterium exchange on the side chain.

Experiments to Mimic Hydrophobic Microenvironment. (a) To a solution of 7-(2-fluoroethyl)guanine (0.025 g, 0.127 mmol) and 18-crown-6 ether (0.335 g, 1.27 mmol) in dimethyl sulfoxide (2.0 mL) was added potassium bromide (0.151 g, 1.27 mmol), in a 3-mL airtight Reactivial equipped with a Teflon septum, thermostated at 37 °C for 24 h. The solution was evaporated to dryness by lyophilization. Water (10 mL) was added and the residue was filtered, washed with cold water, and dried. Recrystallization from methanol afforded only unreacted 7-(2-fluoroethyl)guanine and therefore no halide exchange was observed.

(b) To a stirred solution of 1,3-bis(2-fluoroethyl)urea (0.02 g, 0.127 mmol) and 18-crown-6 ether (0.335 g, 1.27 mmol) in acetonitrile (2.0 mL) was added potassium bromide (0.151 g, 1.27 mmol), in a 3-mL airtight Reactivial equipped with a Teflon septum, thermostated at 37 °C for 24 h. The solution was evaporated to dryness under vacuum, and the residual solid was purified by column chromatography (silica gel, $CHCl_3$) to afford only unreacted 1,3-bis(2-fluoroethyl)urea and therefore no halide exchange was observed.

Chromatography. The HPLC system consisted of a Waters Model 510 LC pump (Waters Associates, Milford, MA) and a Rheodyne Model 7125 injection valve with a 100-μL sample loop. The LC column was reversed-phase Whatman 4.6 mm × 25 cm Partisil 5 ODS-3 (Whatman, Clifton, NJ) connected to a Waters Model 441 fixed-wavelength UV detector (254 nm), which was output to a linear 800 series recorder (Lineman, CA). The eluent flow rate was maintained at 0.8 mL/min with a mobile phase of 10% methanol in 0.1 M ammonium acetate buffered to pH 4.35 with acetic acid. The exit of the UV detector was connected directly to a heated nebulizer LC/MS interface (SCIEX, Thornhill, ON).

Mass Spectrometry. HPLC/MS and HPLC/MS/MS analyses were performed with a SCIEX TAGA 6000E triple-quadrupole mass spectrometer utilizing the atmospheric pressure ion source (API) and the heated nebulizer LC/MS interface.

Eluent flow rates of 0.8 mL/min were continuously introduced into the API under either LC/MS or LC/MS/MS conditions. Typical interface operating conditions were as follows: nebulizer heater temperature, 535 °C; nebulization pressure, 720 kPa; makeup flow rate, 4.0 L/min. In addition, air at atmospheric pressure enters the ion source at a flow rate of 12 L/min by an air pump connected to the plenum chamber (also used to remove the vaporized mobile phase). The interface dry nitrogen gas flow was kept at 200 mL/min. These exact conditions were also used in the flow injection experiments.

The mass spectrometer was operated in the positive-ion mode, since high sensitivity is expected for the analytes under investigation (nitrogen-containing bases).[23c] In this mode, API reagent ions generated in the reaction zone from the HPLC effluent are NH_4^+ clusters with water, methanol, and ammonia molecules. Under LC/MS conditions, conventional positive-ion mass spectra were obtained with quadrupole 1 (Q_1) in the mass filter mode with a resolution of 300 and with quadrupole 3 (Q_3) in the total ion mode. The typical scan rate was 0.2 s/decade, and the m/z 60–350 range was scanned by Q_1 (both data acquisition and analysis are under software control). Background-subtracted spectra are plotted by averaging 4–8 scans (depending on chromatographic peak width) associated with the maxima of a specific peak in the reconstructed ion chromatogram and subtracting an average background spectrum. The background spectrum is obtained by averaging a combination of 6–12 scans taken just before and after elution of the HPLC peak of interest. LC/MS/MS experiments were undertaken by operating the mass spectrometer in the CAD fragment ion mode, using argon as the collision gas (effective target thickness was about 3×10^{14} atoms cm^{-2}; collision gas energy, 48 eV). Unit resolution (full width at half maximum = 0.6 Da) was maintained across the mass range scanned by Q_3 in all LC/MS/MS experiments. CAD spectra are plotted by averaging 5–10 scans taken during elution of the component of interest.

Both the interface and the mass spectrometer were checked daily by running a 10^{-5} M adenosine solution through the system while keeping all other parameters, those of the interface and of the MS, constant. This precaution was necessary to ensure that optimum conditions for signal stability and sensitivity were met.

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