Spectroscopic Characterization of the Initial C8 Intermediate in the Reaction of the 2-Fluorenylnitrenium Ion with 2′-Deoxyguanosine

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Abstract: Irradiation of 2-azidofluorene in an aqueous solution containing 2′-deoxyguanosine (dG) gives good yields of 8-(2-fluorenylamino)dG. This is the C8 adduct implicated in the carcinogenicity of 2-aminofluorene, and formed in vivo from the reaction of DNA-guanine and an ester of *N*-hydroxy-2-aminofluorene. Flash photolysis of the azide reveals two intermediates on the pathway that forms this adduct, the 2-fluorenylnitrenium ion, and a subsequent longer-lived species formed in the reaction of this ion with dG. A number of pieces of evidence identify this later intermediate as the initial C8 adduct derived from addition of the nitrenium ion to the C8 carbon prior to loss of the C8 proton. Both spectroscopic and kinetic analyses show that the latter actually exists in both a cationic acid form and a base form, with a pK_a for the acid of 3.9. The base form is a tautomer of the final product and is the species present at pH 7. There is also evidence that the reaction of the nitrenium ion and dG that forms this intermediate proceeds directly by addition at C8. In other words, the substitution of ArNH$^+$ for H$^+$ at the C8 position of 2′-deoxyguanosine is a straightforward electrophilic aromatic substitution.

2-Aminofluorene has received extensive investigation since it and its *N*-acetyl derivative were found to be potent carcinogens over 50 years ago.[1] These two compounds are representative of a diverse class of carcinogenic aromatic and heteroaromatic amines present in the environment in tobacco smoke, automobile exhaust, broiled meats and fish, and as side products of industrial processing.[2] A common feature of the class is the transfer of the arylamine group to DNA. The predominant site of attachment on DNA is guanine. Within this base, the major adduct is **3** involving the C8 position attaching to the carcinogen's nitrogen (Scheme 1).[1,3]

Although these C8 adducts were first structurally characterized over 30 years ago, mechanistic details have been uncertain. It is now generally accepted that bioactivation through oxidation and (in most cases) O-esterification is required. The esters **1** so formed have been generally assumed to react via an S_N1 pathway, with an an electrophilic intermediate, the arylnitrenium ion **2**, as the species that actually reacts with guanine.[4] Suggestions however have also appeared of a direct interaction at the ester stage, in what is essentially an S_N2 reaction of guanine at the ester nitrogen.[5] Only recently have experiments from the Novak group conclusively established the nitrenium

Scheme 1

R = H, COCH$_3$ X = COCH$_3$, SO$_3^-$
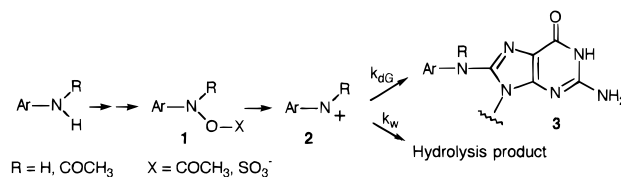
pathway. They found that hydrolysis of three examples of **1** in the presence of 2′-deoxyguanosine (dG) gave substantial amounts of the C8 adduct, but with no change in the rate constant.[6] This is classic evidence for the nucleophile reacting with an intermediate and not in the rate-limiting step of the reaction. A similar observation has recently been reported with a DNA oligomer.[7]

Our group has corroborated this conclusion through laser flash photolysis (LFP) experiments involving the same nitrenium ions.[8,9] In addition to the direct observation of these electrophiles, the absolute rate constants k_w and k_{dG} were measured, the former representing the decay of the cation in the solvent alone and the latter, the reaction with added dG. One of the

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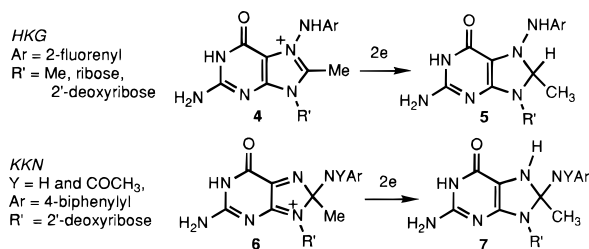(3) Dipple, A. *Carcinogenesis* **1995**, *16*, 437−441.

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Scheme 2

Table 1. Reactivities of Arylnitrenium Ions with Water and 2′-Deoxyguanosine[a]

cation	k_w (s^{-1})[b]	k_{dG} (M^{-1} s^{-1})[c]
$C_6H_5C_6H_4$-4-NH$^+$ (**8**)	1.8×10^6	2.0×10^9
4-MeC$_6$H$_4$C$_6$H$_4$-4-NH$^+$ (**9**)	2.7×10^5	1.5×10^9
fluorenyl-2-NH$^+$ (**10**)	2.7×10^4	7.6×10^8
4-MeOC$_6$H$_4$C$_6$H$_4$-4-NH$^+$ (**11**)	1.6×10^3	3.6×10^7

[a] At 20 °C, in 20% acetonitrile, pH 7. [b] Reference 8d for (**8**)(**10**), 8e for (**8**)(**11**). [c] Reference 8f.

Results

Generation of Nitrenium Ions. The 2-fluorenylnitrenium ion **10** was the principle electrophile employed in these studies; however, to investigate structural effects, a few experiments were performed with the biphenylyl derivatives **8**, **9**, and **11**. The rate constants k_w and k_{dG} for these ions are given in Table 1. All four show a high selectivity for dG. For example, the nucleoside will trap 53, 85, 96, and 96% of **8**−**11**, respectively when its concentration is 1 mM. This means that the intermediate that arises in the nitrenium−dG reaction can be generated in excellent yield even at low dG concentrations.

All four cations were generated photochemically from azide precursors.[8b,d,e] The intermediate that is initially formed is a very short-lived (~100 ps)[8d] singlet nitrene, and the nitrenium ion arises by protonation by the solvent (eq 1). Pathways involving

$$ArN_3 \xrightarrow{h\nu} {}^1[ArN] \xrightarrow{H_2O} ArNH^+ \qquad (1)$$
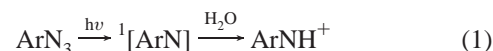
ring expansion to a didehydroazepine or formation of triplet nitrene[12] also compete, so that the yield of nitrenium-derived products is not 100%.[8d,e] A detailed discussion of the photogeneration of arylnitrenium ions via this approach is given in previous publications.[8b−f] An important feature is that for the 4-biphenylyl- and 2-fluorenylnitrenium ions, there is a high chemical and quantum yield even at neutral pH. This arises since the singlet nitrene is quenched very efficiently by solvent.

C8 Adduct from Azidofluorene. Irradiation of 2-azidofluorene in an aqueous solution containing dG gives rise to substantial amounts of the C8 adduct derived from the 2-fluorenylnitrenium ion. The adduct was isolated and purified from a scaled-up reaction and then employed as a standard in quantitative HPLC analysis of experiments at varying dG concentration (Figure 1).
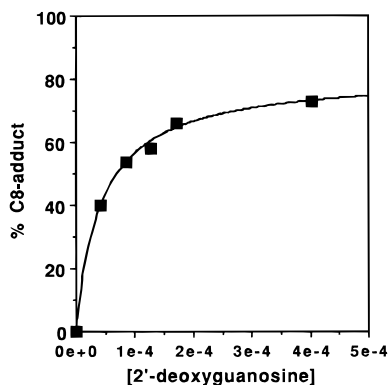
The data were fit to eq 2, which pertains to a competition

$$\%C8\ \text{adduct} = (\%C8_{max})\left(\frac{(k_{dG}{:}k_w)[dG]}{1 + (k_{dG}{:}k_w)[dG]}\right) \qquad (2)$$

between dG and water for the nitrenium ion (Scheme 1), but where the maximum yield of adduct is less than 100%. This provided $k_{dG}{:}k_w = (2.3 \pm 0.4) \times 10^4$ M^{-1}, in good agreement with the ratio from LFP (2.8×10^4, Table 1). The maximum yield of C8 adduct is 81%. The 19% not accounted for probably arises from the other reactions of the singlet nitrene, as just discussed.

Laser Flash Photolysis. Growth of Intermediate. As shown in Figure 2, the 2-fluorenylnitrenium ion is strongly absorbing with a λ_{max} at ~450 nm. A smaller peak is seen at 335 nm, but this is due to some other species formed from the singlet nitrene.[8d,e] In the solvent alone, the OD decreases from 315 to 600 nm, with the same rate constant throughout. There is even

important observations to emerge was that the selectivities k_{dG}:k_w were in excellent agreement with those found by the Novak group in their analysis of the products of the ground-state ester hydrolyses.[8c,f] Thus, there can be no doubt that the transients observed by LFP are ground-state arylnitrenium ions, that these same species are formed in the ground-state hydrolysis of ester precursors, and that these electrophiles do react with guanine derivatives to form the C8 adduct.

The problem then arises that C8 is not regarded as the normal position of electrophilic addition in guanine.[3,10] Experimental evidence for initial bond formation at N7 was claimed by Humphreys, Kadlubar, and Guengerich (HKG). By employing a system that generated the 2-fluorenylnitrenium ion in the presence of 8-methylguanine derivatives, an unstable species and its reduction product were obtained and characterized as N7 adducts **4** and **5** (Scheme 2).[10] Kennedy, Novak, and Kolb (KNK) then performed similar experiments with 8-MedG and precursors to both the *N*-acetyl and the parent 4-biphenylylnitrenium ions. They also observed an unstable species and a reduction product, but in this case assigned C8 structures **6** and **7**.[6b,11] These seemingly contradictory results are difficult to reconcile, particularly considering that both groups included a nitrenium ion ArNH$^+$ reacting with 8-MedG. This uncertainty thus leaves the question as to the initial site of attachment still open.

In our LFP experiments with dG present, we observed a growth of absorbance at 300−370 nm occurring with the same rate as the decay of the nitrenium ion. Since there was no further spectral change, we had assigned this growth to the formation of the final product of the reaction. A lamp flash photolysis apparatus however has revealed that such changes were occurring, only at much longer times. This species therefore is an intermediate, moreover, one that that forms in the reaction of the nitrenium ion and dG. This paper addresses the structure of this intermediate, and the implication for the mechanism for the formation of the final C8 adduct.

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(11) KNK still suggested N7 as the initial site of attack, on the basis of a correlation of reactivity with N7 basicity.[6b] This latter argument breaks down when imidazoles are included in the correlation.[8f]

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Figure 1. Yield of C8-adduct upon 300-nm irradiation (Rayonet) of 2-azidofluorene (20 μM) in 20% acetonitrile (0.002 M Na_2HPO_4:0.002 M NaH_2PO_4) containing varying amounts of 2'-deoxyguanosine. The yield is based on the amount of azide reacted for irradiation times sufficient to cause 30−40% conversion. The curve is drawn on the basis of eq 2, using the parameters given in the text.
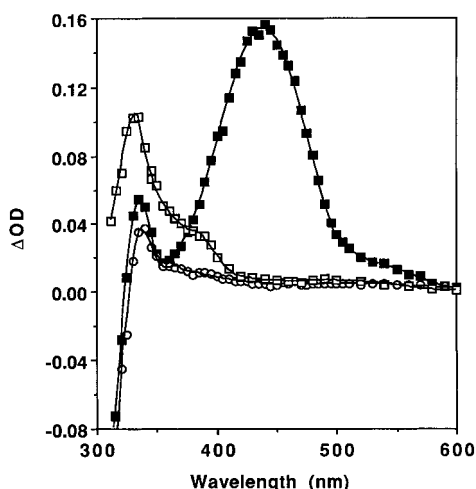

Figure 2. Spectra obtained on 308-nm laser irradiation of 2-azidofluorene (20 μM) in 20% acetonitrile (0.002 M Na_2HPO_4:0.002 M NaH_2PO_4). (■) data after the laser pulse, (□) data at the completion of the first-order process with 0.0012 M 2'-deoxyguanosine present, and (○) data at the completion of the first-order process when there is no 2'-deoxyguanosine present.

a small decay at 315−350 nm, indicating that even here there is a some nitrenium absorbance. At the completion of the decay, there is little OD left, other than the band for the other species at 335 nm.

The decay is accelerated with dG present, by a factor of 30 at the concentration of Figure 2. Now, however, there is a significant growth of absorbance below ∼380 nm, with some residual absorbance even out to 420 nm. This growth is observed whenever there is sufficient dG to compete with the solvent. It always occurs with the same first-order rate constant, within experimental error, as that for the decay of the nitrenium ion (Figure 3).

Growths producing similar final spectra are seen with **8**, **9**, and **11** in the presence of dG, again with rate constants that are identical to those measured for the decay at the λ_{max} of the nitrenium ion.

Lamp Flash Photolysis. Decay of Intermediate. The experiments that demonstrate that the species whose growth is observed by LFP is also an intermediate are shown in Figure 4. The curve labeled Diff in this figure is the spectrum of a sample of the authentic C8 adduct **3** (labeled C8 in the Figure),
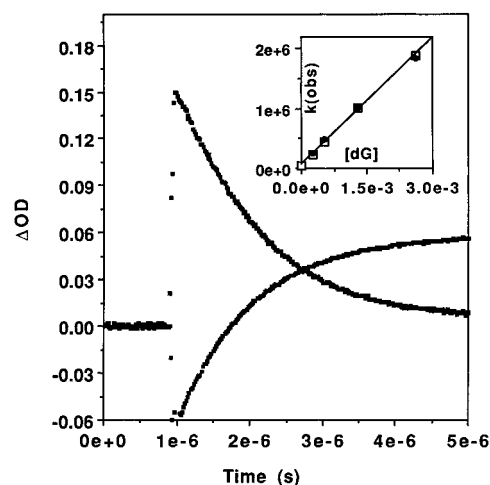

Figure 3. Kinetic traces at 450 and 316 nm for the solution of Figure 2 containing 0.0012 M dG. The insert shows the dependence of the first-order rate constants measured at these two wavelengths on the concentration of dG: (□) 450 nm, (●) 316 nm.
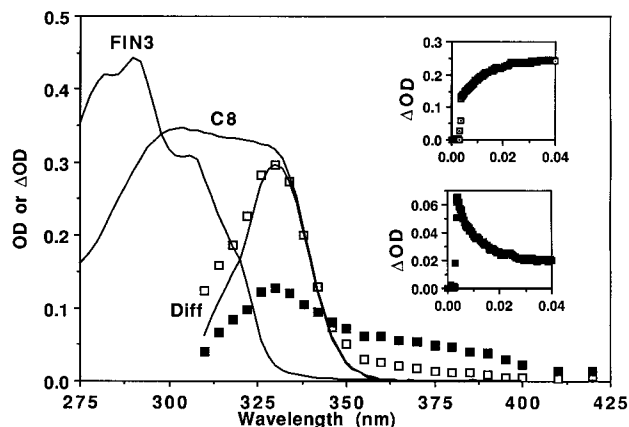
Figure 4. Spectra obtained on flash lamp excitation of the solution of Figure 2 (with 4 μM 2-azidofluorene). (■) ΔOD obtained after the lamp flash; (□) ΔOD after completion of the first-order kinetic process. The inserts show these kinetic traces at 335 nm (rise) and 365 nm (fall). The curves labeled FlN3 and C8 are the spectra (true ODs) in 20% acetonitrile of equal concentrations (17 μM based on extinction coefficients) of 2-azidofluorene and the final C8 adduct. The curve Diff is a ΔOD, the OD of the C8 adduct minus the OD of the azide. The curves for FlN3, C8, and Diff have been placed on the figure by multiplying every absorbance by the same correction factor, calculated so that the ΔOD for Diff at 330 nm is identical to the ΔOD at this wavelength for the open squared point.

corrected by subtracting the spectrum for the same concentration of 2-azidofluorene (FlN3). Since the flash photolysis experiments measure the OD after irradiation minus the OD before, Diff mimics what would be observed if the azide were replaced by the ultimate product, the C8 adduct **3**.[13] Comparing this with the spectrum of the LFP product shows that while both have an apparent maximum near 330 nm, the C8 adduct does not absorb beyond 350 nm, where the LFP product has a pronounced shoulder. This led us to suspect that the latter was not the final product, and we sought conditions where we could observe a further reaction. This proved possible with lamp flash photolysis. The further changes occur on the millisecond time scale, and constitute a decrease in OD above 345 nm, with a quite substantial increase below. The process is first-order, with the

(13) This applies to wavelengths above 310 nm only. Below this wavelength, the dG present in the solution also absorbs.
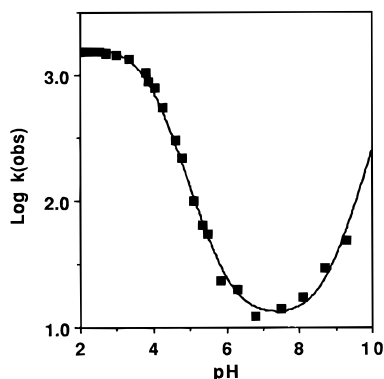
Figure 5. Log k(obs)-pH profile for the reaction of the intermediate from the 2-fluorenylnitrenium ion and 2′-deoxyguanosine. Conditions: 20% acetonitrile, 20 °C, ionic strength 0.1 M (NaClO$_4$). The data below pH 3.5 were obtained in dilute HClO$_4$ solutions, from pH 3.5−5.3 in acetate buffers, from pH 5.5−6.9 in cacodylate buffers, and from pH 7−9 in Tris buffers. In the case of the buffer solutions, the rate constants were obtained by extrapolating to zero buffer concentration. The curve is drawn according to eq 3 (see Discussion) using parameters given in the text.

same rate constant in the increasing and decreasing regions. It can be noted that the "initial" spectrum in Figure 4 is that of the LFP product, since the fast process occurs within the ∼100 μs lamp flash. Although an exact match is not expected because of the other photochemical products, the final spectrum is close to that of the C8 adduct. Moreover, there is no further change, as seen by recording spectra of the irradiated solutions on a diode array spectrometer.

Kinetics of Decay of Intermediate. Kinetic experiments were performed by following the millisecond time scale decay at 365 nm at 20 °C in 20% acetonitrile with the ionic strength at 0.1 M maintained with NaClO$_4$. Initial experiments demonstrated that rate constants measured in the same buffer solution were independent of both the initial azide concentration (from 2 to 20 μM) and the initial dG concentration (from 100 μM to 2 mM). The later experiments then involved 4 μM azide and 500 μM dG. No OD change was observed in the absence of dG or the absence of the azide.

(a) Rate-pH Profile. The intermediate from the 2-fluorenylnitrenium ion was the one investigated in detail, and its rate−pH profile is shown in Figure 5.

(b) Buffer Catalysis. The reaction is catalyzed by buffers, in some cases quite significantly. The data were analyzed by plotting k(obs) at a given buffer ratio versus total buffer concentration. These plots were linear, and the slopes, k(cat), were in turn plotted against $\alpha(A^-)$, the fraction of the buffer in the base form (see Figure 6 for two examples). For cacodylate, phosphate, and Tris buffers, these plots follow the expected linear relation. For acetate however, the plot is curved.

(c) Aryl Substituent Effect. The decrease in OD at 365 nm (and increase at lower wavelengths) is also seen with the intermediates from **8**, **9**, and **11**. Rate constants for all four intermediates were compared in three solutions: (A) 0.001 M HClO$_4$, (B) a pH 7.6 phosphate buffer containing 0.007 M Na$_2$HPO$_4$:0.003 M NaH$_2$PO$_4$, and (C) a pH 4.6 acetate buffer containing 0.005 M HOAc:0.005 M NaOAc. In each solution the rate constants for the four were within ±5%: 1.45 × 10^3 s^{-1} (A), 2.7 × 10^2 s^{-1} (B), 4.9 × 10^2 s^{-1} (C).

(d) C8-Deuterium Isotope Effect. Rate constants for the intermediates from dG and 8-deuterio-dG with the 2-fluorenylnitrenium ion were obtained in the same three solutions. A
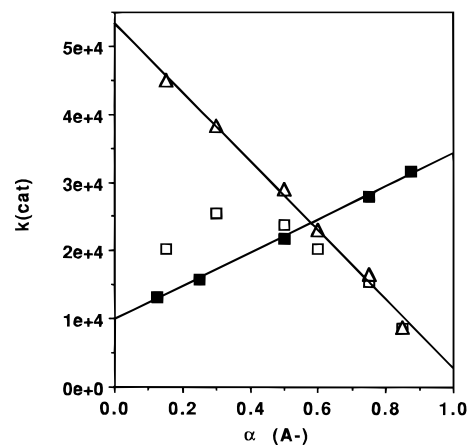
Figure 6. Buffer data for the reaction of Figure 5. k(cat) is the slope of the plot of k(obs) versus total buffer concentration at a given buffer ratio, and $\alpha(A^-)$ is the fraction of buffer in the base form. (■) cacodylate, (□) acetate, (△) corrected acetate data where k(cat) has been divided by $\alpha(B)$ (see discussion).
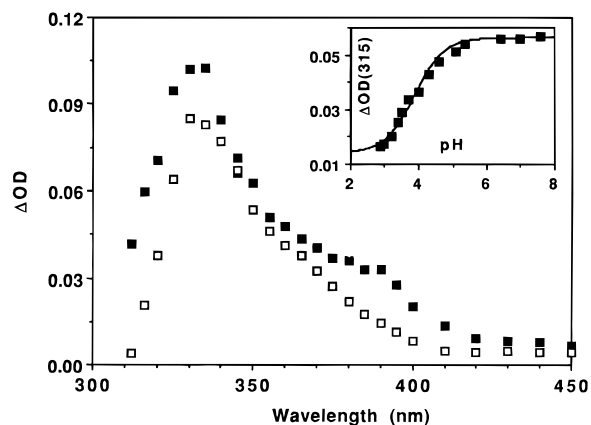
Figure 7. Optical densities at completion of fast kinetic process on 308-nm laser irradiation of 2-azidofluorene (20 μM) in 20% acetonitrile containing 1 mM 2′-deoxyguanosine. The main figure compares spectra at pH 7 (■) and pH 3 (□). The insert shows the pH dependence of the OD at 315 nm.

significant isotope effect is observed in all three: $k_{8H}:k_{8D} =$ 5.6 (A), 7.4 (B), and 6.1 (C).

C8-Deuterium Isotope Effect on the Nitrenium Ion Decay. The isotope effect on k_{dG}, the fast reaction of the nitrenium ion with dG, was $k_{dG-8H}:k_{dG-8D} = 0.98 \pm 0.02$ (**9**), 0.955 ± 0.02 (**10**), and 0.88 ± 0.025 (**11**).

Spectroscopic Acidity Constant. As shown in Figure 7, there is a slight difference in the intermediate's spectra at pH 7 and 3. By working at 315 nm where the difference is quite significant, a titration curve was obtained, and from this, an acidity constant of $(1.3 \pm 0.2) \times 10^{-4}$ M ($pK_a = 3.9$).

Discussion

Kinetic Analysis. Since this has some impact on the question of structure, we begin by presenting an analysis of the kinetics of the reaction of the intermediate. There are two key observations here. The first is the isotope effect which shows that the C8 proton is being removed in the rate-limiting step of the reaction. The second is that the intermediate exists in acid:base forms with a pK_a of 3.9. Combining these with the observed kinetic patterns gives rise to a mechanistic scheme where both the acid and base forms are reacting, and moreover they each

C8 Adduct in Reaction of dG and Nitrenium Ion

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Scheme 3
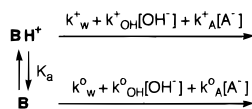
Table 2. Rate Constants for the Reaction of the Intermediate from the 2-Fluorenylnitrenium Ion and 2′-Deoxyguanosine[a]

catalyst	$k_A(M^{-1} s^{-1})$	$k^+_A(M^{-1} s^{-1})$
acetate	3×10^3	2.5×10^5
cacodylate	3.4×10^4	2.5×10^6
phosphate dianion	5.5×10^4	1.5×10^7
tris	1.8×10^4	b
hydroxide	2.1×10^7	
water	1.4×10^1	1.7×10^3

[a] 20% acetonitrile, 20 °C, ionic strength 0.1 M (NaClO$_4$). [b] Acid intercept not statistically significant. Units are s^{-1}.

do so with water, hydroxide, and the buffer base removing the C8 proton (Scheme 3).

This provides the rate expression

$$k(\text{obs}) = \frac{k^+_w[H^+] + (k^+_{OH}K_w + k^o_wK_a) + k^o_{OH}K_wK_a/[H^+]}{[H^+] + K_a} \quad (3)$$

The experimental date were fit to this equation with four adjustable parameters including K_a. The values so obtained were $k^+_w = 1.65 \times 10^3$ s^{-1}, $(k^+_{OH}K_w + k_w{}^oK_a) = 1.53 \times 10^{-3}$ M·s^{-1}, $k^o_{OH}K_wK_a = 2.9 \times 10^{-12}$ M^2·s^{-1}, and $K_a = 1.25 \times 10^{-4}$ M. The kinetic K_a obviously agrees very well with the spectroscopic one. In terms of the rate−pH profile, the acid region (pH < 5.5) is due to the acid form reacting with solvent (k^+_w term), with the break at pH 4 as the equilibrium shifts from acid to base. The dependence at high pH represents hydroxide ion deprotonating the base (k^o_{OH} term). Kinetic ambiguity exists around pH 7, where there is a short region where the rate is approximately pH independent ($k^+_{OH}K_w + k_w{}^oK_a$ term), and which could represent hydroxide reacting with the acid or water with the base. In fact, it must be the latter. Even with $k^+_{OH} = 1 \times 10^{10}$ M^{-1} s^{-1}, the contribution of $k^+_{OH}K_w$ to the observed $(k^+_{OH}K_w + k_wK_a)$ is only 1%.[14]

For the buffer component, $k(\text{cat})$ is given by

$$\frac{k(\text{cat})}{\alpha(\mathbf{B})} = \left(\frac{k^+_AK_{HA}}{K_a}\right)(1 - \alpha(A^-)) + k^o_A\alpha(A^-) \quad (4)$$

where K_{HA} is the acidity constant of the buffer acid and $\alpha(\mathbf{B})$ is the fraction of the intermediate in the base form. With pK_a = 3.9, $\alpha(\mathbf{B})$ is essentially unity for all pHs for the cacodylate, phosphate, and Tris buffers, and the plots of $k(\text{cat})$ versus $\alpha(A^-)$ are linear. Only in acetate buffers does $\alpha(\mathbf{B})$ change, and thus the $k(\text{cat}) - \alpha(A^-)$ plot curves. As can be seen in Figure 6, correcting by dividing by $\alpha(\mathbf{B})$ does produce a reasonably linear plot.

According to eq 4 the plots of $k(\text{cat})$ or $k(\text{cat})/\alpha(\mathbf{B})$ versus $\alpha(A^-)$ have an intercept at unit $\alpha(A^-)$ of k^o_A and an intercept at zero $\alpha(A^-)$ of $k^+_AK_{HA}/K_a$. Table 2 provides the catalytic coefficients so obtained, using pK_a = 3.9 to calculate k^+_A. Values for the water and hydroxide rate constants obtained from

(14) The pK_w in 20% acetonitrile is 14.8.[8d]

(15) Zoltewicz, J. A.; Clark, D. F.; Sharpless, T. W.; Grahe, G. *J. Am. Chem. Soc.* **1970**, *92*, 1741−1750.

(16) Haines, J. A.; Reese, C. B.; Todd, L. *J. Chem. Soc.* 5281−5288. Hecht, S. M.; Adams, B. L.; Kozarich, J. W. *J. Org. Chem.* **1976**, *41*, 2303−2311.
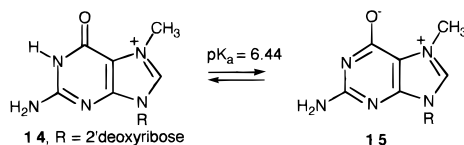
Scheme 4
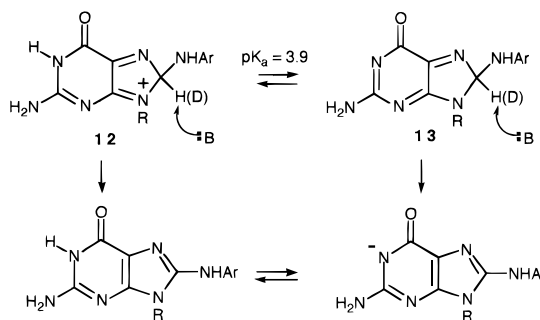
the pH profile are also included. The Bronsted plots using the phosphate, cacodylate, acetate, and water rate constants (with the water constant converted to second-order), are two parallel lines with slopes of 0.6 separated by 2 log units. It is noteworthy that the rate constants for the acid and base form differ by only a factor of about 100-fold.

As mentioned at the start of this section the primary $k_H:k_D$ is an key aspect of this model. This effect was observed in three solutions, and we can now provide a breakdown of the species contributing in each. In the HClO$_4$ solution (A), the rate constant being measured represents 100% of the reaction of water with the acid form, in the phosphate buffer (B), 99% of the reaction of phosphate dianion with the base form, and in the acetate buffer (C) about 50% each of water and acetate reacting with the acid form, with only a small percentage (2%) of acetate reacting with the base form. The observations of the primary $k_H:k_D$ in (A) and (C) are important since there is a kinetically equivalent alternative to the processes responsible for the rate constants k^+_w and k^+_A. This involves processes where H$_3$O$^+$ or the buffer acid reacts directly with the base form. This possibility however seems highly unlikely in terms of the kinetic isotope effect. Particularly considering the nature of the final product, this surely must be indicating a reaction where a base is removing the C8 proton in the rate-limiting step.
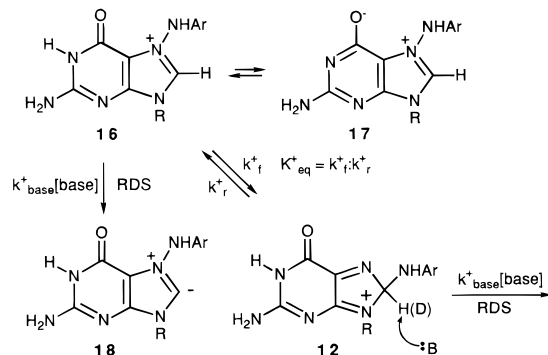
Structure of the Intermediate. There are a number of experimental observations that provide clues as to the structure of the intermediate. (i) It has an absorption spectrum that extends out to 400 nm, especially in its base form. (ii) Its reaction must involve a mechanism where the C8 proton of guanine is being transferred in the rate-limiting step. (iii) There is no effect of the aryl substituent on the rate constant for its decay. (iv) It exists in acid:base forms with a pK_a of 3.9. (v) Both the acid and the base forms are reactive.

The structure that best fits these criteria is the one where the nitrenium ion has added directly to the guanine C8 carbon, so that the acid form is the cation **12** and the base **13** is obtained by loss of the N1 proton (Scheme 4). These structures are consistent with the isotope effect (point ii), since both the acid and the base form can proceed to product by simply losing the C8 proton (point v). There should be little dependence on the Ar substituent, which is well removed from the site of deprotonation (point iii). Both forms are highly conjugated, consistent with the absorption spectra (point i). In terms of the pK_a value (point iv), **12** is required to be a little over 5 log units more acidic than parent dG. There is no direct comparison to show whether this is reasonable. N7 alkylation is known to increase acidity, as shown, for example, by 7-methyl-2′-deoxyguanosine **14** (Scheme 5), which has a pK_a of 6.4.[15] This is less than the effect observed in this study, but **12** might be expected to be more somewhat more acidic, since its conjugate base can be written as the charged neutralized structure **13**. The conjugate base of **14** must be written as a zwitterion (e.g., **15**).

Scheme 5

pK_a = 6.44

1 4, R = 2'deoxyribose   **1 5**

Scheme 6

1 6 ⇌ **1 7**

k^+_{base}[base] | RDS k^+_f $K^+_{eq} = k^+_f : k^+_r$
                                          k^+_r

1 8   **1 2** k^+_{base}[base]
                                       RDS

An N7 structure must also be considered, particularly in light of the HKG results. In this case the acid form is the cation **16** and the base a zwitterion such as **17** (Scheme 6). These two structures are directly analogous to the N7-methylated **14** and **15**, and we would argue that the latter system predicts a pK_a closer to 6. The arylamino at N7 in **16** is electron-withdrawing relative to methyl, but it is difficult to imagine this effect increasing the acidity by the 2.5 log units required to achieve the pK_a observed for the intermediate in this work. The comparison with the N7-methylated system also shows that there is a problem with the spectra, since neither **14** or **15** exhibit much absorbance above 300 nm.[16] There is obviously an arylamino chromophore in **16**, **17** as well, but the parent arylamines also do not absorb much beyond 320−330 nm. Since there is no conjugative interaction between the two chromophores, their combination should not lead to absorbance out to 400 nm. A caveat here is that there could be some form of charge-transfer interaction.

There are two possible mechanisms to explain the isotope effect if the intermediate were N7, and these are illustrated for the acid form in Scheme 6. One is the suggestion by HKG,[10] a rate-limiting deprotonation at C8 to give a zwitterion, followed by rapid transfer of the arylamino group. The second is the KNK proposal,[6b] which has the arylamino group migrating from N7 to C8, followed by deprotonation.

The zwitterion mechanism appears to be immediately dismissable. Guanine derivatives exchange their C8 proton by a mechanism that involves N7 protonation, followed by rate-limiting deprotonation at C8 to give a zwitterion.[17] The kinetics require that hydroxide ion be the base, with no indication of a process where water removes the C8 proton.[17] Moreover, an upper limit of 10^{-5} s^{-1} can be placed on such a water deprotonation.[18] This is 10^8 lower than the 10^3 value of k^+_w, the rate constant that would represent water deprotonating **16** to form the zwitterion **18** if Scheme 6 were the mechanism.

(17) Tomasz, M.; Olson, J.; Mercado, C. M. *Biochemistry* **1972**, *11*, 1235−1241.

(18) At pH 3 and 37 °C, 1-methylguanosine (1-MeG) exchanges its C8 proton with a rate constant of 1.1×10^{-6} s^{-1}.[17] Since the pK_a of N7-protonated 1-MeG is 2.3,[17] the maximum rate constant for water deprotonating the cation to form a zwitterion is 7×10^{-6} s^{-1}. This, moreover, is an upper limit, since the kinetics shows no sign that such a process is occurring.

For the migration mechanism, the rate-limiting deprotonation requires that the migration step be an equilibrium. The observed rate constants in Table 2 would then be the product of an equilibrium constant and a rate constant, i.e., k^+_A of Table 2 = $K^+_{eq}k^+_{base}$ (or $k_A = K^o_{eq}k^o_{base}$). Thus the actual k^+_{base} or k^o_{base} would be larger than the values in Table 2 by a factor of $1/K_{eq}$. To avoid processes occurring faster than diffusion, the smallest value possible for K_{eq} is then 10^{-3}, since some k_A are already of the order of 10^7. The equilibrium condition is satisfied when k_{base}[base], the rate constant for deprotonation, is significantly less than k_r, the rate constant for the reverse C8-to-N7 migration. The largest value of k_A(obs)[base] measured in this work was 1×10^4 s^{-1}. Using a value of K_{eq} of 10^{-3}, the actual value of k_{base}[base] is 1×10^7 s^{-1}, and k_r must then be greater than 10^8 s^{-1}. Although such a rate constant is not impossible, it does seem very large for a reaction in which the migrating group must move from C8 by bonding with the N7 lone pair which is actually pointing in the opposite direction. Finally, it can be noted the larger rate constants for k_{base} seem inconsistent with the Brønsted β value and the near maximal isotope effect, both of which suggest a transition state for the proton transfer that is close to being symmetrical. In summary, although the migration mechanism cannot be unequivocally ruled out, it seems unlikely, particularly when the above considerations are coupled with the evidence from the pK_a and spectra.

Direct Conversion of Nitrenium Ion to C8 Intermediate. There is further evidence in favor of the C8 intermediate in the form of the small inverse isotope effect on k_{dG}, the rate constant for the reaction of the nitrenium ion and dG. The important system here is the 4′-methoxy derivative. The 4′-methylbiphenylyl- and 2-fluorenylnitrenium ions were not expected to show much of an effect, since these react at close to the diffusion limit of $2−2.5 \times 10^9$ M^{-1} s^{-1}.[8f] These were included in the analysis so as to validate the experimental procedure. Indeed their $k_H{:}k_D$ values are close to unity, albeit slightly inverse at 0.98 and 0.955. The 4′-methoxy derivative, on the other hand, reacts well below the diffusion limit (Table 1), and its $k_H{:}k_D$ of 0.88 is significantly less than 1. An inverse secondary isotope effect of this nature is the result expected for a reaction where the isotopic substitution takes place on a carbon undergoing a change of hybridization from sp^2 to sp^3. The implication is that the nitrenium ion must be bonding at C8 in the transition state when it reacts with dG. By combining the information on the intermediate as discussed in the previous section, the most consistent interpretation by far is found to be one where the long-lived species is the C8 intermediate, and this forms directly in the nitrenium-dG reaction.

Conclusions. Irradiation of 2-azidofluorene in aqueous solutions containing dG results in significant yields of the C8 adduct **3**, and flash photolysis has revealed two intermediates of the reaction. One is the nitrenium ion and the second, longer-lived species, is the C8 adduct prior to loss of the C8 proton. This exists in a cationic acid form **12** and a neutral conjugate base form **13**, with a pK_a of 3.9. The base is a tautomer of the final product and is the species present at pH 7. The C8 intermediate appears to form directly in the nitrenium:dG reaction. In other words, substitution of ArNH$^+$ for H$^+$ at the C8 position of a guanine derivative is a straightforward electrophilic aromatic substitution.

One of the arguments against electrophilic addition at C8 is that this position is less nucleophilic than some of the others in guanine. The present results, however, demonstrate that the intermediate cation **12** is relatively very stable, at least in a kinetic sense. This cation is directly analogous to the cyclo-

hexadienyl cation intermediates of electrophilic substitution of benzene derivatives. A relevant comparison here is with **19**, whose rate constant for deprotonation by water is 5.8×10^5 s^{-1}.[19] This cation would be regarded as being relatively stabilized, since it has three methoxy groups correctly positioned to directly conjugate the three $C^{\delta+}$ centers. Less stabilized examples are much shorter-lived, and in fact require less basic solvents for their observation by LFP.[19,20] Even with the stabilizing substituents, **19** is over 2 orders of magnitude more short-lived than **12** (see k^+_w in Table 2). Thus, in terms of kinetic stability, **12** appears as a highly stabilized "cyclohexadienyl" cation. In this respect it is perhaps not so surprising that the nitrenium ions can react readily at C8 to form such a cation. Interestingly, recent experiments in our laboratories have shown that the arylnitrenium ions of this study are quenched by 1,3,5-trimethoxybenzene, with rate constants that are similar to k_{dG}.

MeO — [structure] — OMe, OMe

19
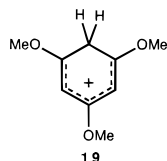
The present results have implications for the reaction with DNA-guanine. In particular the deprotonation of the intermediate that occurs with the monomer at pH 7 involves a proton that is involved in hydrogen bonding in the DNA double helix. We also argue that the cationic form **12** acquires a considerable stabilization by delocalization involving electrons on N1 and the C2-nitrogen.[21] These centers are again involved in hydrogen bonding. In both respects it is therefore interesting that double-stranded DNA appears to be very much less efficient at trapping the *N*-acetyl-2-fluorenylnitrenium, as compared to both single-stranded DNA and the monomer dG.[7] A source of this effect could be the inhibition of the resonance stabilization/deprotonation imposed by the double helix structure.

Experimental Section

The azide precursors were available from previous studies.[8d,e] 2'-Deoxyguanosine was commercially available and used as received. This was converted to its 8-deuterio form by refluxing in D$_2$O overnight.[22] The ^{1}H NMR of this material showed it to be 99% deuterated (integrating the C8-H signal versus the signals for the deoxyribose). 8-(2-Fluorenylamino)-2'-deoxyguanosine was obtained by irradiating (300 nm) 2-azidofluorene (50 mg) in 500 mL of 20% acetonitrile containing 0.5 g of 2'-deoxyguanosine. The volume of the solution was then reduced to 10 mL, and the C8 adduct was isolated by preparative HPLC (0.5-mL injections onto 25 × 250 mm C18 semipreparative column, with 20:80 acetonitrile:water eluting at 20 mL/min, and eluant collected from 25 to 30 min) followed by short column chromatography (silica gel, 50:50 ethyl acetate:hexanes). The sample so obtained had NMR[23] and UV[10] identical to those in the literature.

Laser Flash Photolysis. Experiments involved ca. 20-ns pulses at 308 nm (60−120 mJ per pulse) from a Lumonics excimer laser. A pulsed Xenon lamp provided monitoring light. After passing through a monochromator, the signal from the photomultiplier tube was digitized and sent to a computer for analysis. Solutions were 10−20 μM azide in 20% acetonitrile, with the substrate being added from a stock solution

in acetonitrile just before irradiation. Spectra were constructed point-by-point, with three measurements being made and averaged at each wavelength.

Conventional Flash Photolysis. Experiments were performed using an apparatus previously described.[24] Samples were 4 μM substrate and were irradiated with a broad band flash lamp of ca. 100-μs duration.

Quantitative Analysis of the C8 Adduct. Analysis (Figure 1) was carried out with HPLC. The solution contained 20 μM 2-azidofluorene and was irradiated in a Rayonet reactor at 300 nm for a time sufficient to give 30−50% conversion. The HPLC conditions were as follows: μ-Bondepak C18 column, 2 mL/min of 85:15 water:acetonitrile isocratic for 7 min and then a linear gradient over 7 min to 20:80 water:acetonitrile, monitoring wavelength = 320 nm. The C8 adduct had a retention time of 7 min, and the 2-azidofluorene, 12.5 min. The areas of the peaks were converted to moles using a correction factor obtained by injecting known amounts of the authentic samples. The yield of C8 adduct was calculated as moles C8 adduct divided by moles azide reacted, where the latter was determined from the peaks before and after irradiation.

Determination of Isotope Effect on Decay of Nitrenium Ion in the Presence of dG. A large quantity of a stock buffer containing 20% acetonitrile and 0.001 M Na$_2$HPO$_4$:0.001 M Na$_2$HPO$_4$ was prepared. This was added to two 100-mL volumetric flasks, one of which contained a weighed amount of 2'-deoxyguanosine, and the other contained an equivalent amount of 8-deuterio-2'-deoxyguanosine. Concentrations of the dG derivatives were in the range 0.5−2 mM. The two solutions were divided in three parts (with a small amount being set aside for HPLC analysis), and 10 μM of 4'-methyl-4-azidobiphenyl, 2-azidofluorene, and 4'-methoxy-4-azidobiphenyl were added to each from a 20−40 mM stock solution of the azides in acetonitrile. Three solutions of the three azides in the stock buffer (no dG) were also prepared. The nine solutions were analyzed by laser flash photolysis with 308 nm irradiation, with the first-order rate constants for the decay of the nitrenium ions being determined at the following λ_{max}: 490 nm (**9**), 450 nm (**10**), and 500 nm (**11**). Five to six measurements were made with each solution (with the laser cuvette being replenished with fresh solution after each pulse). The relative dG concentration in the two solutions was accurately determined by HPLC. The HPLC conditions were 95:5 water:acetonitrile at a flow rate of 2 mL/min through a C18 column with the detector set at 270 nm. Exactly 80 μL of each the two solutions was injected alternatively, with four (sometimes five) injections of each being made. The isotope effect was calculated with the equation

$$\frac{k_{dG-8H}}{k_{dG-8D}} = \frac{\left(\dfrac{k_{decay}(dG\text{-}8H) - k_{decay}(\text{no dG})}{\text{HPLC area}(dG\text{-}8H)} \right)}{\left(\dfrac{k_{decay}(dG\text{-}8D) - k_{decay}(\text{no dG})}{\text{HPLC area}(dG\text{-}8D)} \right)} \quad (5)$$

where the rate constants k_{decay} were the average of the measurements made in the three solutions, and the HPLC areas were the average of the four or five injections. It can be noted that the solutions were prepared in such a way that the concentrations of dG-8H and dG-8D were very similar. We felt, however, that the HPLC method was more accurate than the weights in determining the relative concentrations. It can also be noted that the concentrations of dG were such that the rate constant with dG was generally greater than 5 times faster than the rate constant without dG, especially with **10** and **11**. This minimizes the error in subtracting the latter from the former.

In general, we found that the HPLC areas were determined with a standard deviation of ±1.2% and the rate constants with a standard deviation of 1.5%. To tighten the error limits on the measurement, the entire procedure was performed a total of 18 times. The numbers given in the Results are the average of these, with 1 standard deviation.

Spectroscopic Acidity Constant. Solutions were prepared that contained 1.00 mM 2'-deoxyguanosine, 20% acetonitrile, and various combinations of phosphate, acetate, and formate buffers and HClO$_4$ so as to provide solutions of pH varying from 2.5 to 8. Immediately before

(19) Steenken, S.; McClelland, R. A. *J. Am. Chem. Soc.* **1990**, *112*, 9648−9649.

(20) Mathivanan, N.; Cozens, F.; McClelland, R. A.; Steenken, S. *J. Am. Chem. Soc.* **1992**, *114*, 2198−2203.

(21) The delocalization involving the NH$_2$ group explains why inosine is less effective than dG at trapping nitrenium ions at C8.[6b,8f]

(22) Tsang, P.; Vold, R. R.; Vold, R. L. *J. Magn. Reson.* **1987**, *71*, 276−283.

(23) Kriek, E.; Westra, J. G. *Carcinogenesis* **1980**, *1*, 459.

(24) Allen, A. A.; Kresge, A. J.; Schepp, N. P.; Tidwell, T. T. *Can. J. Chem.* **1987**, *65*, 1719.

LFP, 2-azidofluorene was added from a stock solution in acetonitrile so as to give a final concentration of 20 μM. This solution was subjected to 308 nm LFP, with three traces being recorded at both 450 and 315 nm, with fresh solution being employed in each case. The time scale at 315 nm was set such that the first-order increase had reached its final value by the end of the trace. The insert to Figure 7 is this OD plotted against the measured pH of the solution. The acidity constant was obtained by fitting the data to the equation

$$OD(obs) = \frac{OD(base)K_a + OD(acid)[H^+]}{K_a + [H^+]} \quad (6)$$

where OD(base) and OD(acid) are the optical densities of the base form and acid form, respectively. The former was set as the average of the three values at pH > 6.5. Since the flat region in acid could not be reached, the latter was left as an adjustable parameter along with K_a in the fitting process.

This experiment requires that the concentration of the intermediate be the same at each pH, which in turn requires that the same amount of nitrenium ion be produced and that it be trapped to the same extent by dG. To ensure that this was the case, there was the same concentration of the reagents in every solution, the concentration of dG was such that a substantial fraction of the nitrenium ion reacted with the nucleoside, and the experiment was carried out over a short period of time to ensure that the solution received the same laser dose. The requirements were nonetheless verified by the traces recorded at 450 nm. The initial absorbance at this wavelength measures the amount of nitrenium ion that is formed in the laser pulse, and this was indeed constant ($\pm5\%$). The rate constant for the decay provides a measure of the extent of trapping, through the following equation:

$$\text{fraction of reaction with dG} = \frac{k(\text{obs with dG}) - k(\text{obs no dG})}{k(\text{obs with dG})} \quad (7)$$

where k(obs with dG) and k(obs no dG) are the rate constants for the solution with 1 mM dG and a solution at the same pH without dG. Both were effectively constant at $(8.0 \pm 0.6) \times 10^5$ and $(3.0 \pm 0.4) \times 10^4$ s^{-1} from pH 3 to pH 8, respectively, so that throughout this pH range $\sim$96% of the cation is trapped by dG. Below pH 3, the rate constant with dG decreases, presumably because the nucleoside is being converted into its protonated form.[6] In consequence, the OD measurements could not be extended into more acidic solutions.