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Identification of New DNA Adducts of Phenylnitrenium

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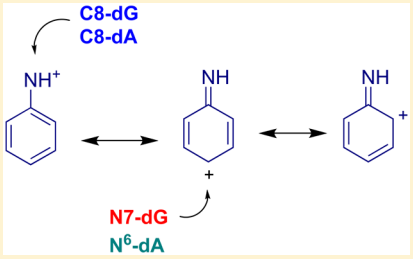
ABSTRACT: Phenylnitrenium ion ($PhNH^+$) may bind to nucleophiles through nitrogen as well as through C2 or C4 carbons. However, only adducts of the former type have been hitherto reported after its reaction with purine nucleosides. In this study, reactions of *N*-acetoxyaniline (PhNHOAc), a precursor to $PhNH^+$, with 2′-deoxyadenosine (dA), 2′-deoxyguanosine (dG), and with DNA *in vitro* at physiological conditions are described. The reaction of PhNHOAc with dA followed by a hydrolytic deribosylation afforded 8-phenylaminoadenine (C8-PhNHA) together with a smaller amount of N^6-(4-aminophenyl)adenine (N^6-4APA). A similar reaction with dG afforded 8-phenylaminoguanine (C8-PhNHG) together with traces of 7-(4-aminophenyl)guanine (N7-4APG). The same adducts were found also in the DNA treated with PhNHOAc, and all of them were identified by comparison of their HPLC retention times and MS^2 spectra with a set of synthesized authentic adenine adducts at C2, C8, *N*7, and N^6 positions and guanine adducts at C8, *N*7, and N^2 positions. The newly identified minor adduct, N7-4APG, represents the first proof of arylnitrenium adduction at the *N*7 position of dG, which is the prominent site of attack by most C-electrophiles.



INTRODUCTION

Arylnitrenium ions are the ultimate mutagens, and carcinogens formed by the dissociation of arylhydroxylamine esters with sulfate and/or acetate in the course of the biotransformation of many carcinogenic aromatic and heteroaromatic amines as well as of nitroaromatics.[1,2] Their main DNA binding site appears to be the C8 position of guanine, but many minor adducts at other sites such as C8 and N^6 of adenine and N^2 of guanine have also been reported.[3−5] In general, the C8 guanine position is a favored site for radical attacks. In contrast, most electrophiles react mainly at the *N*7 position of guanine.[6,7] This certainly holds for C-electrophiles, such as alkylating and arylating agents. To explain the formation of the C8-*N* adducts, several different mechanisms were proposed: (i) attack of the nitrenium ion primarily at the *N*7 position with subsequent migration of the arylamino group to C8 and deprotonation,[8,9] (ii) direct attack of the nitrenium ion at C8 followed by deprotonation,[10] and (iii) primary attack by C2 of the resonance stabilized nitrenium ion at *N*7 followed by an imidazolidine ring closure and deprotonation accompanied by ring reopening to yield the C8-*N* adduct as the end-product.[11] The mechanism (iii), proposed by Parks et al., involves the formation of the 7-(2-aminophenyl)guanine (N7-2APG) intermediate, which in its protonated form rearranges to 8-phenylaminoguanine (C8-PhNHG). According to quantum chemical calculations, C8-PhNHG is the most stable phenylnitrenium adduct of guanine.[11] The attack at *N*7 by an electrophilic carbon atom seems to be very likely as the arylnitrenium ions are resonance stabilized, and the charge is delocalized through the aromatic system. In the case of phenylnitrenium ($PhNH^+$), which is the simplest model arylnitrenium ion, nucleophiles may attack both the *o*- and *p*-positions. Therefore, when reacting with dG two *N*7 adducts, namely, 7-(2-aminophenyl)-2′-deoxyguanosine (N7-2APdG) and 7-(4-aminophenyl)-2′-deoxyguanosine (N7-4APdG), can be expected. Despite numerous reports on arylnitrenium DNA adducts derived from a wide variety of aromatic and heteroaromatic amines as well as from nitroaromatics,[1−5] to our knowledge, no carbon-bound arylnitrenium adduct at the guanine *N*7 position has been reported as yet. The lack of information about this type of adduct may be due to the unavailability of authentic samples of 7-(aminoaryl)guanines, and these adducts may have escaped identification. To test our hypothesis that arylnitrenium ions can form stable adducts also by binding through one of the aromatic carbons at the *N*7 position of guanine and, possibly, also of adenine, we decided to synthesize authentic samples of the expected phenylnitrenium *N*7 adducts as well as some other possible adducts with Ade and Gua. They were used to support our search for adducts formed by the reaction of activated phenylhydroxylamine as a source of $PhNH^+$ with dG, dA, and DNA under physiological conditions. These compounds (Chart 1) are deribosylated analogues of dG and dA adducts and can therefore be used as reference standards for the identification of products obtained by the reaction of PhNHOAc with purine nucleosides followed by hydrolytic treatment.

EXPERIMENTAL PROCEDURES

General. Polymer-bound 1,5,7-triazabicyclo[4.4.0]dec-5-ene (TBD) was purchased from Novabiochem (Germany). 2′-Deoxyadenosine monohydrate and 2′-deoxyguanosine monohydrate (Fluka), 2-phenylaminoadenosine (Sigma), palladium catalyst (10 wt % Pd on charcoal, Degussa type, Aldrich), and acetonitrile, LC/MS grade, were purchased through Sigma-Aldrich, Prague, Czech Republic.

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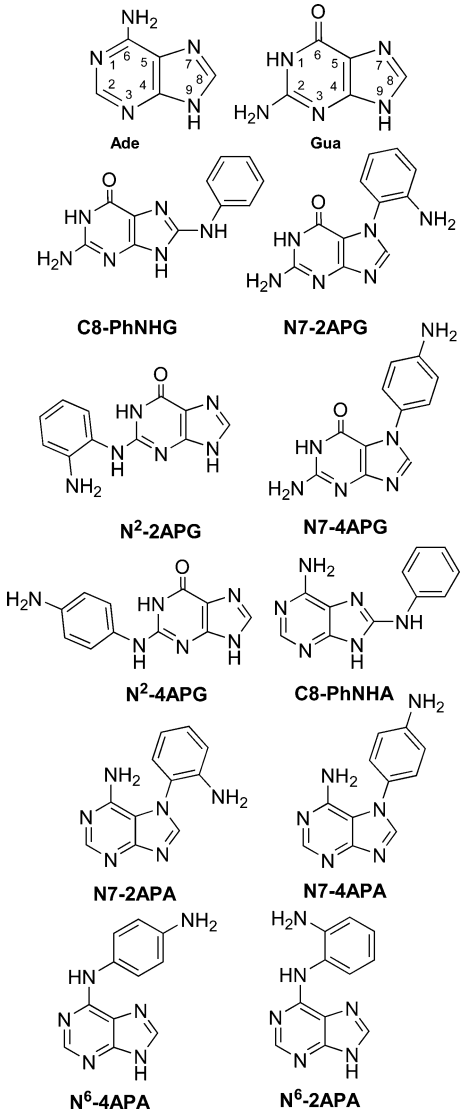
Triethylamine (TEA) was dried over calcium hydride, distilled, and kept over molecular sieves. For LC/MS, distilled water was passed through a Milli-Q water purification system (Millipore, Bedford, MA). Phenylhydroxylamine (PhNHOH) was prepared according to Liu et al.[12] and N^2-(dimethylaminomethylene)guanine (DMF-G) according to Votruba et al.[13] Other chemicals were of analytical or synthetic grade and were used as received. LC/MS analyses were carried out on a Thermo Scientific LXQ linear trap mass spectrometer in tandem with a Janeiro LC system consisting of two Rheos 2200 pumps and a CTC PAL autosampler. Electrospray ionization in the positive ion mode was used. The collision gas was helium, the capillary temperature was set to 300 °C, and the capillary voltage was 4.6 kV. The collision energy was adjusted to achieve a nearly full fragmentation of parent $(M + H)^+$ ions using reference standards of the phenylnitrenium adducts. NMR spectra were taken on a Varian Mercury 300 MHz spectrometer or a Bruker Avance 600 MHz spectrometer. HRMS analyses were performed on an LTQ Orbitrap Velos instrument (Thermo Fisher Scientific) using electrospray ionization (ESI) in the positive mode.

Chart 1. Structures of Potential Adenine and Guanine Adducts with $PhNH^+$ Prepared as Reference Standards[a]



[a]The numbering of the purine ring is also shown.

Preparation of Authentic Purine Adducts. 7-(2-Aminophenyl)-adenine (N7-2APA), 7-(4-aminophenyl)adenine (N7-4APA),[14] 8-phenylaminoadenine (C8-PhNHA),[15] 8-phenylaminoguanine (C8-PhNHG),[16] and 8-phenylamino-2′-deoxyguanosine (C8-PhNHdG)[17] were prepared as reported in the literature.

7-(4-Aminophenyl)guanine (N7-4APG). DMF-G (288 mg, 1.4 mmol), 4-nitrophenylboronic acid (467 mg, 2.8 mmol), and *N,N,N′,N′*-tetramethylethylenediamine (TMEDA) (420 μL, 325 mg, 2.8 mmol) were dissolved in dry methanol (20 mL) and Cu(II) acetate (255 mg; 1.4 mmol), and activated molecular sieves 4 Å (1 g) were then added to the solution. The resulting mixture was stirred at 45 °C in an apparatus protected from moisture by a tube filled with anhydrous calcium chloride. After 24 h, the reaction mixture was diluted with chloroform (100 mL) and filtered through a thin layer of Cellite. Column chromatography on silica gel afforded the crude product containing a 5:1 mixture of isomeric 7 and 9-(4-nitrophenyl)-guanine derivatives. Further chromatographic separation on silica gel using a chloroform−methanol mixture as an eluent (the concentration of methanol was increased gradually from 2 to 10% v/v) afforded 200 mg (44%) of 7-(4-nitrophenyl)-N^2-(dimethylaminomethylene)-guanine (N7-4NPDG) as a yellow powder. ^{1}H NMR (DMSO-d_6): δ = 3.04 and 3.18 (2 s, 3 + 3 H, NCH_3); 7.94 (d, J = 9.1 Hz, 2H, Ar−H); 8.36 (d, J = 9.1 Hz, 2H, Ar−H); 8.53 (s, 1 H, 8-H); 8.69 (s, 1 H, N═CH); 11.63 (s, 1H, NH) ppm. ^{13}C NMR (DMSO-d_6): δ = 110.6 (C5); 125.0 and 125.8 (C2′, C3′, C5′, and C6′); 141.3 (C1′); 146.6 (C4′); 155.3 (C2); 158.1 (C6); 158.7 (C-amidine); 161.4 (C4) ppm. HRMS (m/z): for $C_{14}H_{14}N_7O_3$ $(M + H)^+$ calculated, 328.1153; found, 328.1154; for $C_{14}H_{13}N_7O_3Na$ $(M + Na)^+$ calculated, 350.0972; found, 350.0972.

Heating of N7-4NPDG (200 mg) in 30 mL of 1 M HCl for 5 h at 75 °C followed by neutralization with an aqueous ammonia solution afforded a yellow precipitate, which was filtered off and washed with

Scheme 1. Synthesis of 7-Aminophenylguanines N7-2APG and N7-4APG

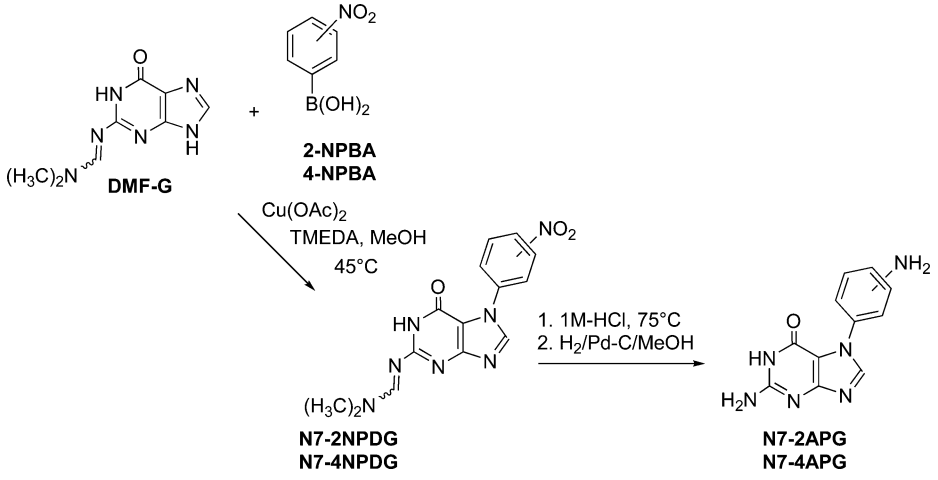


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Table 1. Diagnostic ^{13}C NMR Signals of the *N*7-Arylguanine Derivatives

compd	chemical shift	
	δ(C4) [ppm]	δ(C5) [ppm]
N7-2NPDG	159.7	111.2
N7-4NPDG	161.9	110.6
N7-2NPG	160.9	108.5
N7-4NPG	n.d.[a]	n.d.[a]
N7-2APG	160.6	109.6
N7-4APG	160.9	108.9

[a] ^{13}C NMR spectrum could not be obtained due to the very low solubility of N7-4NPG in DMSO.

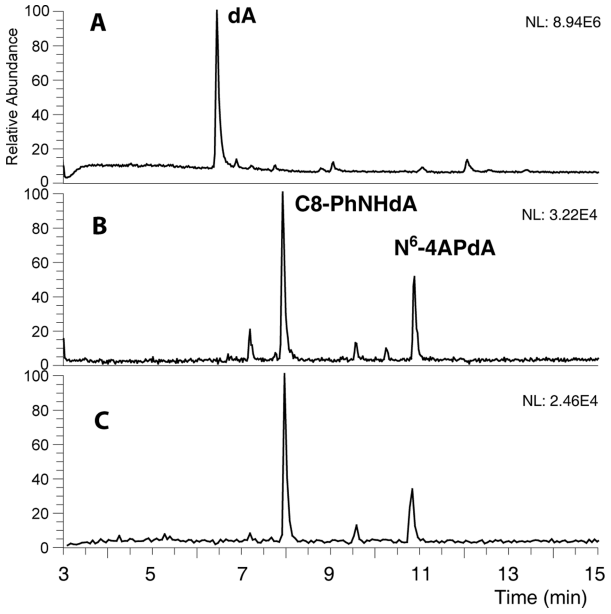

Figure 1. LC-ESI-MS analyses of phenylnitrenium adducts arising from the reaction of PhNHOAc with dA. Full scan ion chromatogram of untreated reaction mixture (trace A) and extracted ion chromatograms at *m*/*z* 343 for MH^+ of phenylnitrenium adducts with dA taken before (trace B) and after hydrolysis by heating the reaction mixture for 20 min at pH 7.4 to 100 °C (trace C).

water. After drying over P_4O_{10}, 140 mg (86%) of 7-(4-nitrophenyl)-guanine (N7-4NPG) as a yellow powder was obtained. ^1H NMR (DMSO-d_6): δ = 6.33 (s, 2H, NH_2); 7.91 (d, J = 9.1 Hz, 2H, Ar−H); 8.34 (d, J = 9.1 Hz, 2H, Ar−H); 8.44 (s, 1 H, 8-H); 10.99 (s, 1 H, NH) ppm. HRMS (*m*/*z*): for $C_{11}H_9N_6O_3$ $(M + H)^+$ calculated, 273.0731; found, 273.0733; for $C_{11}H_8N_6O_3Na$ $(M + Na)^+$ calculated, 295.0550; found, 295.0551.

The nitro group of N7-4NPG was reduced by transfer hydrogenation on palladium. The palladium catalyst (20 mg 10% Pd−C, 0.018 mmol) was activated by heating to 95 °C in a vacuum for 30 min. Dry methanol (12 mL), N7-4NPG (33 mg, 0.12 mmol), and ammonium formate (500 mg, 8 mmol) were added, and the reaction mixture was refluxed under argon. After 12 h, another portion of ammonium formate was added (500 mg, 8 mmol), and the reaction was continued for another 12 h. After cooling, the catalyst was filtered off, washed thoroughly with hot water to remove excess ammonium formate, and then extracted with DMSO. The extract was evaporated to dryness in a vacuum, the residue triturated with water, filtered off, and dried over P_4O_{10}. A yellowish powder (9 mg, 31%) was identified as N7-4APG. ^1H NMR (DMSO-d_6): δ = 5.29 (s, 2H, NH_2); 6.12 (s, 2H, NH_2); 6.58 (d, J = 8.6 Hz, 2H, Ar−H); 7.13 (d, J = 8.2 Hz, 2H, Ar−H); 7.97 (s, 1H, 8-H); 10.71 (s, 1H, NH) ppm.

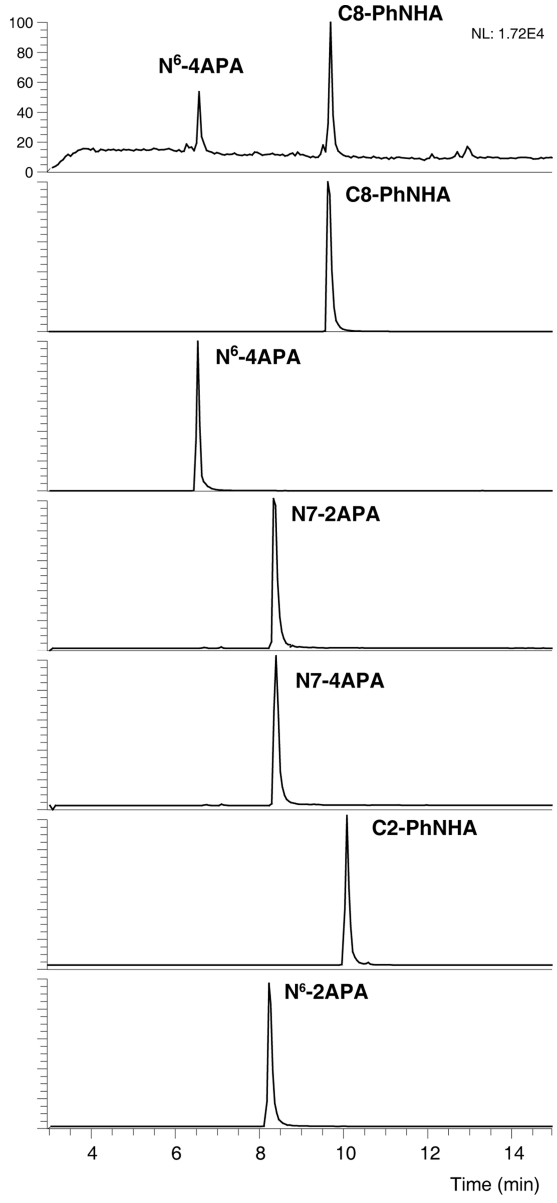

Figure 2. LC-ESI-MS^2 of phenylnitrenium adducts with adenine obtained by the reaction of PhNHOAc with dA followed by acidic hydrolysis (0.1 M HCl, 100 °C) taken at *m*/*z* 227 (top trace) as compared with authentic C8-PhNHA, N^6-4-APA, N^6-2-APA, N7-2APA, N7-4APA, and C2-PhNHA.

^{13}C NMR (DMSO-d_6): δ = 108.6 (C5); 113.7 (C2′ and C6′); 125.0 (C4′); 125.9 (C3′ and C5′); 143.3 (C1′); 148.0 (C8); 153.2 (C2); 154.3 (C6); 160.9 (C4) ppm.

7-(2-Aminophenyl)guanine (N7-2APG). Synthesis of this compound was accomplished in three steps as described for N7-4APG. The ratio of 7- to 9-(2-nitrophenyl)guanine derivatives obtained was 5:1. The yield of 7-(2-nitrophenyl)-N^2-(dimethylaminomethylene)guanine (N7-2NPDG) obtained as a yellow powder was 165 mg (35%). ^1H NMR (DMSO-d_6): δ = 3.00 and 3.15 (2 s, 3 + 3H, NCH_3); 7.79 and 7.90 (m, 1 + 1H, C4′-H and C5′-H); 8.24 and 8.27 (d, J = 9.1 Hz, 1 + 1H, C3′-H and C6′-H); 8.31 (s, 1 H, 8-H); 8.64 (s, 1 H, N═CH); 11.47 (s, 1H, NH) ppm. ^{13}C NMR (DMSO-d_6): δ = 111.2 (C5); 123.1 (C2′); 129.9, 130.1, and 130.4 (C3′, C4′, and C5′); 135.1 (C6′); 143.7 (C1′); 144.5 (C8); 155.3 (C2); 157.5 (C6); 158.4 (C-amidine); 159.7 (C4) ppm. HRMS (*m*/*z*): for $C_{14}H_{14}N_7O_3$ $(M + H)^+$ calculated, 328.1153; found, 328.1154; for $C_{14}H_{13}N_7O_3Na$ $(M + Na)^+$ calculated, 350.0972; found, 350.0974.

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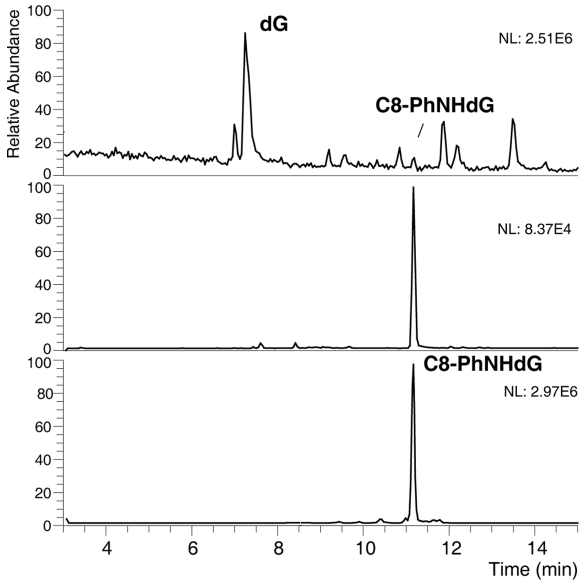

Figure 3. LC-ESI-MS analyses of phenylnitrenium adducts arising from the reaction of PhNHOAc with dG. Full scan ion chromatogram (top trace) and extracted ion chromatogram at m/z 359 for MH^+ of phenylnitrenium adducts with dG (middle trace) as compared with authentic 8-PhNHdG (bottom trace).

Acid hydrolysis of N7-2NPDG (1 M HCl, 5 h, 75 °C) afforded 7-(2-nitrophenyl)guanine (N7-2NPG) as an orange powder in 87% yield. ^{1}H NMR (DMSO-d_6): δ = 6.63 (br s, 2H, NH_2); 8.13 (d, J = 9.1 Hz, 2H, Ar−H); 8.24 (s, 1H, 8-H); 8.37 (d, J = 9.1 Hz, 2H, Ar−H); 10.81 (br s, 1H, NH) ppm. ^{13}C NMR (DMSO-d_6): δ = 108.5 (C5); 126.1 (C2′); 129.9, 130.1, and 130.4 (C3′, C4′, and C5′); 135.2 (C6′); 143.4 (C1′); 144.4 (C8); 153.6 (C2); 154.5 (C6); 157.0 (C-amidine); 160.9 (C4) ppm. HRMS (m/z): for $C_{11}H_9N_6O_3$ $(M + H)^+$ calculated, 273.0731; found, 273.0732; for $C_{11}H_8N_6O_3Na$ $(M + Na)^+$ calculated, 295.0552; found, 295.0556.

Transfer hydrogenation of N7-2NPG under the same conditions as those described for N7-4NPG afforded 6 mg (21%) of yellowish powder, which was identified as N7-2APG. ^{1}H NMR (DMSO-d_6): δ = 4.94 (s, 2H, NH_2); 6.12 (s, 2H, NH_2); 6.55−6.59 (m, 1H, Ar−H); 6.78 (d, J = 7.4 Hz, 1H, Ar−H); 6.98−7.01 (m, 1H, Ar−H); 7.09−7.13 (m, 1H, Ar−H); 7.85 (s, 1H, 8-H); 10.76 (s, 1H, NH) ppm. ^{13}C NMR (DMSO-d_6): δ = 109.6 (C5); 116.1 and 116.2 (C3′, C6′); 121.9 (C2′); 128.5 and 129.8 (C4′ and C5′); 144.1 (C6′); 143.4 (C1′); 145.1 (C8); 153.4 (C2); 154.3 (C6); 160.6 (C4) ppm.

***N*^{6}-(4-Aminophenyl)adenine (N^6-4APA).** 6-Chloropurine (310 mg, 2 mmol), *p*-phenylenediamine (430 mg, 4 mmol), and TEA (556 μL, 404 mg, 4 mmol) were refluxed in 15 mL of anhydrous 1-propanol for 3 h. The starting material was dissolved upon heating, and 30 min later, the product began to precipitate. After 3 h, the reaction mixture was allowed to cool, and the greyish precipitate was filtered off, washed with ethanol, and dried over P_4O_{10}. The crude product (434 mg, 96%) was recrystallized from aqueous ethanol to give 290 mg of (63%) of N^6-4APA. ^{1}H NMR (DMSO-d_6): δ = 6.55 (d, J = 8.5 Hz, 2H, Ar−H); 7.42 (d, J = 8.5 Hz, 2H, Ar−H); 8.15 (s, 1H, C2−H); 8.21 (s, 1H, C8−H); 4.90 (bs, 2H, NH_2); 9.25 (bs, 1H, NH); 13.0 (bs, 1H, NH) ppm. ^{13}C NMR (DMSO-d_6): δ = 114.2 (C2′ and C6′); 119.5 (C5); 123.3 (C3′ and C5′); 129.1 (C4′); 139.9 (C4); 145.0 (C1′); 150.4 (C2); 152.5 (C8) ppm.

***N*^{6}-(2-Aminophenyl)adenine (N^6-2APA).** The reaction of 6-chloropurine with *o*-phenylenediamine under the conditions described above for N^6-4APA proceeded more slowly, so that it was complete after 8 h. No precipitate was spontaneously formed. Therefore, the solvent was evaporated in a vacuum, and the residue triturated with water. The product (230 mg, 51%), which remained undissolved, was filtered off, washed with ethanol, and dried over P_4O_{10}. Crystallization from ethanol afforded 85 mg (19%) of yellowish powder, which was identified as N^6-2APA. ^{1}H NMR (DMSO-d_6): δ = 6.60 (t, J = 7.7 Hz, 1H, Ar−H); 6.76 (d, J = 8.2 Hz, 1H, Ar−H); 6.93 (t, J = 7.6 Hz, 1H, Ar−H); 7.26 (d, J = 7.9 Hz, 1H, Ar−H); 8.17 (C2−H); 8.18 (C8−H); 5.82 (bs, 2H, NH_2); 8.90 (bs, 1H, NH) ppm. ^{13}C NMR (DMSO-d_6): δ = 116.7 and 117.1 (C3′ and C5′); 120.0 (C5); 125.2 (C2′); 126.5 and 127.4 (C4′ and C6′); 143.9 (C1′); 150.5 (C2); 152.8 (C8); 153.9 (C6) ppm.

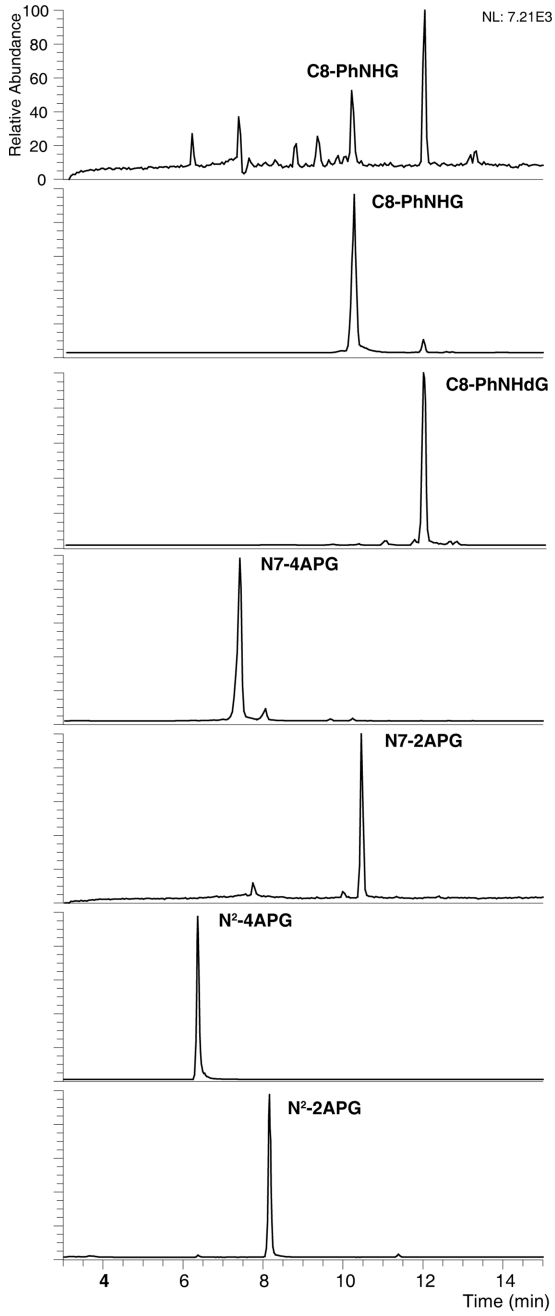

Figure 4. LC-ESI-MS^2 of phenylnitrenium adducts with guanine obtained by the reaction of PhNHOAc with dG followed by neutral hydrolysis (100 °C, 20 min). Extracted ion chromatogram taken at m/z 243 (top trace) as compared with authentic C8-PhNHG, C8-PhNHdG, N7-4APG, and N7-2APG, and N^2-4APG and N^2-2APG.

***N*^{2}-(4-Aminophenyl)guanine (N^2-4APG).** 2-Bromohypoxanthine (215 mg, 1 mmol), *p*-phenylenediamine (324 mg, 3 mmol), and TEA (73 μL, 101 mg, 1 mmol) were dissolved in 10 mL of dry 1-propanol, and the reaction mixture was refluxed for 4 h. After cooling, the precipitate formed was filtered off and washed thoroughly with aqueous ethanol followed by pure ethanol. After drying over P_4O_{10},

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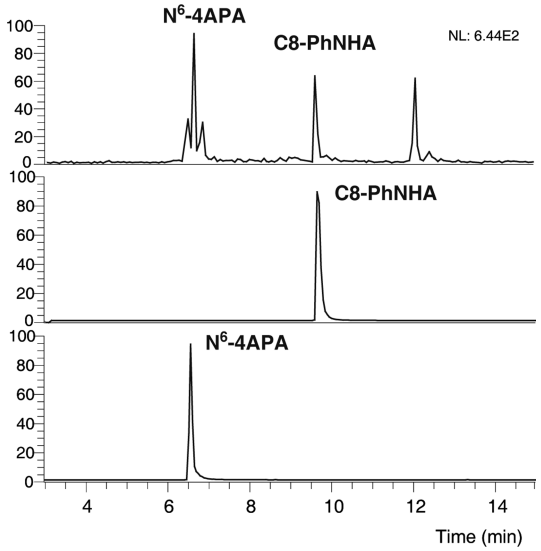


Figure 5. LC-ESI-MS^2 analyses of phenylnitrenium adducts with adenine in the DNA reacted with PhNHOAc. Extracted ion chromatogram of acid hydrolyzed DNA (top trace) taken at m/z 210 from MS^2 of the parent ion m/z 227 (MH$^+$) as compared with with authentic N^6-4APA and C8-PhNHA.

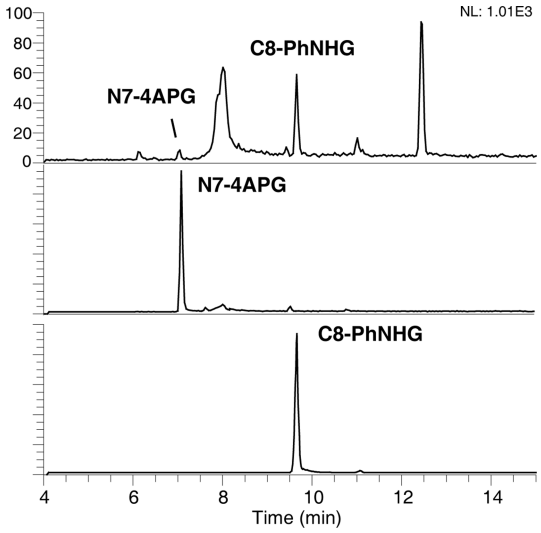
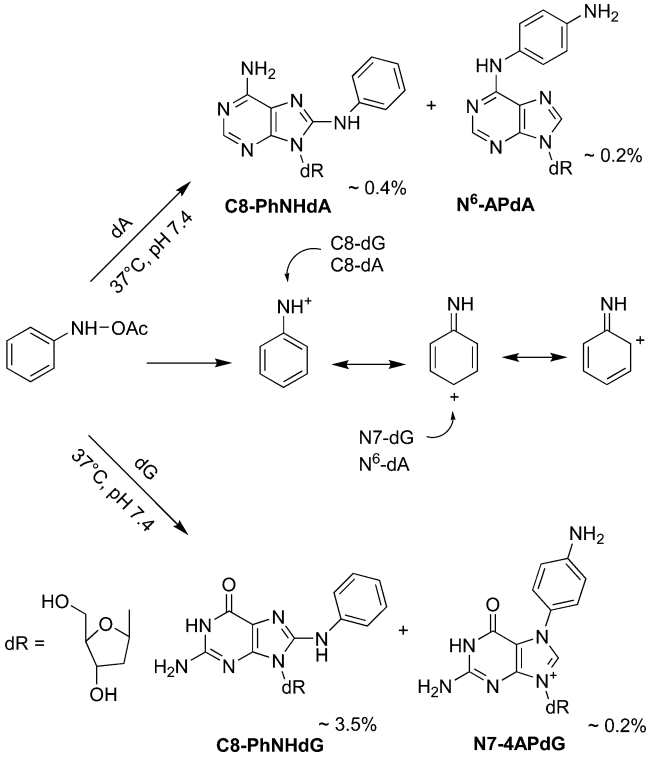


Figure 6. LC-ESI-MS^2 analyses of phenylnitrenium adducts with guanine in the DNA reacted with PhNHOAc. Extracted ion chromatogram of acid hydrolyzed DNA (top trace) taken at m/z 226 from MS^2 of the parent ion m/z 243 (MH$^+$) as compared with authentic N7-4APG and C8-PhNHG.

the product was obtained as a slightly greyish powder (160 mg, 66%). ^{1}H NMR (DMSO-d_6): δ = 4.95 (bs, 2H, NH_2); 6.52 (d, J = 8.3 Hz, 2H, C3′-H and C5′-H); 7.15 (d, J = 8.0 Hz, 2H, C2′-H and C6′-H); 8.1 (bs, 1H, NH); 10.3 (bs, 1H, NH) ppm. ^{13}C NMR (DMSO-d_6-DCl-D_2O): δ = 109.5 (C5); 121.6 and 124.4 (Ar−CH); 126.5 (C1′); 137.1 (C2); 138.3 (C4′); 149.3, 151.7, and 153.3 (C4, C6 and C8) ppm.

***N*^{2}-(2-Aminophenyl)guanine (N^2-2APG).** N^2-2APG was prepared as described for N^2-4APG using *o*-phenylenediamine instead of its *p*-isomer and 1-butanol instead of 1-propanol. The crude product obtained (70 mg, 33%) was recrystallized from ethanol to afford 20 mg (9%) of a beige powder. ^{1}H NMR (DMSO-d_6): δ = 4.95 (bs, 2H, NH_2); 6.55 (t, J = 7.9 Hz, 1H, C5′-H); 6.72 (d, J = 7.1 Hz, C6′-H); 6.90 (t, J = 8.1 Hz, C3′-H); 7.38 (d, J = 7.1 Hz, C3′-H); 7.62 (s, 1H, C8−H); 7.64 (bs, 1H, NH); 10.45 (bs, 1H) ppm. ^{13}C NMR (DMSO-d_6): δ = 108.4 (C5); 111.6; 116.3 and 116.8 (C3′-6′); 126.3 (C1′); 151.7 and 153.3 (C4 and C6) ppm.

Scheme 2. Formation of Phenylnitrenium Adducts by the Reaction of PhNHOAc with dG and dA



2-Phenylaminoadenine (C2-PhNHA). 2-Phenylaminoadenosine (10 mg, 0.028 mmol) was dissolved in 0.1 M HCl (4 mL) and stirred at 80 °C for 4 h. The solution was then allowed to stand overnight at room temperature. The white precipitate, which was formed upon neutralization with NaOH to pH 7, was filtered off, washed with water, and dried over P_4O_{10}. The product was obtained as a white powder (4 mg, 63%). ^{1}H NMR (DMSO-d_6): δ = 6.82 (t, J = 7.2 Hz, 2H, Ar−H); 7.20 (t, J = 7.6 Hz, 2H, Ar−H); 7.82 (d, J = 7.8 Hz, 1H, Ar−H); 8.74 (s, 1H, C8−H); 6.79 (s, 1H, NH); 7.79 (s, 1H, NH); 12.4 (s, 1H, NH) ppm. ^{13}C NMR (DMSO-d_6): δ = 114.0 (C5); 118.0 (C2′ and C6′); 119.8 (C4′); 128.1 (C3′ and C5′); 136.4 (C8); 141.8 (Ar−C); 151.4 (C4); 155.7 (C2); 156.7 (C6) ppm.

Reaction of *N*-Acetoxyaniline (PhNHOAc) with Purine Nucleosides. PhNHOH (4 mg, 0.04 mmol) was dissolved in 2 mL of dry tetrahydrofuran (THF), polymer-bound TBD (40 mg, 0.116 mmol) and pyruvonitrile (20 μL, 19 mg, 0.28 mmol) were added, and the resulting mixture was stirred for 10 min at room temperature. An aliquot of this solution (0.8 mL) containing activated PhNHOAc was added to a solution of corresponding nucleoside, i.e., dA (5.2 mg, 0.02 mmol) or dG (6 mg, 0.02 mmol) in 0.1 M phosphate buffer, pH 7.4 (6 mL). The reaction mixture was incubated at 37 °C for 1 h. In the course of incubation, the other two PhNHOAc aliquot solutions of 0.6 mL were subsequently added after 15 and 30 min. Samples of the reaction mixture were then hydrolyzed under neutral and acidic conditions. For the neutral hydrolysis, an aliquot of 2 mL was refluxed for 20 min. For the acidic hydrolysis, aliquots were mixed with equal volumes of 0.1 M HCl and heated at 60 or 100 °C for 20 min.

Reaction of *N*-Acetoxyaniline (PhNHOAc) with DNA. Calf thymus DNA (2.5 mg) was dissolved in 3 mL of a buffer solution consisting of 10 mM TRIS/HCl and 1 mM EDTA (pH 7.4). PhNHOAc solution in THF (1.5 mL), which was prepared from 3 mg (0.03 mmol) of PhNHOH, 15 mg (0.22 mmol) of pyruvonitrile, and 30 mg of polymer-bound TBD as described above, was added

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Table 2. Mass Spectra (ESI) of Potential Adenine and Guanine Adducts of Phenylnitrenium[a]

	(M+H)$^+$ (m/z)		formula	fragments (m/z)		abundance	formula
compd	found	Δ mmu		found	Δ mmu	[%]	
C8-PhNHA	227.1039	−0.1	$C_{11}H_{11}N_6$	210		100	$C_{11}H_8N_5$
				200		30	$C_{10}H_{10}N_5$
				185		5	$C_{10}H_9N_4$
N7-2APA	227.1040	0.0	$C_{11}H_{11}N_6$	210	210	100	$C_{11}H_8N_5$
				200	200	5	$C_{10}H_{10}N_5$
				185	185	7	$C_{10}H_9N_4$
N7-4APA	227.1040	+0.0	$C_{11}H_{11}N_6$	210.0774	−0.1	100	$C_{11}H_8N_5$
				200.0931	0.0	10	$C_{10}H_{10}N_5$
				185.0822	−0,2	5	$C_{10}H_9N_4$
N^6-2APA	227.1041	+0.1	$C_{11}H_{11}N_6$	210.0774	+0.7	100	$C_{11}H_8N_5$
				200.0931	+0.6	28	$C_{10}H_{10}N_5$
N^6-4APA	227.1042	+0.2	$C_{11}H_{11}N_6$	210.0781	+0.7	100	$C_{11}H_8N_5$
				200.0937	+0.6	30	$C_{10}H_{10}N_5$
				186.0781	+0.7	7	$C_9H_8N_5$
				134.0717	+0.5	22	$C_7H_8N_3$
				108.0688	+0.6	7	$C_6H_8N_2$
C2-PhNHA	227.1044	+0.4	$C_{11}H_{11}N_6$	210		100	$C_{11}H_8N_5$
				200		3	$C_{10}H_{10}N_5$
C8-PhNHG	243.0991	+0.2	$C_{11}H_{11}N_6O$	226		100	$C_{11}H_8N_5O$
				201		18	$C_{10}H_9N_4O$
N7-2APG	243.0986	−0.3	$C_{11}H_{11}N_6O$	226.0723	−0.1	100	$C_{11}H_8N_5O$
				201.0771	0.0	42	$C_{10}H_9N_4O$
				200.0934	+0.3	15	$C_{10}H_{10}N_5$
				158.0712	−0.1	8	$C_9H_8N_3$
N7-4APG	243.0989	0.0	$C_{11}H_{11}N_6O$	226.0723	0.0	100	$C_{11}H_8N_5O$
				201.0770	0.0	33	$C_{10}H_9N_4O$
N^2-2APG	243.0985	−0.5	$C_{11}H_{11}N_6O$	226.0716	−0.7	100	$C_{11}H_8N_5O$
				134.0708	−0.5	10	$C_7H_8N_3$
N^2-4APG	243.0983	−0.7	$C_{11}H_{11}N_6O$	226.0717	−0.6	100	$C_{11}H_8N_5O$

[a]Quasimolecular ions (MH^+) and product ions in the collision induced MS^2 taken at m/z of respective MH^+ parent ions.

subsequently in three equal portions, and the reaction mixture was incubated for 1 h under gentle stirring. The time interval between individual additions was 15 min. To release the adducts formed, the solution was refluxed for 40 min and analyzed by LC/MS.

Analyses of the Adducts with PhNH$^+$ by LC/MS. Samples hydrolyzed under acidic conditions were neutralized to pH 7. Both hydrolyzed and nonhydrolyzed samples of the reaction mixtures were diluted 10 times with 10% aqueous acetonitrile, and aliquots of 10 μL were injected onto a 150 × 2 mm Phenomenex Aqua C18 column, 5 μm particle size, which was eluted with 0.1% formic acid in aqueous acetonitrile at the flow rate of 0.2 mL/min. For dA and Ade adducts, the concentration of acetonitrile was increased linearly from 0 to 60% within 20 min. For dG and Gua adducts, a two-step gradient of acetonitrile was used, i.e., from 0 to 30% within 15 min and thereafter to 50% in another 5 min. The mass analyzer was set to monitor MS^2 spectra at m/z values corresponding to $(M + H)^+$ of the parent ions (m/z 343.1, 359.1, 243.1, and 227.1 for dA, dG, Gua, and Ade adducts, respectively). In addition, full scan spectra were also taken in the range of m/z 100−400. Conversion of dA to the major adducts found was estimated from the peak areas taken from extracted mass chromatograms at respective m/z values of the $(M + H)^+$ ions.

RESULTS AND DISCUSSION

Synthesis of the *N*7-Guanine Adducts. To prove whether *N*7-guanine adducts with phenylnitrenium ion are formed upon the reaction of dG with PhNHOAc, authentic samples of the expected adducts were needed. These samples were obtained by arylation of protected guanine (DMF-G) with 2- and 4-nitrophenylboronic acids followed by hydrolytic removal of the amidino protecting group and reduction of the nitro group (Scheme 1). The protecting group at N^2 was required to redirect the arylation reaction from *N*9 to *N*7 position. This reaction has been previously described for methyl- and vinylphenylboronic acids to give mainly *N*7-arylguanines accompanied by minor amounts of *N*9-isomer (*N*7/*N*9 isomer ratios up to 6:1).[18] We have newly observed a similar selectivity (5:1) for arylation with nitrophenylboronic acids, although this reaction required a somewhat elevated temperature. To distinguish between *N*7- and *N*9-substituted products, diagnostic signals of C4 and C5 in ^{13}C NMR spectra were used (Table 1). For *N*7-arylguanines, C4 and C5 resonances should be around 160 and 110 ppm, respectively, as a result of an anisotropic effect caused by aryl substitution.[14,18]

Reaction of dA with *N*-Acetoxyaniline (PhNHOAc). PhNHOH can be activated for reactions with nucleophiles by acetate ester formation. Arylhydroxylamine esters are also formed in the course of the metabolism of many mutagenic and carcinogenic arylamines as well as nitroaromatics. Their reactions with nucleosides are relevant simplified models of DNA adduct formation *in vivo*. Although, to our knowledge, the reaction of PhNHOAc or another active ester of PhNHOH with dA itself has not been reported in the literature, it is known that similar reactions, such as that of PhNHOH benzoate ester with adenosine[19] or other arylhydroxylamine esters with dA, give mainly corresponding C8- and/or N^6-adenine adducts.[20,21] We have detected two major adducts at m/z 343 arising from the reaction of PhNHOAc with dA at pH 7.4 and 37 °C (Figure 1). Conversion of dA to these

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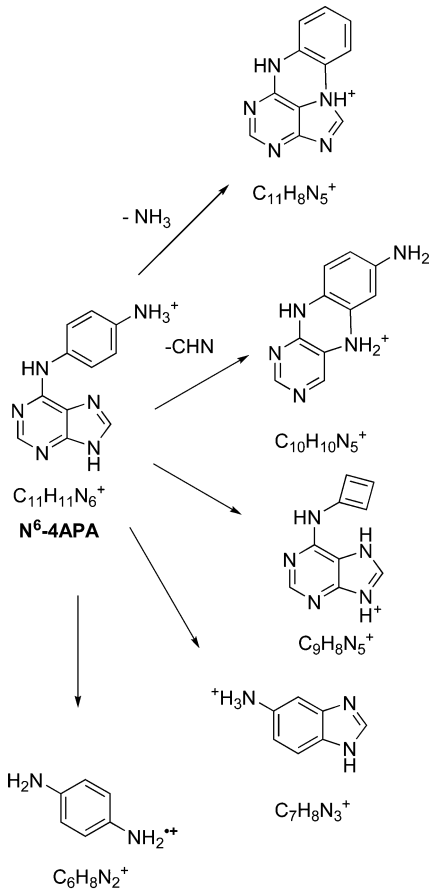
adducts was approximately 0.6%, and their ratio was about 2:1. After acidic hydrolysis, two major deribosylated adducts were obtained, which were identified as C8-PhNHA and N^6-4APA by LC/MS analysis by comparing their retention time and MS^2 spectra with those of synthesized reference standards (Figure 2). Other synthesized authentic adducts, i.e., C2-PhNHA, N7-2APA, N7-4APA, and N^6-2APA, were also searched for, but none of them coeluted with any of the minor peaks detected at m/z 227 (Figure 2). The formation of N^6-4APA can be explained by the S_N1 mechanism proceeding via the phenylnitrenium ion rather than by S_N2 reaction of PhNHOAc with dA. Although it is known that $PhNH^+$ has a very short lifetime of 0.25 ns in aqueous solutions,[22,23] it is most likely the ultimate electrophile attacking the nucleophilic amino group at C6. Surprisingly, despite its short lifetime, it is able to react rather selectively with the most nucleophilic reaction center of dA (Scheme 2).

Reaction of dG with PhNHOAc. In accordance with previous reports,[1,24] the reaction of PhNHOAc with dG under physiological conditions afforded C8-PhNHdG as the predominating adduct. Its identity was confirmed by a comparison of its elution time and MS^2 spectra with an authentic sample (Figure 3). The conversion of dG to 8-PhNHdG amounted to about 3.5% as estimated by comparing corresponding peak areas in extracted ion chromatograms. After hydrolysis by heating at 60 or 100 °C for 20 min, several peaks at m/z 243 corresponding to MH^+ of deribosylated $PhNH^+$ adducts were detected. Among them, C8-PhNHG and N7-4APG were identified using authentic standards (Figure 4). Another expected *N*7-guanine adduct, N7-2APG, has not been detected. Deoxyguanosine adducts at the *N*7 position are prone to hydrolytic deribosylation due to the weakening of the glycosidic bond caused by the positively charged imidazolium ring. Therefore, unlike C8-arylamino adducts, the *N*7-adducts can be effectively deribosylated even under the conditions of mild neutral hydrolysis.[7,25] It is therefore not surprising that after neutral hydrolysis, only a portion of C8-PhNHdG was deribosylated to C8-PhNHG (Figure 4). Complete deribosylation of dG adducts can be usually achieved by acidic hydrolysis. When heating the reaction mixture in 0.05 M HCl at 60 or 100 °C, the adducts with dG underwent a degradation, so that at least 10 peaks at m/z 243 (MH^+ of $PhNH^+$ adducts with Gua) were found in the extracted ion mass chromatograms. These results show that PhNHOAc can react with dG not only at C8 position but also at other nucleophilic sites including the *N*7 position. However, only a small amount of the *N*7-adduct is formed as compared with the C8-adduct, which was the only major adduct formed (Scheme 2). Adducts at the exocyclic NH_2 group, namely, N^2-2APG and N^2-4APG, have not been detected (Figure 3). This type of adduct is known to be formed by some other arylnitrenium ions.[1]

Reaction of PhNHOAc with DNA. The same $PhNH^+$ adducts as those identified in the reactions of PhNHOAc with dA and dG, albeit at low concentrations, were detected after its reaction with the DNA *in vitro* under physiological conditions followed by neutral hydrolysis. These adducts were also identified by comparison of their retention times and MS^2 spectra with those of reference standards. Mass chromatograms are shown in Figures 5 and 6. Neutral hydrolysis was chosen because the *N*7-guanine adducts are known to be easily cleaved from the DNA due to destabilization of the *N*-glycosidic bond.[7,25] However, after mild neutral hydrolysis we could detect not only N7-4APG but also all other adducts previously found in the reactions with dA and dG, namely, C8-PhNHG, C8-PhNHA, and N^6-4APA. It cannot be excluded that at least a part of these adducts might have originated from binding to nucleoside and/or nucleotide fragments formed in the course of incubation rather than from untouched DNA.

Mass Spectra of Adducts. The deribosylated adducts formed by the above-mentioned reaction of PhNHOAc were identified by a comparison with synthesized authentic standards. Retention times and MS^2 spectra of the reference standards were used to support identification. Despite certain common features in MS fragmentation, isomeric adducts also showed some distinctive fragments, which facilitated structural assignment. Compositions of the key fragment ions were determined unequivocally by HRMS. Results are listed in Table 2, and proposed fragment structures for N^6-4APA are shown in Scheme 3 and those of N7-2APG and N7-4APG in Scheme 4. A strong tendency toward cyclization leading to heteroaromatic structures seems to be a characteristic feature of all tested adducts.

Scheme 3. Fragmentation of N^6-4APA in Collision Induced MS^2 Taken at m/z 227 for MH^+



Mechanistic Considerations. A detailed mechanism of the reaction of arylhydroxylamine esters with purine nucleosides has been a subject to dispute over the years. Several proposals were based on the primary attack of the positively charged nitrogen of the nitrenium ion at the nucleophilic *N*7 position of dG or other nucleosides.[1] More recently, McClelland et al. proposed that this reaction proceeds as a straightforward electrophilic substitution, i.e., substitution of $ArNH^+$ for H^+ at the C8 position of dG. This mechanism was strongly

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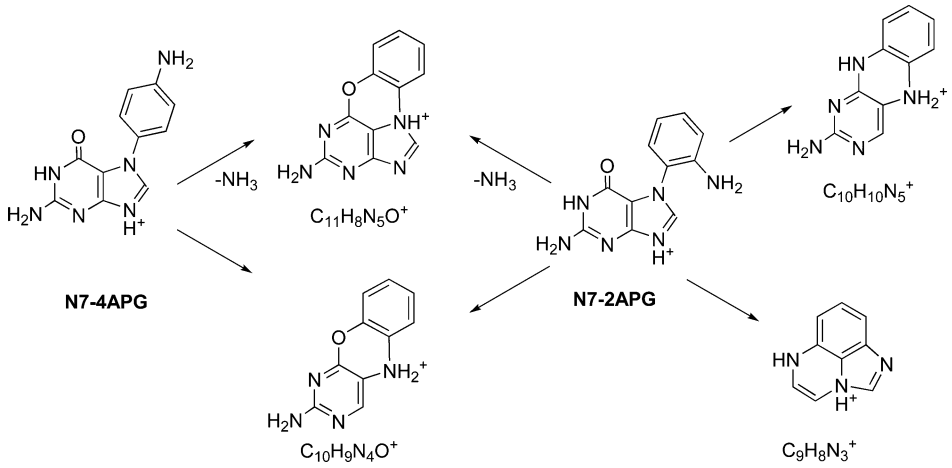
Scheme 4. Fragmentation of N7-APG in Collision Induced MS^2 Taken at m/z 243 for MH^+



supported by identification of the key intermediate, a primary arylnitrenium adduct at the C8 position of dG.[10] These adducts were found to be the predominating DNA adducts for all arylnitrenium ions tested, but other minor ones, namely, those at C8 and N^6 of adenine and N^2 of guanine, have also been reported.[1] Binding of arylnitrenium ions through both nitrogen and carbon atoms can be explained by a distribution of positive charge so that the attack by nucleophiles may take place at the electrophilic carbon atoms as well.

Unlike many arylnitrenium ions derived from carcinogenic arylamines, $PhNH^+$ has a very short lifetime of 125−240 ps in aqueous solutions.[23] Even a lower lifetime of 110 ps was reported recently in a study describing the first spectroscopic observation of this species in 100% formic acid.[26] Nonetheless, our results show that despite its short lifetime, $PhNH^+$ is able to react rather selectively, if not effectively, with both dG and dA. Theoretically, adducts can be formed through two different electrophilic carbon atoms of phenylnitrenium, i.e., C2 and C4. However, only the latter type of adducts has been detected in our study.

CONCLUSIONS

The phenylnitrenium ion generated from PhNHOAc can react under physiological conditions with dA and dG as well as with nucleophilic sites in the DNA through both the charged nitrogen and the C4 position. Identification of N7-4APG, which was found as a minor DNA adduct formed by these reactions, represents the first evidence of arylnitrenium adduct formation at the *N*7 position of dG, which is known to be a prominent site of attack by other electrophilic species. Further studies are needed to learn whether significant amounts of *N*7-guanine adducts are formed from arylnitrenium ions derived from strongly mutagenic arylamines and/or nitroaromatics with long lifetime in aqueous solutions and to determine their biological significance.

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Notes

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ABBREVIATIONS

C2-PhNHA, 2-phenylaminoadenine; C8-PhNHA, 8-phenylaminoadenine; C8-PhNHdA, 8-phenylamino-2′-deoxyadenosine; C8-PhNHG, 8-phenylaminoguanine; C8-PhNHdG, 8-phenylamino-2′-deoxyguanosine; dA, 2′-deoxyadenosine; dG, 2′-deoxyguanosine; DMF-G, N^2-(dimethylaminomethylene)guanine; EDTA, ethylendiaminetetraacetic acid; ESI, electrospray ionization; N7-2APA, 7-(2-aminophenyl)adenine; N7-4APA, 7-(4-aminophenyl)adenine; N7-2APdG, 7-(2-aminophenyl)-2′-deoxyguanosine; N7-4APdG, 7-(4-aminophenyl)-2′-deoxyguanosine; N7-2APG, 7-(2-aminophenyl)guanine; N7-4APG, 7-(4-aminophenyl)guanine; N^6-2APA, N^6-(2-aminophenyl)adenine; N^6-4APA, N^6-(4-aminophenyl)adenine; N^2-2APG, N^2-(2-aminophenyl)guanine; N^2-4APG, N^2-(4-aminophenyl)guanine; N7-2NPDG, 7-(2-nitrophenyl)-N^2-(dimethylaminomethylene)guanine; N7-4NPDG, 7-(4-nitrophenyl)-N^2-(dimethylaminomethylene)guanine; N7-2NPG, 7-(2-nitrophenyl)guanine; N7-4NPG, 7-(4-nitrophenyl)guanine; 2-NPBA, 2-nitrophenylboronic acid; 4-NPBA, 4-nitrophenylboronic acid; $PhNH^+$, phenylnitrenium; PhNHOAc, *N*-acetoxyaniline; PhNHOH, phenylhydroxylamine; TBD, 1,5,7-triazabicyclo[4.4.0]dec-5-ene; TEA, triethylamine; TMEDA, *N*,*N*,*N*′,*N*′-tetramethylethylenediamine; TRIS, tris(hydroxymethyl)aminomethane

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