Synthesis, Characterization, and Conformational Analysis of DNA Adducts from Methylated Anilines Present in Tobacco Smoke

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Received March 10, 1995[⊗]

The ability of a series of aromatic amines present in tobacco smoke (2-, 3-, and 4-methylaniline, 2,3- and 2,4-dimethylaniline) to bind to DNA has been investigated by reacting *N*-(acyloxy)arylamines with dG, dG nucleotides, and DNA. The predominant products from reactions with dG and the nucleotides were characterized as *N*-(deoxyguanosin-8-yl)arylamines by spectroscopic and HPLC methods. HPLC and spectroscopic analyses of the modified DNA indicated the same adducts. Analyses of the ^{1}H and ^{13}C NMR spectra suggested that the adducts containing a methyl substituent *ortho* to the arylamine nitrogen had a higher percentage of *syn* conformers. This observation was supported by theoretical simulation studies that indicated substantial percentages of low energy *syn* conformers, increasing with the substitution pattern in the order *para* < *meta* < *ortho* < *ortho,para* < *ortho,meta*. The results demonstrate that, although single-ring arylamines are considered weak carcinogens, their electrophilic *N*-acetoxy derivatives, which are plausible metabolic intermediates, react with DNA to yield covalent adducts structurally identical to those derived from carcinogenic polyarylamines, such as 2-aminofluorene and 4-aminobiphenyl. Furthermore, the conformational perturbation induced in DNA by the formation of the monoarylamine−DNA adducts, especially those with an *ortho* substituent, may contribute to the biological activities of these compounds.

Introduction

Aromatic amines and amides are a class of chemical carcinogens for which there is ample evidence of human exposure (*1*). Although legal restrictions have curtailed the magnitude of occupational hazard, the general population remains exposed to aromatic amines from a variety of ubiquitous sources. Cigarette smoke is one of the major contemporary origins of exposure to these chemicals, which are found in nanogram quantities in the mainstream smoke and in higher concentrations in the sidestream smoke (*2, 3*).

A wide range of epidemiological data indicates that tobacco usage is causally related to human cancer in a variety of organs, including the urinary bladder (*4*−*6*). Compared to nonsmokers, cigarette users have been estimated to be 2- to 10-fold more likely to contract bladder cancer, depending on average daily usage and cumulative consumption (*7*). Likewise, histologic changes in the urinary bladder epithelium have been found to correlate with smoking habits in a dose-responsive manner (*8*). Among the profusion of chemical constituents of tobacco smoke, only aromatic amines (*e.g.*, 4-aminobiphenyl and 2-naphthylamine) have been implicated as human bladder carcinogens (*9*); therefore, arylamines are regarded as determinant etiological factors in the induction of bladder cancer in smokers (*10*).

An extensive amount of evidence has demonstrated that to generate their biological effects, aromatic amines must undergo metabolic activation, *via N*-hydroxylation, to reactive electrophilic intermediates that bind to DNA, yielding covalent adducts (*11*, *12*). *In vitro* and *in vivo* studies have indicated that aromatic amine carcinogens produce C8-deoxyguanosine−arylamine derivatives (dG-C8-Ar)[1] as predominant DNA adducts in nearly all circumstances; N^2-deoxyguanosine− and N^6- and C8-deoxyadenosine−arylamine adducts have also been reported as minor products (*11, 12*). Arylamine−DNA adducts are potential genotoxic lesions that have been shown to induce mutations in bacterial and mammalian test systems (refs 12 and 13 and references therein), as well as in oncogenes of experimental animals (reviewed in ref 12). Conformational perturbations associated with a change from the normal *anti* to an abnormal *syn* orientation about the glycosyl bond, accompanied by stacking of the arylamine moiety with the nearby bases, are thought to be decisive factors in the mutagenic and tumorigenic properties of specific arylamine−DNA adducts (refs 14−19 and references therein).

DNA adducts related to cigarette smoking have been detected in various human tissues by sensitive techniques, such as ^{32}P-postlabeling (reviewed in ref 20). Among these, several putative arylamine−DNA adducts

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⊗ Abstract published in *Advance ACS Abstracts,* December 1, 1995.

[1] Abbreviations: Ar, arylamine; Bis-Tris, (bis[2-hydroxyethyl]iminotris[hydroxymethyl])methane; diMeA, dimethylaniline; dG, 2′-deoxyguanosine; dG3′p, 2′-deoxyguanosine 3′-monophosphate; dG5′p, 2′-deoxyguanosine 5′-monophosphate; dG3′,5′p, 2′-deoxyguanosine 3′,5′-bisphosphate; dG-C8-Ar, *N*-(deoxyguanosin-8-yl)arylamine; DEPT, distortionless enhancement by polarization transfer; DMF, *N,N*-dimethylformamide; dR, 2′-deoxyribosyl; dR5′p, 2′-deoxyribosyl 5′-phosphate; FAB, fast atom bombardment; MeA, methylaniline; MD, molecular dynamics; MM, molecular mechanics; NOE, nuclear Overhauser effect.

0893-228x/96/2709-0099$12.00/0

have been found in analyses of exfoliated cells and in surgical autopsy and biopsy samples of human bladder (*21*−*24*); however, with the exception of *N*-(deoxyguanosin-8-yl)-4-aminobiphenyl (*22*−*24*), none of the tobacco-related adducts could be identified. Significantly higher levels of 4-aminobiphenyl−hemoglobin adducts were also found in blood samples from smokers, as compared to nonsmokers (*25*).

The major contribution to the arylamine content of cigarette smoke comes from mononuclear arylamines, such as aniline and its methyl and dimethyl derivatives (*2*, *3*). Although single-ring aromatic amines are generally regarded as weak carcinogens (*26*−*28*), the results of a recent study of occupational carcinogenesis indicate that chronic exposure to monoarylamines, in particular, 2-methylaniline (*o*-toluidine), may induce bladder cancer in humans (*29*). Furthermore, hemoglobin adducts of several single-ring arylamines present in tobacco smoke have been detected in human blood samples, and in certain cases (*e.g.*, 2- and 4-methylaniline, 2,4-dimethylaniline, and 2-ethylaniline), the adduct levels were significantly higher in smokers than in nonsmokers (*30*, *31*). The formation of these monoarylamine−hemoglobin adducts implies the bioavailability of the corresponding *N*-arylhydroxylamines (*25*, *30*), thus suggesting that single-ring arylamines undergo initial metabolic activation in a manner that parallels the activation of their binuclear analogues. Therefore, mononuclear aromatic amines may account for some of the putative arylamine−DNA adducts detected in bladder samples from smokers and may play a role in tobacco-induced carcinogenesis. The preparation of well characterized monoarylamine−DNA adduct standards would be of obvious importance to test this hypothesis by allowing the unambiguous identification and quantification of trace amounts of potential adducts formed *in vivo* through comparison with the synthetic standards (*e.g.*, by ^{32}P-postlabeling). However, the difficulty in producing substantial quantities of DNA adducts from single-ring arylamines is reflected in the limited number of successful syntheses that have been reported (*32*−*35*).

In the present study, we have investigated the ability of several single-ring arylamines present in tobacco smoke (2-, 3-, and 4-methylaniline, 2,3- and 2,4-dimethylaniline) to form DNA adducts. We have characterized the predominant products of reaction between electrophilic synthons and dG, dG nucleotides, and DNA as dG-C8-Ar adducts. In addition, we have conducted spectroscopic studies and energy minimization calculations to establish the preferred conformations, which may determine the potential genotoxic effects of each adduct. The results are discussed in relation to the number and position of methylated substituents on the arylamine ring.

Materials and Methods

Instrumentation. Melting temperatures were measured with a Köfler hot-stage apparatus and are uncorrected. Reversed-phase HPLC analyses and separations were conducted using a μBondapak C_{18} column (0.39 × 30 cm; Waters Associates, Milford, MA) on a Waters Associates system consisting of two Model 510 pumps, a U6K injector, and a Model 660 automated gradient controller; the peaks were monitored at 280 nm with a Hewlett-Packard 1040 diode array spectophotometric detector.

UV spectra were recorded with either a Beckman DU-40 or a Shimadzu 1202 UV/vis spectrophotometer.

Fast atom bombardment (FAB) mass spectra were obtained on a Finnigan TSQ 70 instrument, using a source temperature of either 90 or 120 °C; the samples were dispersed in a thioglycerol matrix.

^{1}H NMR spectra were recorded on a Varian Unity 300 or a Bruker AM 500 spectrometer, operating at 300 and 500 MHz, respectively. ^{13}C NMR spectra were recorded on a Varian Unity 300 spectrometer, operating at 75.43 MHz. The samples were dissolved in Me_2SO-d_6 (2′-deoxyguanosine adducts) or Me_2SO-d_6/D_2O (2′-deoxyguanosine 5′-phosphate adducts). Proton chemical shifts were referenced by assigning the residual Me_2SO resonance to 2.49 ppm, and carbon chemical shifts, by assigning the solvent resonance to 39.50 ppm. ^{1}H resonance assignments were based on comparison with literature data for 2′-deoxyguanosine (dG) and related dG adducts (36−*38*), combined with homonuclear decoupling experiments, chemical exchange of the labile protons with D_2O, and observation of the nuclear Overhauser effect (NOE) enhancement patterns upon irradiation of specific protons. ^{13}C resonance assignments were based on published data for dG (*39*) and related dG adducts (*40*), as well as on the use of a distortionless enhancement by polarization transfer (DEPT) pulse sequence and analysis of the ^{13}C−^{1}H coupling patterns.

Chemicals. 2′-Deoxyguanosine was obtained from US Biochemicals (Cleveland, OH). 2′-Deoxyguanosine 3′-monophosphate (dG3′p), 2′-deoxyguanosine 5′-monophosphate (dG5′p), 2′-deoxyguanosine 3′,5′-bisphosphate (dG3′,5′p), salmon testes DNA, and (bis[2-hydroxyethyl]iminotris[hydroxymethyl])methane (Bis-Tris) were purchased from Sigma Chemical Co. (St. Louis, MO). All other commercially available reagents were obtained from Aldrich, through either Aldrich Chemical Co. (Milwaukee, WI) or Sigma-Aldrich Química, S. A. (Madrid, Spain), and were used as received.

Syntheses. Caution: *N-Arylhydroxylamines and their O-acyl derivatives are potentially carcinogenic. They should be handled with protective clothing in a well-ventilated fume hood.*

Starting Materials. Pivaloyl cyanide [bp 119−120 °C, lit. (*41*) 119−120 °C] was prepared according to the procedure of Herrmann and Simchen (*41*).

N-Arylhydroxylamines [*N*-(2-methylphenyl)hydroxylamine, mp 42−44 °C, lit. (*42*) 44 °C; *N*-(3-methylphenyl)hydroxylamine, mp 67−69 °C, lit. (*43*) 68 °C; *N*-4-(methylphenyl)hydroxylamine, mp 92−94 °C, lit. (*43*) 93−94 °C; *N*-(2,3-dimethylphenyl)-hydroxylamine, mp 73−75 °C, lit. (*42*) 74 °C; and *N*-(2,4-dimethylphenyl)hydroxylamine, mp 62−64 °C, lit. (*44*) 64.5 °C] were synthesized in 50−60% yields by reduction of the corresponding nitroarenes (10 mmol) at 50 °C with zinc dust (20−30 mmol) in aqueous ammonium chloride containing 30% ethanol (*42*−*45*).

The *N*-arylhydroxylamines were converted to their *O*-acetyl derivatives by an adaptation of the procedure of Lobo *et al.* (*46*, *47*). Specifically, an argon-purged 200 mM solution of each *N*-arylhydroxylamine in dry THF (2−5 mL), containing 1.1 molar equiv of triethylamine, was cooled to between −30 and −40 °C. Acetyl cyanide (1.1 molar equiv) was then added, and the mixture was stirred under argon for 0.5−1 h, while maintaining the temperature below −30 °C. The unstable *N*-acetoxyarylamines thus generated were kept in solution and used immediately for the adduct syntheses. *N*-Pivaloxyarylamines were prepared in a similar manner, using pivaloyl cyanide as the acylating agent and a reaction time of 1.5−2 h at −30 °C. As with the *N*-acetoxyarylamines, the *N*-pivaloxyarylamines were used immediately after synthesis for the preparation of arylamine−dG adducts.

***N*-(Deoxyguanosin-8-yl)arylamines. Method 1: By Reaction of dG with *N*-Acetoxyarylamines.** Typically, a cold (−30 to −40 °C) THF solution containing the *N*-acetoxyarylamine (generated from 1.1−1.2 molar equiv of the corresponding *N*-arylhydroxylamine) was added to an argon-purged 120 mM solution of dG in DMF/water (2/1), kept at 0−5 °C. The mixture was further purged with argon and then stirred at room temperature for 6−18 h. Following evaporation to dryness, the residue was resuspended in water (10 mg of starting dG/mL) and extracted 4−6 times with 1 volume of water-saturated diethyl ether. The ether layer was discarded, and the aqueous phase was extracted with 3 × 1 volume of water-saturated 1-butanol. The butanol extracts were combined and extracted

with 1 volume of 1-butanol-saturated water to remove unreacted dG. Following evaporation of the 1-butanol, the resulting material was redissolved in 20% aqueous methanol. The dG-C8-Ar adducts were subsequently isolated, in 1−1.5% average yields, either by reversed-phase HPLC, using a 20-min linear gradient of 20−70% aqueous methanol, followed by 5 min at 70% methanol, or by column chromatography on Sephadex LH-20 (Pharmacia/PL Biochemicals, Piscataway, NJ), using a 20−80% step gradient of aqueous methanol. The spectral properties of the products were fully consistent with the assigned structures (*vide infra*).

Method 2: By Reaction of dG with *N*-Pivaloxyarylamines. Typically, a 1.5 M THF solution (3−6 mL) containing the *in situ* generated *N*-pivaloxyarylamine was maintained at −30 to −40 °C for 3−4 h. During this period, aliquots were added at 30-min intervals to an argon-purged 200 mM solution of dG in DMF/10 mM sodium citrate, pH 6 (2.5/1), kept at room temperature. Upon addition of 4−8 molar equiv of the *N*-pivaloxyarylamine, the mixture was allowed to react at room temperature for an additional 16−18 h. After solvent extractions, the products were isolated by Sephadex LH-20 column chromatography as described above. The following adducts, structurally identical to those generated in method 1, were obtained from the corresponding *N*-pivaloxyarylamines:

***N*-(Deoxyguanosin-8-yl)-2-methylaniline (dG-C8-2-MeA).** η 3%; mp >220 °C dec; UV (EtOH) λ_{max} 279 nm (log ϵ 4.09); ^{1}H NMR (Me_2SO-d_6) δ 2.03 (1H, m, H2″), 2.16 (3H, s, CH_3), 2.69 (1H, m, H2′), 3.66 (2H, m, H5′,5″), 3.87 (1H, m, H4′), 4.37 (1H, m, H3′), 5.30 (1H, bs, 3′-OH), 5.49 (1H, bs, 5′-OH), 6.26 (1H, bs, H1′), 6.29 (2H, bs, N^2H_2), 6.98 (1H, t, *J* 7.5 Hz, ArH4), 7.12 (1H, t, *J* 7.5 Hz, ArH5), 7.17 (1H, d, *J* 7.2 Hz, ArH3), 7.31 (1H, d, *J* 7.2 Hz, ArH6), 7.92 (1H, bs, ArNH), 10.48 (1H, bs, N1H); ^{13}C NMR (Me_2SO-d_6) δ 17.86 (CH_3), 38.02 (C2′), 61.23 (C5′), 71.02 (C3′), 82.88 (C1′), 87.19 (C4′), 112.35 (C5), 123.17 (ArCH), 126.02 (ArCH), 130.33 (ArCH), 139.13 (C8), 145.12 ($ArC_{1/2}$), 147.79 (C4), 152.49 (C2), 155.56 (C6); *m*/*z* (FAB) 373 (MH^+, 40), 257 [(MH_2 − dR)$^+$, 84].

***N*-(Deoxyguanosin-8-yl)-3-methylaniline (dG-C8-3-MeA).** η 3%; mp >250 °C dec; UV (EtOH) λ_{max} 283 nm (log ϵ 3.87); ^{1}H NMR (Me_2SO-d_6) δ 1.98 (1H, m, H2″), 2.26 (3H, s, CH_3), ∼2.50 (m, H2′, partially obscured by the solvent resonance), 3.73 (2H, m, H5′,5″), 3.90 (1H, m, H4′), 4.40 (1H, m, H3′), 5.32 (1H, bs, 3′-OH), 5.90 (1H, bs, 5′-OH), 6.30 (1H, bs, H1′), 6.34 (2H, bs, N^2H_2), 6.43 (1H, d, *J* 7.5 Hz, ArH4), 7.12 (1H, t, *J* 7.8 Hz, ArH5), 7.48 (1H, s, ArH2), 7.54 (1H, d, *J* 7.5 Hz, ArH6), 8.53 (1H, bs, ArNH), 10.53 (1H, bs, N1H); ^{13}C NMR (Me_2SO-d_6) δ 21.37 (CH_3), 38.34 (C2′), 61.25 (C5′), 71.22 (C3′), 82.74 (C1′), 87.12 (C4′), 112.16 (C5), 114.62 (ArCH), 117.78 (ArCH), 121.41 (ArCH), 128.39 (ArCH), 137.63 (C8), 140.76 ($ArC_{1/3}$), 143.32 ($ArC_{1/3}$), 149.48 (C4), 152.79 (C2), 155.68 (C6); *m*/*z* (FAB) 395 [(M + Na)$^+$, 8], 373 (MH^+, 26), 279 [(MH + Na − dR)$^+$, 24], 257 [(MH_2 − dR)$^+$, 85].

***N*-(Deoxyguanosin-8-yl)-4-methylaniline (dG-C8-4-MeA).** η 7%; mp >250 ° dec; UV (EtOH) λ_{max} 284 nm (log ϵ 4.42); ^{1}H NMR (Me_2SO-d_6) δ 1.98 (1H, m, H2″), 2.22 (3H, s, CH_3), ∼2.50 (m, H2′, partially obscured by the solvent resonance), 3.73 (2H, m, H5′,5″), 3.90 (1H, m, H4′), 4.39 (1H, m, H3′), 5.32 (1H, bs, 3′-OH), 5.89 (1H, bs, 5′-OH), 6.29 (1H, bs, H1′), 6.33 (2H, bs, N^2H_2), 7.04 (2H, d, *J* 7.8 Hz, ArH3 + H5), 7.61 (2H, d, *J* 7.8 Hz, ArH_2 + H_6), 8.53 (1H, bs, ArNH), 10.49 (1H, bs, N1H); ^{13}C NMR (Me_2SO-d_6) δ 20.35 (CH_3), 38.40 (C2′), 61.35 (C5′), 71.36 (C3′), 82.85 (C1′), 87.21 (C4′), 112.14 (C5), 117.47 ($ArC_{2,6/3,5}$), 128.92 ($ArC_{2,6/3,5}$), 138.35 (C8), 143.62 ($ArC_{1,4}$), 149.54 (C4), 152.82 (C2), 155.75 (C6); *m*/*z* (FAB) 373 (MH^+, 33), 257 [(MH_2 − dR)$^+$, 72].

***N*-(Deoxyguanosin-8-yl)-2,3-dimethylaniline (dG-C8-2,3-diMeA).** η 4%; mp >220 °C dec; UV (EtOH) λ_{max} 280 nm (log ϵ 4.07); ^{1}H NMR (Me_2SO-d_6) δ 2.03 (4H, m + s, H2″ + 2-CH_3), 2.23 (3H, s, 3-CH_3), 2.66 (1H, m, H2′), 3.65 (2H, m, H5′,5″), 3.87 (1H, m, H4′), 4.37 (1H, m, H3′), 5.30 (1H, bs, 3′-OH), 5.50 (1H, bs, 5′-OH), 6.27 (3H, bs, H1′ + N^2H_2), 6.92 (1H, d, *J* 7.2 Hz, ArH4), 7.00 (1H, t, *J* 7.5 Hz, ArH5), 7.08 (1H, d, *J* 7.2 Hz, ArH6), 7.98 (1H, bs, ArNH), 10.50 (1H, bs, N1H); ^{13}C NMR (Me_2SO-d_6) δ 14.11 (CH_3), 20.30 (CH_3), 38.04 (C2′), 61.30 (C5′), 71.21 (C3′), 82.87 (C1′), 87.22 (C4′), 112.38 (C5), 122.17 (ArCH), 125.27 (ArCH), 125.42 (ArCH), 130.11 ($ArC_{1/2/3}$), 136.85 (C8), 138.96 ($ArC_{1/2/3}$), 145.83 ($ArC_{1/2/3}$), 149.90 (C4), 152.50 (C2), 155.63 (C6); *m*/*z* (FAB) 409 [(M + Na)$^+$, 8], 387 (MH^+, 100), 293 [(MH + Na − dR)$^+$, 6], 271 [(MH_2 − dR)$^+$, 96].

***N*-(Deoxyguanosin-8-yl)-2,4-dimethylaniline (dG-C8-2,4-diMeA).** η 9%; mp >220 °C dec; UV (EtOH) λ_{max} 277 nm (log ϵ 4.32); ^{1}H NMR (Me_2SO-d_6) δ 2.03 (1H, m, H2″), 2.12 (3H, s, 2-CH_3), 2.23 (3H, s, 4-CH_3), 2.69 (1H, m, H2′), 3.65 (2H, m, H5′,5″), 3.87 (1H, m, H4′), 4.37 (1H, m, H3′), 5.29 (1H, bs, 3′-OH), 5.49 (1H, bs, 5′-OH), 6.26 (1H, bs, H1′), 6.26 (2H, bs, N^2H_2), 6.92 (1H, d, *J* 7.8 Hz, ArH5), 6.98 (1H, s, ArH3), 7.17 (1H, d, *J* 8.1 Hz, ArH6), 7.86 (1H, bs, ArNH), 10.45 (1H, bs, N1H); ^{13}C NMR (Me_2SO-d_6) δ 17.71 (CH_3), 20.32 (CH_3), 38.00 (C2′), 61.24 (C5′), 71.08 (C3′), 82.84 (C1′), 87.17 (C4′), 112.33 (C5), 123.83 (ArCH), 126.46 (ArCH), 130.84 (ArCH + $ArC_{1/2/4}$), 132.45 ($ArC_{1/2/4}$), 136.42 (C8), 145.59 ($ArC_{1/2/4}$), 149.80 (C4), 152.42 (C2), 155.55 (C6); *m*/*z* (FAB) 409 [(M + Na)$^+$, 6], 387 (MH^+, 40), 293 [(MH + Na − dR)$^+$, 4], 271 [(MH_2 − dR)$^+$, 100].

***N*-(Deoxyguanosin-8-yl)arylamine n-Phosphates (n = 3′, 5′, or 3′,5′).** Each nucleotide (dG3′p, dG5′p, or dG3′,5′p) was dissolved in water to a concentration of 120 mM and reacted overnight with 1.1−1.2 molar equiv of each specific *N*-acetoxyarylamine, as described for the dG adducts. Following evaporation of the THF, the aqueous solutions were further diluted with water to a concentration of 30−40 mM and extracted 4−6 times with 1 volume of water-saturated diethyl ether to remove solvolysis products. For isolation of the dG5′p adducts, the aqueous solutions were evaporated to dryness, redissolved in 100 mM ammonium acetate (pH 5.7), and loaded on Waters Sep-Pak C_{18} reversed-phase cartridges. The adducts were subsequently separated by elution with a 0−50% step gradient of acetonitrile in 100 mM ammonium acetate (pH 5.7). The dG3′p adducts were isolated from the aqueous phase by reversed-phase HPLC, using a 30-min linear gradient of 5−60% acetonitrile in 100 mM ammonium acetate (pH 5.7). The dG3′,5′p adducts were isolated by reversed-phase HPLC using a similar gradient of 5−60% acetonitrile in 100 mM ammonium acetate and 10 mM ammonium phosphate (pH 5.7). The average adduct yields were estimated to be approximately 1% on the basis of UV absorbance measurements and the molar extinction coefficients determined for the corresponding dG adducts. The dG5′p adducts, each prepared from 20 mg of the starting nucleotide, were characterized by ^{1}H NMR and mass spectral analyses, as described below. In addition, these adducts were treated with alkaline phosphatase for 3 h at 37 °C, and the HPLC retention times and UV spectra of the resultant dG adducts were compared to those of the dG adducts described above. The dG3′p and dG3′,5′p adducts were prepared from 2.5 mg of the starting nucleotide. These adducts were identified through comparison of their UV spectra with the spectra of the dG and dG5′p adducts. The dG3′p and dG3′,5′p adducts were also converted to dG adducts by similar incubations with alkaline phosphatase, for HPLC and spectroscopic analyses.

***N*-(Deoxyguanosin-8-yl)-2-methylaniline 5′-Phosphate (dG5′p-C8-2-MeA).** ^{1}H NMR (Me_2SO-d_6/D_2O) δ 2.04 (1H, m, H2″), 2.15 (3H, s, CH_3), 2.90 (1H, m, H2′), 3.75 (1H, m, H5″), 3.85 (1H, m, H4′), 4.16 (1H, m, H5′), 4.54 (1H, m, H3′), 6.12 (1H, m, H1′), 6.87 (1H, t, *J* 7.3 Hz, ArH4), 7.08 (1H, t, *J* 7.7 Hz, ArH5), 7.12 (1H, d, *J* 7.3 Hz, ArH3), 7.29 (1H, d, *J* 7.9 Hz, ArH6); *m*/*z* (FAB) 475 [(M + Na)$^+$, 5], 453 (MH^+, 14), 257 [(MH_2 − dR5′p)$^+$, 100].

***N*-(Deoxyguanosin-8-yl)-3-methylaniline 5′-Phosphate (dG5′p-C8-3-MeA).** ^{1}H NMR (Me_2SO-d_6/D_2O) δ 2.04 (1H, m, H2″), 2.18 (3H, s, CH_3), 2.78 (1H, m, H2′), 3.81 (1H, m, H5″), 3.83 (1H, m, H4′), 4.16 (1H, m, H5′), 4.65 (1H, m, H3′), 6.17 (1H, m, H1′), 6.65 (1H, d, *J* 7.7 Hz, ArH4), 7.08 (1H, t, *J* 7.7 Hz, ArH5), 7.40 (1H, s, ArH2), 7.41 (1H, d, *J* 8.2 Hz, ArH6); *m*/*z* (FAB) 491 [(M + K)$^+$, 10], 475 [(M + Na)$^+$, 14], 453 (MH^+, 16), 257 [(MH_2 − dR5′p)$^+$, 100].

***N*-(Deoxyguanosin-8-yl)-4-methylaniline 5′-Phosphate (dG5′p-C8-4-MeA).** ^{1}H NMR (Me_2SO-d_6/D_2O) δ 2.02 (1H, m, H2″), 2.21 (3H, s, CH_3), 2.80 (1H, m, H2′), 3.81 (1H, m, H5″), 3.86 (1H, m, H4′), 4.14 (1H, m, H5′), 4.62 (1H, m, H3′), 6.19 (1H, m, H1′), 7.01 (2H, d, *J* 8.4 Hz, ArH3 + H5), 7.54 (2H, d, *J* 8.4 Hz, ArH2 + ArH6); *m*/*z* (FAB) 475 [(M + Na)$^+$, 10], 453 (MH^+, 24), 257 [(MH_2 − dR5′p)$^+$, 100].

***N*-(Deoxyguanosin-8-yl)-2,3-dimethylaniline 5′-Phosphate (dG5′p-C8-2,3−diMeA).** ^{1}H NMR (Me_2SO-d_6/D_2O) δ 2.01 (4H, s + m, H2″+ 2-CH_3), 2.22 (3H, s, 3-CH_3), 2.93 (1H, m, H2′), 3.73 (1H, m, H5″), 3.83 (1H, m, H4′), 4.14 (1H, m, H5′), 4.56 (1H, m, H3′), 6.10 (1H, m, H1′), 6.82 (1H, d, *J* 7.5 Hz, ArH4), 6.96 (1H, t, *J* 7.5 Hz, ArH5), 7.02 (1H, d, *J* 7.5 Hz, ArH6); *m*/*z* (FAB) 489 [(M + Na)$^+$, 3], 467 (MH$^+$, 37), 271 [(MH_2 − dR5′p)$^+$, 100].

***N*-(Deoxyguanosin-8-yl)-2,4-dimethylaniline 5′-Phosphate (dG5′p-C8-2,4-diMeA).** ^{1}H NMR (Me_2SO-d_6/D_2O) δ 2.03 (1H, m, H2″), 2.10 (3H, s, 2-CH_3), 2.20 (3H, s, 4-CH_3), 2.90 (1H, m, H2′), 3.74 (1H, m, H5″), 3.85 (1H, m, H4′), 4.54 (1H, m, H3′), 6.10 (1H, m, H1′), 6.89 (1H, t, *J* 8.2 Hz, ArH5), 6.93 (1H, s, ArH3), 7.18 (1H, d, *J* 8.2 Hz, ArH6); *m*/*z* (FAB) 505 [(M + K)$^+$, 7], 489 [(M + Na)$^+$, 8], 467 (MH$^+$, 10), 271 [(MH_2 − dR5′p)$^+$, 100].

DNA Modification Reactions. Chemical modification of DNA with each of the arylamines was conducted using 500 μL aliquots of a 1 mg/mL stock solution of salmon testes DNA in 5 mM Bis-Tris and 0.1 mM EDTA (pH 7.1). In some instances, the DNA was dissolved in DMF/5 mM Bis-Tris and 0.1 mM EDTA, pH 7.1 (65/35). Each aliquot was purged with argon and reacted with 120 μL of a THF solution containing the *N*-acetoxyarylamine, generated from 6−24 μmol of the corresponding *N*-arylhydroxylamine, as described above. The mixture was then incubated at 37 °C for 16−18 h. Following evaporation of the THF, the aqueous solution was extracted sequentially with 3 × 1 volume of 1-butanol and 3 × 1 volume of diethyl ether, both saturated with the same Bis-Tris buffer. The DNA was precipitated by addition of 0.1 volume of 5 M NaCl and 1.1 volumes of absolute ethanol, washed with 70% ethanol, and redissolved in 5 mM Bis-Tris and 0.1 mM EDTA (pH 7.1) to a concentration of approximately 1 mg/mL. The arylamine−DNA adducts in the treated samples were identified by comparison of their HPLC retention times and UV spectra to those of the corresponding dG-C8-Ar adducts, following standard enzymatic hydrolysis of the DNA to nucleosides (*48*).

Theoretical Simulations. Energy minimization calculations were conducted with the molecular simulation program Discover (version 2.9) from Biosym Technologies Inc., San Diego, CA, using the AMBER forcefield, together with MOPAC charges and distance-dependent dielectric constants to simulate the solvent. The Biosym graphics program, Insight II (version 2.2.0), was used to design and display the structures. Molecular dynamics (MD) was employed to generate different conformations, and molecular mechanics (MM) was used for refinement. In each MD run, the temperature was 1000 K and the time step was 1 fs. Two hundred structures were saved at intervals of 100 steps and then minimized using MM. No constraints were imposed during the MD simulation to allow maximum freedom of motion. Before each MD run, the starting structures for the adduct were designed by using dG subunits in standard B−DNA conformations and then minimized by MM and MOPAC. Hydrogen bonds within the adduct molecules were also investigated. Since hydrogen positions are easily monitored in theoretical studies, the hydrogen (H).. acceptor (A) distance was monitored, instead of the donor (D)...acceptor (A) distance. The normal criterion of 2.5 Å was taken as the upper limit for effective hydrogen bonding, although less restrictive conditions (*e.g.*, 2.7 Å) have been used in some DNA simulations (*49*).

The calculated dihedral angles were defined as follows: α, N9−C8−N(Ar)−C1(Ar); β, C8−N(Ar)−C1(Ar)−C2(Ar); χ, O4′−C1′−N9−C4; γ, O5′−C5′−C4′−C3′ (Figure 1).

Results and Discussion

Synthesis of Monoarylamine−DNA Adducts. *N*-(Deoxyguanosin-8-yl)arylamine (dG-C8-Ar) adducts are the major persistent species resulting from *in vivo* covalent binding of electrophilic metabolites of carcinogenic arylamines to DNA (*11, 12*) and are also the predominant products formed *in vitro* by reaction of DNA, dG, or dG nucleotides with model electrophilic synthons (reviewed in ref 12). Despite their presence as environmental contaminants (*1*) and their potential role as human carcinogens (*9, 29*), little is known about the ability of mononuclear arylamines to undergo similar transformations. Therefore, we selected a series of single-ring arylamines present in tobacco smoke (2-, 3-, and 4-methylaniline, 2,3- and 2,4-dimethylaniline) to examine the capacity of suitably activated derivatives of these amines to undergo DNA binding. Since the *O*-acetylation of *N*-arylhydroxylamines has been implicated as a major metabolic activation pathway for aromatic amines (*11*), we tested *N*-acetoxyarylamines and their synthetic equivalents, *N*-pivaloxyarylamines, as key reactive intermediates. This strategy has been applied previously by Boche and co-workers to the synthesis of dG-C8-Ar adducts from the polyarylamine carcinogens 4-aminobiphenyl, 2-naphthylamine, and 2-aminofluorene in low to moderate yields (*50−52*). The same investigators have shown that the procedure can be extended to some monoarylamines, specifically aniline (*33*) and anilines monosubstituted in the *para* position (*34*).

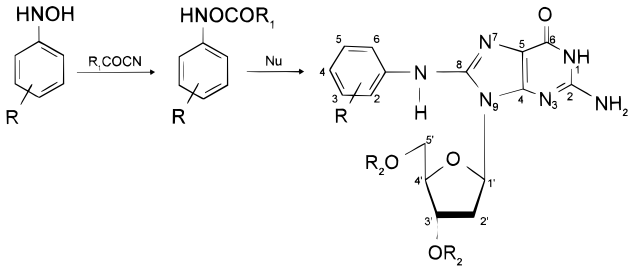

Figure 1. Scheme for the synthesis of monoarylamine−DNA adducts. R = 2-Me, 3-Me, 4-Me, 2,3-diMe, or 2,4-diMe; R_1 = Me or Me_3C; R_2 = H or PO_3^{2-}; Nu = dG, dGnp (n = 3′, 5′, or 3′,5′), or DNA.

We prepared *N*-arylhydroxylamines by the classical reduction of their nitro precursors with zinc dust (*42−45*), and converted the reduced species to their *N*-acetoxy and *N*-pivaloxy derivatives by reaction with acetyl cyanide and pivaloyl cyanide in dry THF at low temperature (-30 °C to −40 °C). Although no attempts were made to isolate the unstable *N*-(acyloxy)arylamines, which were kept in solution and used immediately, the selective *O*-acylation of *N*-arylhydroxylamines by acyl cyanides is an established procedure in this temperature range (*46, 47*). As indicated in Figure 1, the *N*-(acyloxy)arylamines were subsequently reacted with dG, dGnp (n = 3′, 5′, or 3′,5′), and DNA. Following solvent extractions, to remove side products of lower polarity, the adducts formed upon reaction with dG (Figure 2a) and with the mononucleotides were isolated by chromatographic techniques (*vide supra*). The adducts formed upon reaction with DNA were analyzed by HPLC (Figure 2b), after enzymatic hydrolysis of the DNA to nucleosides. They were identified on the basis of their HPLC retention times and UV spectra, by comparison with those of the adducts formed upon reaction with dG. For all the monoarylamines investigated, dG-C8-Ar adducts (*vide infra*) were identified systematically as the major products of reaction with either the nucleoside, the nucleotides, or DNA. The successful isolation of dG-C8-Ar adducts, using a synthetic approach that simulates a plausible metabolic pathway, suggests that methylated single-ring arylamines have the potential to bind to DNA *in vivo*.

As a synthetic tool, the methodology followed in this work is limited by the highly competitive solvolytic decomposition of the *N*-(acyloxy)arylamines (*53*), which restricts the adduct yields (*33, 34, 50−52*). We attempted to overcome this obstacle by using a substantial molar

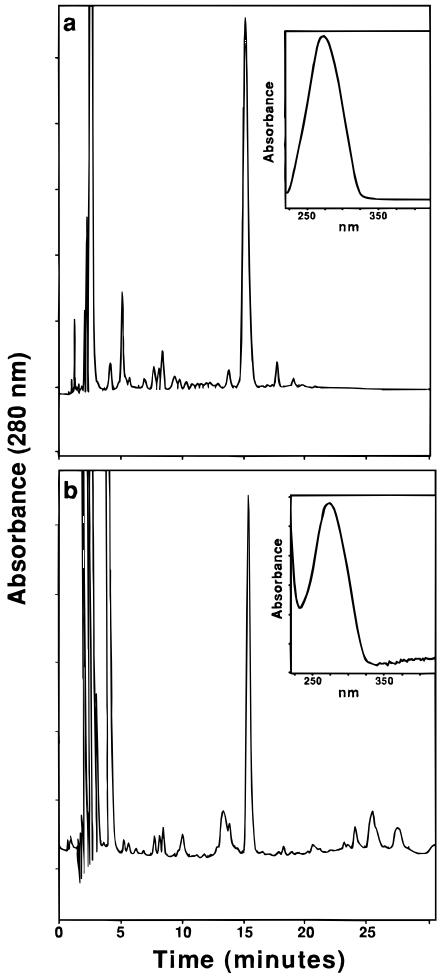

Figure 2. HPLC of products obtained from (a) reacting *N*-acetoxy-2,4-dimethylaniline with dG and (b) enzymatic hydrolysate of DNA after reaction with *N*-acetoxy-2,4-dimethylaniline. The material eluting at 15 min was characterized as *N*-(deoxyguanosin-8-yl)-2,4-dimethyaniline. The insets show the UV spectra of the 15-min peaks.

excess of the *N*-(acyloxy)arylamines (up to ~10 fold) but experienced increased difficulties in the extraction of the solvolysis products and the subsequent purification of the adducts as the concentration of the *N*-(acyloxy)arylamines was raised. Near-optimum conditions were reached for dG modifications in DMF/water using a 4−8 molar excess of *N*-pivaloxyarylamines, presumably due to lower rates of solvolysis in the presence of DMF and to a slightly lower leaving ability of the bulky pivalate group compared to acetate; even so, the adduct yields (3−9%) were modest. Interestingly, the presence of a *p*-methyl substituent in the arylamine ring (*e.g.*, 4-methyl- and 2,4-dimethylaniline) resulted in the more efficient adduct syntheses, which suggests that the electron-donating methyl substituent has a better ability to stabilize the putative nitrenium/carbenium ion intermediate when located *para* to the electrophilic nitrogen. This observation is consistent with previous theoretical work, in which the enthalpies of nitrenium ion formation for a series of alkylated anilines were estimated to be lower for *para*-substituted anilines compared to their *ortho*- and *meta*-substituted isomers (*54*).

Despite producing relatively low adduct yields, the synthetic approach employed in this study emerges as the only method currently available for the preparation of monoarylamine−DNA adduct standards at the nucleoside/nucleotide level. The use of more stable electrophilic synthons, such as *N*-acetoxy-*N*-(trifluoroacetyl)arylamines

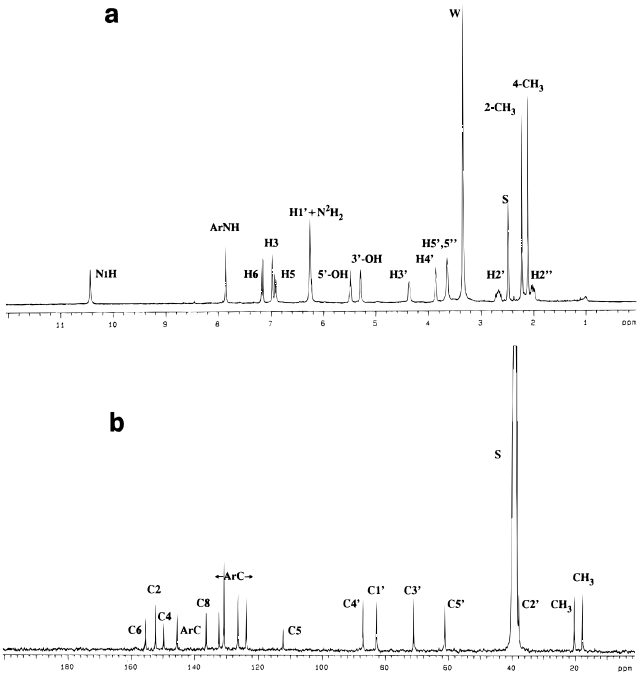

Figure 3. NMR spectra of *N*-(deoxyguanosin-8-yl)-2,4-dimethylaniline recorded at 22 °C. (a) 300 MHz ^{1}H spectrum in Me_2SO-d_6. The acquisition conditions were as follows: sweep width, 6000 Hz; pulse width, 7 μs; acquisition time, 3 s; relaxation delay, 0 s. "S" represents the residual Me_2SO resonance and "W" the water resonance. (b) 75.43 MHz ^{13}C spectrum in Me_2SO-d_6. The acquisition conditions were as follows: sweep width, 40 000 Hz; pulse width, 6 μs; acquisition time, 1 s; relaxation delay, 2 s. Low power broad-band decoupling was accomplished by WALTZ-16 modulation. The time domain data were treated with an exponential multiplication, using a line broadening of 7 Hz. "S" represents the Me_2SO resonance.

and *N*-acetyl-*N*-(acyloxy)arylamines, which is quite successful for the synthesis of dG-C8-Ar adducts from carcinogenic polyarylamines (*55*−*57*), failed to produce more than trace quantities of adducts from the monoarylamines under investigation,[2] possibly due to insufficient stabilization of the presumed nitrenium/carbenium ion intermediates by a single aromatic ring. On the other hand, although an alternate method, based upon the synthesis of *N*-(purin-8-yl)tolylamines from thiourea derivatives (*35*), should have general applicability, the problem of building the β-glycosyl bond to generate the corresponding nucleosides/nucleotides is not straightforward. For example, the chemical coupling of adducted bases to activated deoxyribose derivatives would require multiple protecting steps (*58*), and previous attempts to achieve enzymatic coupling have failed for guanine C8−arylamine adducts (*59*).

Characterization of the Adducts. The major products of reaction between dG and the *N*-(acyloxy)arylamines investigated were characterized as covalent adducts on the basis of UV, ^{1}H and ^{13}C NMR, and mass spectral analyses (*vide supra*). Thus, the UV spectra showed bathochromic shifts of 23−30 nm compared to dG, as a result of increased conjugation, while the FAB mass spectra were fully consistent with binding of each arylamine fragment to the nucleoside. Likewise, the ^{13}C NMR spectra indicated the presence of all the dG and the arylamine carbon resonances, as illustrated in Figure 3 (panel b) for the 2,4-dimethylaniline adduct (dG-C8-2,4-diMeA).

[2] L. L. G. Mourato and M. M. Marques, unpublished observations.

The structures of the dG adducts were established upon analysis of their ^{1}H NMR spectra, which indicated that every isolated adduct was a dG-C8-Ar product. A representative example is shown in Figure 3 (panel a) for dG-C8-2,4-diMeA. In each case, all the dG proton resonances were present, with the exception of the nonexchangeable H8 at ~δ 8.0; in addition, all the arylamine ring proton resonances were observed, as well as a 1-proton exchangeable resonance at δ 7.8−8.6 assigned to the arylamine NH. Moreover, the spectral data for the 4-methylaniline adduct (dG-C8-4-MeA) were in full agreement with those reported previously (*38*).

All the dG5′p adducts had FAB mass spectra in full agreement with binding of arylamine fragments to the nucleotide and ^{1}H NMR spectra indicative of substitution of the amine nitrogen at the C8 of the guanine moiety. In addition, upon treatment with alkaline phosphatase, the dG5′p adducts were converted into products with HPLC retention times and UV spectra identical to those of the corresponding dG-C8-Ar adducts.

The quantities of isolated dG3′p and dG3′,5′p adducts were insufficient for NMR analyses; nonetheless, the UV spectra recorded for these products were identical to those of the corresponding nucleoside and 5′-nucleotide C8 adducts. Furthermore, the dG3′p and dG3′,5′p adducts were converted into corresponding nucleoside C8 adducts, as indicated by HPLC and UV spectral analyses, upon incubation with alkaline phosphatase.

The arylamine−DNA adducts were characterized after enzymatic hydrolysis of the samples to nucleosides. In each instance, the resultant products had HPLC retention times and UV spectra identical to the corresponding dG-C8-Ar adducts (Figure 2).

Conformational Properties of the Adducts. Among the approaches applied for the estimation of the dynamic *anti* ⇄ *syn* equilibria in nucleosides and nucleotides, those based on inspection of the NMR chemical shifts of the sugar H2′ and C2′ have been widely favored (*36, 40, 60*−*62*). Generally, a downfield shift of the H2′ resonance and an upfield shift of the C2′ resonance are regarded as indicators of an increased contribution by *syn* conformer populations, with the H2′ chemical shifts being more accurate for quantification (*61*). Quantitative analyses have also been attempted on the basis of the vicinal ^{13}C−^{1}H coupling constants between the sugar H1′ and the base carbons (*40, 61, 63*). Since the predominant conformations about the glycosyl bond are thought to be decisive factors for the mutagenic and carcinogenic properties of specific dG-C8-Ar adducts, we conducted a comparative analysis of the NMR spectral data for the dG and the dG5′p adducts to search for any arylamine-related conformational trends. Although the C8−H1′ and C4−H1′ coupling constants were not sufficiently resolved to allow systematic conclusions based on Karplus-type relationships, some qualitative indications could be drawn from the H2′ and C2′ chemical shifts, which are summarized in Table 1.

Compared to dG, no changes were detected in the H2′ resonances of dG-C8-3−MeA and dG-C8-4-MeA. This observation suggests that both adducts prefer *anti* conformations, in agreement with previous findings for the 4-methylaniline guanosine adduct (*38*). For all the remaining dG adducts, the H2′ resonances exhibited slight downfield shifts of similar magnitude (~0.17 ppm) relative to the analogous resonances in dG (Table 1). Since these adducts have a methyl substituent *ortho* to the amine function as a common structural feature, the NMR data strongly indicate that *ortho* substitution in the arylamine moiety induces a higher *syn* population. The ^{1}H NMR data for the dG5′p adducts (Table 1) are fully consistent with this inference; thus, compared to dG5′p, the H2′ resonances of the dG5′p adducts of 2-methylaniline, 2,3-dimethylaniline, and 2,4-dimethylaniline were shifted downfield by ~0.4 ppm, while the analogous resonances for the adducts not containing an *o*-methyl substituent were shifted in the same direction to a lesser extent.

Table 1. Selected NMR Data for the *N*-(Deoxyguanosin-8-yl)arylamine (dG-C8-Ar) and *N*-(Deoxyguanosin-8-yl)arylamine 5′-Phosphate (dG5′p-C8-Ar) Adducts[a]

adduct		δ (ppm)	
Ar		H2′	C2′
	dG	~2.50[b]	40.33
	dG5′p	~2.56[b]	ND[c]
2-MeA	dG	2.69	38.02
	dG5′p	2.90	ND[c]
3-MeA	dG	~2.50[b]	38.34
	dG5′p	2.78	ND[c]
4-MeA	dG	~2.50[b]	38.40
	dG5′p	2.80	ND[c]
2,3-diMeA	dG	2.66	38.04
	dG5′p	2.93	ND[c]
2,4-diMeA	dG	2.69	38.00
	dG5′p	2.90	ND[c]

[a] The selected resonances correspond to the deoxyribose protons and carbons that are sensitive to the glycosydic torsion angle. The spectra were recorded at 22 °C in Me_2SO-d_6 (dG adducts) or Me_2SO-d_6/D_2O (dG5′p adducts). Proton chemical shifts were referenced by assigning the residual Me_2SO resonance to 2.49 ppm and carbon chemical shifts by assigning the solvent resonance to 39.5 ppm. [b] Partially obscured by the solvent resonance. [c] ND, not determined.

^{13}C NMR spectra were obtained on the dG adducts to confirm the trends indicated by the ^{1}H NMR spectra (Table 1). Compared to dG, the C2′ resonance of dG-C8-3-MeA and dG-C8-4-MeA was shifted upfield by ~2 ppm, while the analogous resonance in the other adducts, all of which contained a methyl substituent *ortho* to the amine function, was upfield by ~2.3 ppm. The greater upfield shift detected with the latter adducts is again consistent with a greater contribution of *syn* conformers.

Theoretical Simulations. MD/MM techniques were used to search for the minimum energy conformers of each dG-C8-Ar adduct, with the purpose of probing the conformational trends detected by NMR. Analysis of the trajectory files (Figure 4) suggests that all the adducts are quite flexible, as indicated in each case by a substantial number of conformers having energies within 2.5 kcal/mol of the global minimum. The preferred geometries of the glycosyl bonds, expressed as the frequencies of occurrence of *anti* (χ = 217−227°) and *syn* (χ = 41−45°) conformers found for the individual adducts in the MD/MM simulations, are summarized in Table 2. Taking into account conformers calculated along each MD/MM run, the adducts had similar *anti*/*syn* populations, with *anti* conformers contributing in each case to approximately 60% of the total. However, differences became apparent when the global minimum and the distributions of the next lowest energy conformers (*i.e.*, within 2 or 3 kcal/mol of the global minimum) were compared. Thus, the 4-methylaniline adduct was found to adopt predominantly an *anti* conformation, having both an *anti* global minimum and a high proportion (*ca.* 80%, within 3 kcal/mol of the minimum) of low energy *anti* conformers. This is consistent with previous calculations by Meier and

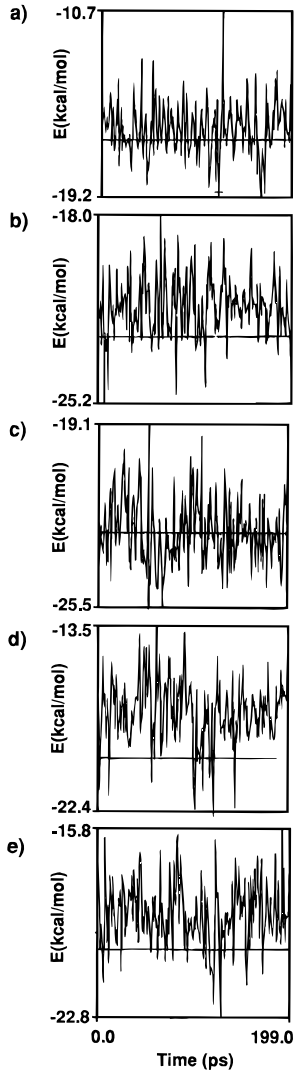

Figure 4. Trajectory files of the total potential energy (kcal/mol) *versus* time (ps) along the MD/MM runs for the *N*-(deoxyguanosin-8-yl)arylamine (dG-C8-Ar) adducts. Ar = (a) 2-MeA; (b) 3-MeA; (c) 4-MeA; (d) 2,3-diMeA; and (e) 2,4-diMeA. The horizontal lines in each panel are drawn 2.5 kcal/mol above the corresponding global minimum. Note that the energy scale differs between panels.

Table 2. Frequencies of Occurrence of *Anti* and *Syn* Conformers for the *N*-(Deoxyguanosin-8-yl)arylamine (dG-C8-Ar) Adducts as Determined by MD/MM Simulations

adduct (dG-C8-Ar)	conformations						
		E_{min}+2[b] (%)		E_{min}+3[c] (%)		total[d] (%)	
Ar	E_{min}[a]	*syn*	*anti*	*syn*	*anti*	*syn*	*anti*
2-MeA	syn	58	42	36	64	40	60
3-MeA	syn	53	47	28	72	40	60
4-MeA	anti	14	86	19	81	35	65
2,3-diMeA	syn	100	0	79	21	37	63
2,4-diMeA	syn	67	33	42	58	43	57

[a] Conformation corresponding to the global minimum energy. [b] Conformations with energies within 2 kcal/mol of the global minimum. [c] Conformations with energies within 3 kcal/mol of the global minimum. [d] Total conformations calculated along each MD/MM run.

Boche (*38*), who determined an *anti* global minimum for the analogous *N*-(guanosin-8-yl)-4-methylaniline adduct. By contrast, both the 2- and 3-methylaniline adducts were found to adopt *syn* global minima, although the contribution of low energy *anti* conformers (64% and 72%, respectively, within 3 kcal/mol of the global minimum) was still predominant.

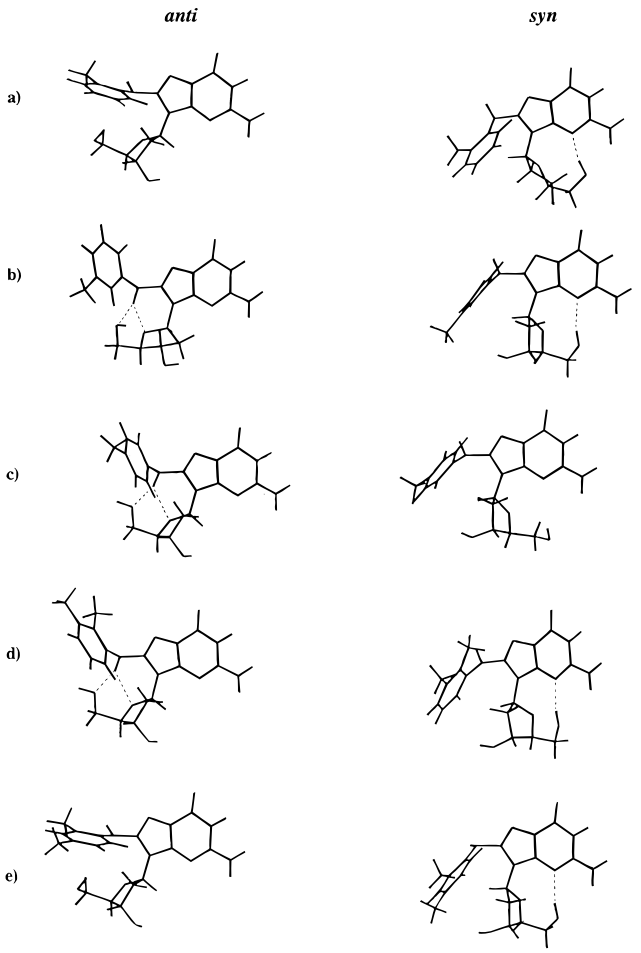

Figure 5. Diagrams of the lowest *anti* and *syn* energy conformers calculated by MD/MM for the *N*-(deoxyguanosin-8-yl)arylamine (dG-C8-Ar) adducts. Ar = (a) 2-MeA; (b) 3-MeA; (c) 4-MeA; (d) 2,3-diMeA; and (e) 2,4-diMeA. The dashed lines represent the hydrogen bonds discussed in the text. See Table 3 for the energies and structural data.

The introduction of a second methyl substituent (*e.g.*, 2,3- and 2,4-dimethylaniline) resulted in global minima having *syn* conformations and much higher percentages of low energy *syn* contributors than those observed for the monomethylated adducts. For instance, in the 2,4-dimethylaniline adduct, only 58% of the conformers with energies within 3 kcal/mol of the global minimum had an *anti* geometry. This number decreased to 21% in the 2,3-dimethylaniline adduct, for which no *anti* conformers were found within 2 kcal/mol of the global minimum. Hence, the data in Table 2 suggest that the dG-C8-Ar adducts derived from mono- and dimethylanilines will exist as a weighted average of *anti* and *syn* conformers, with the major contributors being clearly *anti* for the 4-methylaniline adduct and the percentage of *syn* conformers depending on the number of methyl substituents and their position relative to the arylamine nitrogen according to the sequence *para* < *meta* < *ortho* < *ortho, para* < *ortho,meta*. These findings support the qualitative predictions drawn from analysis of the ^{1}H and ^{13}C NMR spectra. Interestingly, the results shown in Table 2 are not substantially different from those obtained by computational methods for dG-C8-Ar adducts of bulkier aromatic amines, such as the carcinogens 2-aminofluorene and 4-aminobiphenyl, in which the theoretical prediction of dynamic states containing a variety of low energy *anti* and *syn* conformers (*17, 64*−*69*) has received experimental confirmation from NMR studies of arylamine-modified oligonucleotides (*15, 16, 18, 19, 70*−*72*).

Table 3. Energies and Structural Data Calculated by MD/MM and Corresponding to the Global Minima of both the *Anti* and *Syn* Conformations of the *N*-(Deoxyguanosin-8-yl)arylamine Adducts (dG-C8-Ar) Investigated

adduct (dG-C8-Ar)	conformation	E_{total} (kcal/mol)	dihedral angle[a] (deg)				$P_1 \wedge P_2$[b]	A...HB[c] (Å)		
			χ	γ	α	β	π	d1 (O4′...HNAr)	d2 (N3...HO5′)	d3 (O5′...HNAr)
2-MeA	*syn*	-19.21	38.38	57.25	−60.38	−51.03	87.47	5.27	**1.96**	
	anti	-18.05	226.04	−176.67	−94.06	24.94	80.04	3.96	6.59	4.60
3-MeA	*syn*	−25.23	43.48	82.69	−61.76	−57.88	87.66	5.23	**1.94**	
	anti	−23.62	218.11	83.25	−119.12	−90.93	72.06	**2.37**	7.33	**1.98**
4-MeA	*anti*	−25.51	217.02	83.27	−117.33	74.59	75.97	**2.36**	7.32	**1.96**
	syn	−24.89	40.70	79.52	−61.69	−56.90	88.10	5.25	3.15	
2,3-diMeA	*syn*	−22.44	42.70	57.80	−55.84	−59.81	75.87	5.27	**1.93**	
	anti	−20.19	219.03	54.38	−118.32	72.88	87.52	**2.40**	7.33	**1.95**
2,4-diMeA	*syn*	−22.85	44.78	57.20	−57.07	−51.68	83.74	4.38	**1.94**	
	anti	-21.37	227.45	−176.70	−91.75	20.70	79.45	3.98	6.51	4.75

[a] The dihedral angles are defined as follows: α, N9−C8−N(Ar)−C1(Ar); β, C8−N(Ar)−C1(Ar)−C2(Ar); χ, O4′−C1′−N9−C4; γ, O5′−C5′−C4′−C3′ (Figure 1). [b] Angle between the arylamine (P_1) and the guanine (P_2) planes. [c] Hydrogen (H)...acceptor (A) distances are shown for the bonds indicated. Effective hydrogen bonding distances (*i.e.*, <2.5 Å) are shown in bold.

The main structural parameters calculated for the lowest energy *anti* and *syn* conformers of each adduct are summarized in Table 3, and diagrams corresponding to these conformers are displayed in Figure 5. The calculated data suggest that the occurrence of a stabilizing effect due to hydrogen bonding between the sugar C4′-oxygen and the arylamine NH will favor glycosydic torsion angles in the *anti* domain, whenever the steric hindrance is not too severe. This is the case with the 4-methylaniline adduct and the lowest *anti* conformer of the 3-methylaniline adduct. However, *ortho* methylation in the arylamine ring clearly introduces an unstabilizing factor, which determines the preferred geometry of the adduct and shifts the lowest energy conformers to the *syn* domain. In addition, there is a clear relationship between the order of increasing contribution of low energy *syn* conformers (*e.g.*, the monomethylated series) and the order of increasing total energy. Interestingly, even the geometry of the lowest energy *anti* conformers is affected by *ortho* substitution. This is indicated by the changes in the β dihedral angle observed for the 2-methylaniline and 2,4-dimethylaniline adducts (Table 3), which result in removal of the *ortho* methyl from the vicinity of the sugar and prevent the establishment of the hydrogen bond between O4′ and the arylamine NH (Figure 5).

An additional intramolecular hydrogen bond, involving the C5′-oxygen and, again, the arylamine NH, was detected in the lowest *anti* conformers of the 3-methylaniline, 4-methylaniline, and 2,3-dimethylaniline adducts (Table 3; Figure 5). This hydrogen bond, which has also been found for the lowest energy conformer of *N*-(guanosin-8-yl)-4-methylaniline (*38*) and for *N*-(deoxyguanosin-8-yl)-2-aminofluorene (*36*), appears to be responsible for the observed synclinal conformation of the exocyclic C4′−C5′ bond that is normally preferred in purine nucleotides. By contrast, in the lowest *anti* conformers of the 2-methylaniline and 2,4-dimethylaniline adducts, for which such hydrogen bonding is not possible, the C4′−C5′ bond adopts an antiperiplanar conformation. For all the *syn* conformers, with the exception of the 4-methylaniline adduct, the calculations indicated the establishment of an intramolecular hydrogen bond between the guanine N3 and the 5′-OH (Table 3). Such an interaction will obviously be absent at the nucleotide level.

Conclusions

Aromatic amine carcinogens are typically activated through *N*-hydroxylation, followed in some instances by esterification, which creates a more reactive electrophile (*11, 12*). In this study, we have demonstrated that *N*-hydroxy derivatives of methylated anilines can be converted synthetically into metabolically plausible *N*-acetoxy products (and their *N*-pivaloxy equivalents) that readily react with nucleosides (*i.e.*, dG), nucleotides (*i.e.*, dG3′p, dG5′p, and dG3′5′p), and DNA. In all cases, the major adducts were characterized as C8-substituted dG derivatives, which is consistent with what has been observed with more carcinogenic aromatic amines, such as 2-aminofluorene and 4-aminobiphenyl (*11, 12*). Although C8-substituted dG adducts were the predominant products in every instance, spectroscopic and theoretical data indicated conformational differences depending upon the location of the methyl substituent. Thus, adducts containing a methyl group *ortho* to the amine function (*e.g.*, 2-methylaniline) had greater percentage of *syn* conformers about the glycosyl bond than those not bearing such a group. With other aromatic amines, the occurrence of *syn* conformers has been associated with both mutagenic and tumorigenic responses (*14*−*19*). In this regard, it should be noted that aromatic amines containing *o*-methyl substituents tend to be more mutagenic (*73, 74*) and tumorigenic (*75, 76*) than analogues with no substituent in the *ortho* position. This increase in biological response may be due in part to the greater propensity of *ortho* methylated adducts to adopt a *syn* conformation.

Acknowledgment. This work was supported in part by NATO (Collaborative Research Grant CRG 910561) and by Junta Nacional de Investigação Científica e Tecnológica, Portugal (Contract PBIC/CEN/1154/92). We thank Joanna Deck for some of the NMR spectra, J. Pat Freeman for the FAB mass spectra, and Cindy Hartwick for helping to prepare the manuscript. Part of this study has appeared as a preliminary report (*77*).

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