Synthesis, Characterization, and Comparative ^{32}P-Postlabeling Efficiencies of 2,6-Dimethylaniline−DNA Adducts

Luísa L. Gonçalves,[†] Frederick A. Beland,*[,‡] and M. Matilde Marques*[,†]

Centro de Química Estrutural, Complexo I, Instituto Superior Técnico, Av. Rovisco Pais, 1049-001 Lisboa, Portugal, and Division of Biochemical Toxicology, National Center for Toxicological Research, Jefferson, Arkansas 72079

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2,6-Dimethylaniline (2,6-diMeA) is a ubiquitous environmental pollutant that is used in industry as a synthetic intermediate. It is also found in tobacco smoke and as a major metabolite of lidocaine. Although the potential carcinogenicity of 2,6-diMeA in humans is presently uncertain, this aromatic amine has been classified as a rodent carcinogen. In addition, it is known to form hemoglobin adducts in humans, which indicates a profile of metabolic activation similar to that of typical arylamine carcinogens. Like other aromatic amines, 2,6-diMeA has been shown to yield *N*-(deoxyguanosin-8-yl)-2,6-dimethylaniline (dG-C8-2,6-diMeA) as a major DNA adduct in vitro. In this study, we show that 2,6-diMeA yields an unusual pattern of DNA adducts. In addition to dG-C8-2,6-diMeA, we have isolated two new adducts, 4-(deoxyguanosin-N^2-yl)-2,6-dimethylaniline (dG-N^2-2,6-diMeA) and 4-(deoxyguanosin-O^6-yl)-2,6-dimethylaniline (dG-O^6-2,6-diMeA), from the reaction of *N*-acetoxy-2,6-dimethylaniline with deoxyguanosine. A similar reaction conducted with deoxyadenosine yielded 4-(deoxyadenosin-N^6-yl)-2,6-dimethylaniline (dA-N^6-2,6-diMeA). All four adducts were detected in DNA reacted with *N*-acetoxy-2,6-dimethylaniline, with the relative yields being 46% for dA-N^6-2,6-diMeA, 22% for dG-N^2-2,6-diMeA, 20% for dG-O^6-2,6-diMeA, and 12% for dG-C8-2,6-diMeA. This product profile contrasts markedly with the usual pattern of adducts obtained with aromatic amines, where C8-substituted deoxyguanosine products typically predominate. We further analyzed the kinetics of the T_4 polynucleotide kinase (PNK)-catalyzed phosphorylation of the C8 and N^2 deoxyguanosine 3′-phosphate adducts from 2,6-diMeA. The kinetic parameters obtained with these two structurally different adducts are compared to those determined with the parent nucleotide (dG3′p), and with (±)-*anti*-10-(deoxyguanosin-N^2-yl)-7,8,9-trihydroxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene 3′-phosphate, the major adduct derived from the environmental pollutant benzo[*a*]pyrene. The results indicate that all the adducts were labeled with lower efficiencies than dG3′p, stressing the likely underestimation of adduct levels in typical ^{32}P-postlabeling protocols. Nonetheless, the N^2 adducts derived from 2,6-diMeA and benzo[*a*]pyrene were both labeled with higher efficiencies than the C8 adduct derived from 2,6-diMeA, with the benzo[*a*]pyrene adduct being the best substrate for PNK. Thus, the data suggest that N^2 adducts from dG3′p are intrinsically better substrates than their C8 analogues for PNK, and that bulkier aromatic fragments may favor the enzyme−substrate interaction during the labeling step.

Introduction

2,6-Dimethylaniline (2,6-diMeA)[1] is a ubiquitous environmental pollutant that is used in industry as an intermediate in the manufacture of several products, including pesticides, dyestuffs, and synthetic resins (*1*). In addition, 2,6-diMeA is present in nanogram amounts in tobacco smoke (*2*, *3*) and is also a major metabolite of the potent anesthetic and antiarrythmic drug lidocaine (*4*). Although the potential carcinogenicity of 2,6-diMeA in humans is presently uncertain, results from a National Toxicology Program bioassay have indicated that it is a rodent carcinogen (*5*).

Like other aromatic amines (*6*−*8*), 2,6-diMeA has the potential to undergo metabolic activation through cytochrome P450-mediated *N*-hydroxylation, followed by *O*-

* To whom correspondence should be addressed. F.A.B.: HFT-110, NCTR, Jefferson, AR 72079; telephone, (870) 543-7205; fax, (870) 543-7136; e-mail, fbeland@nctr.fda.gov. M.M.M.: CQE, Complexo I, IST, Av. Rovisco Pais, 1049-001 Lisboa, Portugal; telephone, 351-21-841-9200; fax, 351-21-846-4455; e-mail, matilde.marques@ist.utl.pt.

[†] Instituto Superior Técnico.

[‡] National Center for Toxicological Research.

[1] Abbreviations: Ar, arylamine; BPDE, (±)-*r*-7,*t*-8-dihydroxy-*t*-9,-10-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene; dA, 2′-deoxyadenosine; dA-N^6-2,6-diMeA, 4-(deoxyadenosin-N^6-yl)-2,6-dimethylaniline; dG, 2′-deoxyguanosine; dG3′p, 2′-deoxyguanosine 3′-phosphate; dG3′,5′p, 2′-deoxyguanosine 3′,5′-bisphosphate; dG-C8-Ar, *N*-(deoxyguanosin-8-yl)arylamine; dG-C8-2,6-diMeA, *N*-(deoxyguanosin-8-yl)-2,6-dimethylaniline; dG-N^2-2,6-diMeA, 4-(deoxyguanosin-N^2-yl)-2,6-dimethylaniline; dG-N^2-BPDE, (±)-*anti*-10-(deoxyguanosin-N^2-yl)-7,8,9-trihydroxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene; dG-O^6-2,6-diMeA, 4-(deoxyguanosin-O^6-yl)-2,6-dimethylaniline; dG3′p-C8-4-ABP, *N*-(deoxyguanosin-8-yl)-4-aminobiphenyl 3′-phosphate; dG3′p-C8-Ar, *N*-(deoxyguanosin-8-yl)-arylamine 3′-phosphate; dG3′p-C8-2,6-diMeA, *N*-(deoxyguanosin-8-yl)-2,6-dimethylaniline 3′-phosphate; dG3′p-N^2-2,6-diMeA, 4-(deoxyguanosin-N^2-yl)-2,6-dimethylaniline 3′-phosphate; dG3′p-N^2-BPDE, (±)-*anti*-10-(deoxyguanosin-N^2-yl)-7,8,9-trihydroxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene 3′-phosphate; 2,6-diMeA, 2,6-dimethylaniline; DMF, *N*,*N*-dimethylformamide; dR, 2′-deoxyribosyl; dT3′p, thymidine 3′-phosphate; ESI, electrospray ionization; *N*-AcO-2,6-diMeA, *N*-acetoxy-2,6-dimethylaniline; *N*-OH-2,6-diMeA, *N*-hydroxy-2,6-dimethylaniline; PEI, polyethyleneimine; PNK, T_4 polynucleotide kinase; RAL, relative adduct labeling.

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esterification to a reactive derivative capable of forming DNA adducts. Indirect evidence for the formation of *N*-hydroxyarylamines in vivo can be obtained from the detection of hemoglobin adducts, which are formed by a sequence involving oxidation of the *N*-hydroxyarylamines to nitrosoarenes, covalent binding of the nitrosoarenes to a cysteine SH residue in the β-chain of hemoglobin, and rearrangement of the intermediate species to a sulfinamide (*9*). This redox process occurs with concomitant production of methemoglobin (*10*, *11*). Hemoglobin adducts from 2,6-diMeA have been found in cigarette smokers and in nonsmokers with no known exposure to this arylamine (*12*) and are also present at significant levels in patients treated with therapeutic doses of lidocaine (*13*). These findings, together with the fact that lidocaine has been reported to induce severe methemoglobinemia in some patients (*14*, *15*), indicate that humans have the ability to metabolize 2,6-diMeA to *N*-hydroxy-2,6-dimethylaniline (*N*-OH-2,6-diMeA).

Aniline derivatives bearing two alkyl substituents ortho to the nitrogen atom have been reported to be nongenotoxic (*16*), and it has been hypothesized that steric hindrance in the vicinity of the nitrogen could prevent metabolic activation. 2,6-diMeA is only a weak mutagen in *Salmonella typhimurium* TA100 in the presence of S9 (*17*); however, its *N*-hydroxylated metabolite, *N*-OH-2,6-diMeA, is the most mutagenic of the entire series of *N*-(hydroxymethyl)-, *N*-(hydroxydimethyl)-, and *N*-(hydroxyethyl)aniline derivatives in the same tester strain in the absence of S9 (*18*, *19*). These data show that *N*-OH-2,6-diMeA has genotoxic properties and suggest that it may be involved in the potential carcinogenicity of 2,6-diMeA.

Aromatic amine carcinogens are known to yield *N*-(deoxyguanosin-8-yl)arylamines (dG-C8-Ar) as the major persistent adducts in vivo, although minor adducts through the N^2 and O^6 atoms of deoxyguanosine (dG) or the C8 and N^6 atoms of deoxyadenosine (dA) may also be present in some instances (*6*−*8*). Although DNA adducts from single-ring aromatic amines have yet to be identified in vivo, several in vitro studies have confirmed that these arylamines can form such adducts. The most common strategy for the synthesis of DNA adduct standards from aniline derivatives involves the reaction of dG, dG nucleotides, or DNA with *N*-acyloxyanilines (*19*−*23*). These procedures, which mimic the metabolic activation pathway for arylamines, give dG-C8-Ar adducts as the major products, but the yields are typically very low, precluding the isolation of presumed minor adducts. More recently developed strategies, involving palladium-catalyzed C−N bond formation (*24*) or the use of arylnitrenes as adduct precursors (*25*), have the potential to become suitable routes for certain types of minor arylamine adducts, but their use has yet to become generalized.

Previous reports (*19*, *23*) have shown that, like other *N*-acyloxyarylamines, *N*-acetoxy-2,6-diMeA (*N*-AcO-2,6-diMeA) yields *N*-(deoxyguanosin-8-yl)-2,6-dimethylaniline (dG-C8-2,6-diMeA) as a major DNA adduct in vitro. However, we have observed that the reaction profile is considerably more complex than with other alkylaniline derivatives, and that the isolation of additional putative adducts requires thorough and efficient separation from a variety of hydrophilic solvolysis products. We now provide evidence that *N*-AcO-2,6-diMeA yields an unusual mixture of DNA adducts in comparable yields, and report the isolation and characterization of two new adducts from dG through the exocyclic N^2 and O^6 atoms, and one new adduct from dA through the exocyclic N^6 atom. Possible implications of this uncommon adduct pattern in the biological response elicited by 2,6-diMeA are discussed. Furthermore, in view of the current relevance of the ^{32}P-postlabeling methodology for the detection of carcinogen−DNA adducts, and of the uncertainties regarding the influence of structural factors on the relative labeling efficiencies of specific adducts (*26*−*29*), we have also examined the kinetics of the T_4 polynucleotide kinase (PNK)-catalyzed phosphorylation of the C8 and N^2 deoxyguanosine 3′-phosphate adducts from 2,6-diMeA. The kinetic parameters obtained with these two structurally different adducts are compared to those determined with the parent nucleotide, deoxyguanosine 3′-phosphate (dG3′p), and also to those we reported previously using other *N*-(deoxyguanosin-8-yl)-arylamine 3′-phosphate (dG3′p-C8-Ar) adducts (*29*). For comparative purposes, the kinetics of phosphorylation of (±)-*anti*-10-(deoxyguanosin-N^2-yl)-7,8,9-trihydroxy-7,8,9,-10-tetrahydrobenzo[*a*]pyrene 3′-phosphate (dG3′p-N^2-BPDE), the major adduct (*30*) derived from the environmental pollutant benzo[*a*]pyrene, was also analyzed.

Materials and Methods

Caution: *N-OH-2,6-diMeA and N-AcO-2,6-diMeA are potentially carcinogenic. They should be handled with protective clothing in a well-ventilated fumehood. Exposure to ^{32}P should be kept as low as possible, by working in a confined laboratory area, with protective clothing, plexiglass shielding, Geiger counters, and body dosimeters. Waste materials must be discarded according to appropriate safety procedures.*

Chemicals. 2′-Deoxyguanosine (dG) was acquired from Amersham U.S. Biochemical (Cleveland, OH). 2-Nitro-*m*-xylene was obtained from Aldrich Chemical Co. (Milwaukee, WI). (±)-*r*-7,*t*-8-Dihydroxy-*t*-9,10-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene (BPDE) was purchased from ChemSyn Laboratories (Lenexa, KS). ATP was acquired from Boehringer Manheim Gmbh (Indianapolis, IN). Carrier-free [γ-^{32}P]ATP was purchased from ICN Pharmaceuticals (Costa Mesa, CA) and diluted 500−1000-fold before being used in the labeling reactions. PNK (90 090 units/mg; 35 000 mg/mmol of monomer) was acquired from Amersham U.S. Biochemical at a concentration of 30 units/μL. Dilutions of the stock solution were performed as needed. All other reagents and enzymes were purchased from Sigma Chemical Co. (St. Louis, MO).

Instrumentation. Melting temperatures were measured with a Leica Galen III hot-stage apparatus and are uncorrected.

Reversed-phase HPLC analyses and separations were conducted using a μBondapak C_{18} column (0.39 cm × 30 cm, Waters Associates, Milford, MA), either on a Varian system consisting of a Star 9012 ternary gradient pump and a Polychrom 9065 diode array spectrophotometric detector (Varian, Inc., Palo Alto, CA), equipped with a Rheodyne injector (Rheodyne, Cotati, CA), or on a Waters Associates system consisting of two model 510 pumps and a model 660 automated gradient controller, equipped with a Rheodyne injector and a Hewlett-Packard 1050 diode array spectrophotometric detector. The peaks were monitored at 280 nm.

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

NMR spectra were obtained at 23 °C on a Varian Unity 300 spectrometer, operating at 300 MHz (^{1}H spectra) or 75.43 MHz (^{13}C spectra). The adduct samples were dissolved in Me_2SO-d_6. The proton chemical shifts were referenced to the residual Me_2SO proton resonance (2.49 ppm) and the carbon chemical shifts to the Me_2SO carbon resonance (39.50 ppm). ^{1}H NMR

assignments were based on comparisons with the spectra of the parent nucleosides and of 2,6-diMeA, combined with homonuclear decoupling experiments, chemical exchange of the labile protons with D_2O, and observations of nuclear Overhauser effect (NOE) enhancement patterns. ^{13}C NMR assignments were based on published data for the parent deoxynucleosides dG and dA, and for polysubstituted aniline derivatives (*31*), combined with the use of a distortionless enhancement by polarization transfer (DEPT) sequence, employing a pulse angle of 135°, and with analysis of the $^{13}C-^{1}H$ coupling patterns in heteronuclear coupled spectra.

Positive ion electrospray ionization (ESI) mass spectra were recorded on a Finnigan TSQ-7000 spectrometer, using flow injection analysis in 50% methanol containing 0.1% ammonium formate (pH 3.5), at a flow rate of 0.1 mL/min.

Liquid scintillation counting was performed on a Packard Tri-Carb model 1600TR instrument (Packard Instrument Co., Meriden, CT), using Ultima Gold (Packard) as the scintillation fluid. TLC plates were exposed to intensifying screens at room temperature for variable periods of time (1−2 h) in Kodak X cassettes. Visualization and quantitation of the chromatograms were subsequently conducted with a phosphorimager (Storm 860 Imager, Molecular Dynamics, Sunnyvale, CA).

Synthesis of Nucleoside Adducts. The nucleoside adducts from 2,6-diMeA were prepared by reacting dG or dA with *N*-AcO-2,6-diMeA, essentially as described for other alkylaniline adducts (*19*, *22*). Briefly, a 200 mM solution of the nucleoside in a 1/2 mixture of 10 mM sodium citrate (pH 6.5) and DMF was prepared, purged with argon, and kept at 0−5 °C. To this solution were added aliquots of 2 M *N*-AcO-2,6-diMeA in THF, which had been generated from *N*-OH-2,6-diMeA and acetyl cyanide (*19*) and kept at −30 °C. Following addition of 5 molar equiv of *N*-AcO-2,6-diMeA over a period of 2 h, the reaction mixture was maintained under argon and stirred overnight at room temperature. Upon evaporation to dryness, the mixture was resuspended in water, and the lower-polarity solvolysis products were removed by extraction with methylene chloride. The adducts were subsequently partitioned into *n*-butanol; the *n*-butanol was evaporated, and the residue was dissolved in a small volume of methanol and separated by column chromatography on Sephadex LH-20 (Pharmacia/PL Biochemicals, Piscataway, NJ), using a 0 to 100% step gradient (in 5% steps) of aqueous methanol, followed by fractionation of the 15−50% MeOH eluates using RP_{18} modified silica (E. Merck, Darmstadt, Germany). The adducts derived from dG were finally purified by reversed-phase HPLC, using a 20 min linear gradient of 5 to 50% aqueous methanol, followed by a 5 min linear gradient to 70% methanol.

The following adducts were isolated from dG.

***N*-(Deoxyguanosin-8-yl)-2,6-dimethylaniline (dG-C8-2,6-diMeA, 1):** η 1%; mp >285 °C dec; UV (methanol) λ_{max} 269 nm ($\log \epsilon = 4.2$); ^1H NMR (DMSO-d_6) δ 2.02 (1H, m, H2″), 2.13 (6H, s, $ArCH_3$), 2.71 (1H, m, H2′), 3.67 (2H, m, H5′,5″), 3.91 (1H, m, H4′), 4.39 (1H, m, H3′), 5.32 (1H, bs, exchanged with D_2O, 3′-OH), 5.56 (1H, bs, exchanged with D_2O, 5′-OH), 6.32 (1H, m, H1′), 6.33 (2H, bs, exchanged with D_2O, N^2H_2), 7.04 (3H, m, $ArH_{3,4,5}$), 7.84 (1H, s, exchanged with D_2O, ArNH), 10.56 (1H, s, exchanged with D_2O, N1H); ^{13}C NMR (DMSO-d_6) δ 18.12 ($ArCH_3$), 37.83 (C2′), 61.30 (C5′), 71.46 (C3′), 82.78 (C1′), 87.17 (C4′), 112.57 (C5), 125.40 (ArC_4), 127.81 ($ArC_{3,5}$), 135.01 ($ArC_{2,6}$), 137.32 (C8/ArC_1), 145.85 (ArC_1/C8), 149.89 (C4), 152.41 (C2), 155.52 (C6); MS (ESI) (relative intensity) *m*/*z* 387 (MH^+, 8%), 271 (MH_2^+ − dR, 100%).

4-(Deoxyguanosin-N^2-yl)-2,6-dimethylaniline (dG-N^2-2,6-diMeA, 2): η 0.5%; mp >160 °C dec; UV (methanol) λ_{max} 284 nm ($\log \epsilon = 4.0$); ^1H NMR (DMSO-d_6) δ 2.08 (6H, s, $ArCH_3$), 2.22 (1H, m, H2″), 2.64 (1H, m, H2′), 3.44 (2H, m, H5′,5″), 3.79 (1H, m, H4′), 4.29 (1H, m, H3′), 4.6−5.1 (1H, bs, exchanged with D_2O, OH), 5.1−5.4 (1H, bs, exchanged with D_2O, OH), 6.12 (1H, t, J = 6.9 Hz, H1′), 7.00 (2H, s, $ArH_{3,5}$), 7.95 (1H, s, H8), 8.26 (1H, s, exchanged with D_2O, N^2H), 10.34 (1H, s, exchanged with D_2O, N1H); ^{13}C NMR (DMSO-d_6) δ 18.34 ($ArCH_3$), ∼39.5 (C2′, partially obscured by the solvent resonance), 62.01 (C5′), 71.14 (C3′), 83.69 (C1′), 87.91 (C4′), 117.92 (C5), 121.46 ($ArC_{3,5}$), 122.11 ($ArC_{2,6}$), 127.94 (ArC_4), 137.03 (C8), 140.85 (ArC_1), 150.66 (C4), 155.21 (C2/C6), 156.36 (C6/C2); MS (ESI) (relative intensity) *m*/*z* 409 [$(M + Na)^+$, 18%], 387 (MH^+, 100%), 271 (MH_2^+ − dR, 44%).

4-(Deoxyguanosin-O^6-yl)-2,6-dimethylaniline (dG-O^6-2,6-diMeA, 3): η 0.3%; mp >300 °C dec; UV (methanol) λ_{max} 237 ($\log \epsilon = 4.0$), 286 nm ($\log \epsilon = 3.8$); ^1H NMR (DMSO-d_6) δ 2.08 (6H, s, $ArCH_3$), 2.24 (1H, m, H2″), 2.57 (1H, m, H2′), 3.52 (2H, m, H5′,5″), 3.82 (1H, m, H4′), 4.35 (1H, m, H3′), 4.44 (2H, s, exchanged with D_2O, $ArNH_2$), 4.98 (1H, t, J = 5.1 Hz, exchanged with D_2O, 5′-OH), 5.27 (1H, d, J = 2.7 Hz, exchanged with D_2O, 3′-OH), 6.21 (1H, t, J = 6.3 Hz, H1′), 6.35 (2H, s, exchanged with D_2O, N^2H_2), 6.65 (2H, s, $ArH_{3,5}$), 8.14 (1H, s, H8); ^{13}C NMR (DMSO-d_6) δ 17.90 ($ArCH_3$), 39.37 (C2′), 61.76 (C5′), 70.81 (C3′), 82.81 (C1′), 87.65 (C4′), 114.12 (C5), 120.75 ($ArC_{3,5}$), 121.60 ($ArC_{2,6}$), 128.38 (ArC_4), 138.31 (C8), 142.25 (ArC_1), 154.30 (C4), 159.88 (C2/C6), 160.66 (C6/C2); MS (ESI) (relative intensity) *m*/*z* 409 [$(M + Na)^+$, 16%], 387 (MH^+, 100%), 271 (MH_2^+ − dR, 93%).

The following adduct was isolated from dA.

4-(Deoxyadenosin-N^6-yl)-2,6-dimethylaniline (dA-N^6-2,6-diMeA, 4): η 2%; mp >220 °C dec; UV (methanol) λ_{max} 306 nm ($\log \epsilon = 4.2$); ^1H NMR (DMSO-d_6) δ 2.08 (6H, s, $ArCH_3$), 2.26 (1H, m, H2″), 2.74 (1H, m, H2′), 3.60 (2H, m, H5′,5″), 3.88 (1H, m, H4′), 4.38 (2H, exchanged with D_2O, $ArNH_2$), 4.42 (1H, m, H3′), 5.25 (1H, bs, exchanged with D_2O, OH), 5.36 (1H, bs, exchanged with D_2O, OH), 6.36 (1H, t, J = 6.8 Hz, H1′), 7.20 (2H, s, $ArH_{3,5}$), 8.24 (1H, s, H8), 8.40 (1H, s, H2), 9.33 (1H, s, exchanged with D_2O, N^6H); ^{13}C NMR (DMSO-d_6) δ 18.25 ($ArCH_3$), 39.67 (C2′), 62.01 (C5′), 71.12 (C3′), 84.29 (C1′), 88.15 (C4′), 119.96 (C5), 121.0 ($ArC_{2,6}$), 122.23 ($ArC_{3,5}$), 128.05 (ArC_4), 140.03 (C8), 140.82 (ArC_1), 148.79 (C4), 152.49 (C2), 152.76 (C6); MS (ESI) (relative intensity) *m*/*z* 371 (MH^+, 100%), 255 (MH_2^+ − dR, 44%).

Synthesis of dG3′p Adducts. (1) 2,6-Dimethylaniline Adducts. A solution of dG3′p (10 mg) in 10 mM sodium citrate (pH 6.0, 300 μL) was allowed to react overnight with *N*-AcO-2,6-diMeA (1.2 molar equiv in 200 μL of THF), generated as described above. The THF was evaporated; water was added to a final volume of 1 mL, and the solvolysis products were extracted with diethyl ether (5 × 500 μL). The adducts were separated by reversed-phase HPLC, using a 30 min linear gradient of 5 to 50% acetonitrile in 100 mM ammonium acetate (pH 5.7). The isolated adducts were *N*-(deoxyguanosin-8-yl)-2,6-dimethylaniline 3′-phosphate (dG3′p-C8-2,6-diMeA) and 4-(deoxyguanosin-N^2-yl)-2,6-dimethylaniline 3′-phosphate (dG3′p-N^2-2,6-diMeA). For characterization purposes, aliquots of both adducts were treated with alkaline phosphatase for 3 h at 37 °C. In both instances, the UV spectra and HPLC retention times of the dephosphorylated products were identical to those of the corresponding dG adducts.

(2) (±)-*anti*-10-(Deoxyguanosin-N^2-yl)-7,8,9-trihydroxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene 3′-Phosphate (dG3′p-N^2-BPDE). An 11 mM solution of (±)-*r*-7,*t*-8-dihydroxy-*t*-9,10-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene (BPDE) in THF (300 μL) was added to an 8-fold molar excess of dG3′p, dissolved in the same volume of 10 mM sodium citrate (pH 6.0), and the mixture was stirred overnight at room temperature. Following evaporation of the THF, water was added to a final volume of 1 mL, and the unbound BPDE derivatives were removed by repeated extractions with diethyl ether. The dG3′p-N^2-BPDE was isolated by reversed-phase HPLC using a 40 min nonlinear gradient (Waters curve 2) of 20 to 56% methanol in 100 mM ammonium acetate (pH 5.7). Upon treatment with alkaline phosphatase, the HPLC retention time and UV spectrum of the adduct were identical to those of (±)-*anti*-10-(deoxyguanosin-N^2-yl)-7,8,9-trihydroxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene obtained by reacting BPDE with dG. The concentration of dG3′p-N^2-BPDE was determined using the extinction coefficient reported by Pulkrabek et al. (*32*).

DNA Modification. The modification of DNA with 2,6-diMeA was conducted by reacting salmon testes DNA with *N*-AcO-2,6-diMeA as described previously (*19*). Upon standard enzymatic hydrolysis of the DNA to nucleosides (*33*), the HPLC retention times and UV spectra of the 2,6-diMeA−DNA adducts were compared to those of the corresponding dG and dA adducts.

Kinetic Studies. (1) Phosphorylation Reactions. The effect of the substrate concentration on the PNK-catalyzed ^{32}P-postlabeling of dG3′p adducts was studied for dG3′p-C8-2,6-diMeA, dG3′p-N^2-2,6-diMeA, and dG3′p-N^2-BPDE using the methodology described by Mourato et al. (*29*). Briefly, aliquots (5 μL) of a labeling solution containing 600 milliunits/μL (50 fmol/μL) PNK and 955 μM ATP in 80 mM bicine-NaOH, 40 mM $MgCl_2$, 40 mM dithiothreitol, and 4 mM spermidine (pH 9.4) buffer were added to 15 μL of various substrate solutions (20−4000 pmol, 1.3−267 μM). The mixtures were vortexed, centrifuged for 5−10 s, and incubated at 37 °C for 5 min. Each reaction was quenched by mixing with 2 μL of 673 μM thymidine 3′-phosphate (dT3′p) and 30 milliunits of apyrase (1.5 μL), and the mixtures were further incubated for 30 min at 37 °C. In each instance, the adduct concentrations were determined by UV spectroscopy, using the extinction coefficients of the corresponding nucleoside adducts (vide supra).

(2) Chromatography. Aliquots of each mixture (15 μL) were spotted on Macherey-Nagel polyethyleneimine cellulose (PEI-cellulose) TLC plates (20 cm in length), purchased from Alltech Associates, Inc. (Deerfield, IL), that had been washed with deionized water and dried prior to use. The elution conditions were as follows.

(i) dG3′,5′p-C8-2,6-diMeA. The elution was conducted onto a Whatman #1 paper wick (direction D1) with 2.5 M sodium phosphate (pH 6.0) and then in the opposite direction (D2) with 1.8 M lithium formate and 4.2 M urea (pH 3.5).

(ii) dG3′,5′p-N^2-2,6-diMeA. The elution was conducted onto a Whatman #1 paper wick (direction D1) with 900 mM sodium phosphate (pH 6.8) and then in direction D2 with 1.8 M lithium formate and 4.2 M urea (pH 3.5).

(iii) dG3′,5′p-N^2-BPDE. The elution was conducted onto a Whatman #1 paper wick (direction D1) with 900 mM sodium phosphate (pH 6.8) and then in direction D2 with 3.6 M lithium formate and 8.5 M urea (pH 3.5).

(3) Calculations. The specific activity of the [γ-^{32}P]ATP in each labeling mixture was established by liquid scintillation counting of diluted aliquots, whose concentration had been determined by UV spectroscopy. Following elution and prior to exposure of each TLC plate to the intensifying screen, an aliquot of the labeling mixture was spotted on the plate to serve as a quantitation standard. Corrections for ^{32}P decay were performed whenever necessary. The kinetic parameters (V_{max} and K_m) were calculated using the weighted least-squares fitting program Leonora (*34*).

Results

Characterization of the 2,6-diMeA Adducts. One of the goals of this work was to elucidate the structures of several putative 2,6-diMeA DNA adducts that are produced from *N*-acyloxy-2,6-diMeA species but have eluded isolation and characterization due to the complexity of the reaction mixtures. Toward this purpose, *N*-AcO-2,6-diMeA (*19*) was used as the electrophilic synthon to generate 2,6-diMeA adducts from dG, dG3′p, dA, and DNA. The adducts formed upon reaction with the nucleosides or dG3′p were isolated by chromatography following solvent extractions to remove the less polar solvolysis products of *N*-AcO-2,6-diMeA. The adducts formed upon reaction with DNA were identified after enzymatic hydrolysis of the modified DNA to nucleosides.

Adducts from the Reaction with dG. In agreement with previous reports (*19*, *23*), the major adduct from the reaction between dG and *N*-AcO-2,6-diMeA was found

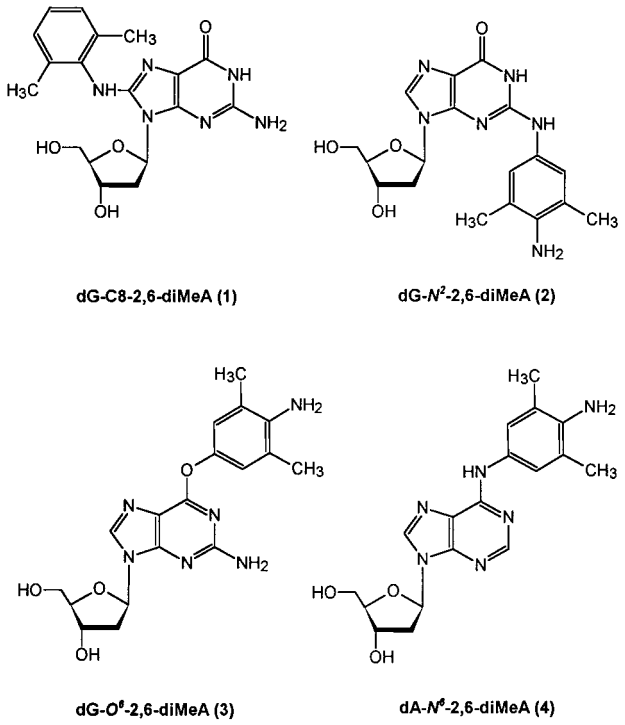

Figure 1. Structures of the adducts isolated from the reaction of *N*-AcO-2,6-diMeA and nucleosides.

to be dG-C8-2,6-diMeA (**1**, Figure 1), the product of C8 substitution through the arylamine nitrogen. This adduct, which has been characterized previously (*19*, *23*), eluted in the 40−50% methanol fraction from Sephadex LH-20 and was obtained in 1% yield. However, as indicated above, *N*-AcO-2,6-diMeA yielded a complex profile upon reaction with dG. In contrast to what we observed with other methyl- and dimethylaniline derivatives (*19*, *22*), additional putative adducts were present in levels comparable to that of dG-C8-2,6-diMeA. These adducts had to be thoroughly separated from hydrophilic side products, stemming from hydrolysis of *N*-AcO-2,6-diMeA. Following partitioning into *n*-butanol, we were able to isolate two additional dG adducts, which eluted in the 30−40% methanol fraction from Sephadex LH-20. The purification of both species required further chromatographic separation on RP_{18} modified silica, followed by HPLC. The new adducts (Figure 1) were characterized as 4-(deoxyguanosin-N^2-yl)-2,6-dimethylaniline (dG-N^2-2,6-diMeA, **2**, 0.5%) and 4-(deoxyguanosin-O^6-yl)-2,6-dimethylaniline (dG-O^6-2,6-diMeA, **3**, 0.3%) on the basis of spectroscopic evidence.

(1) dG-N^2-2,6-diMeA (2). The ESI mass spectrum of adduct **2** in the positive ion mode indicated the presence of the protonated (*m*/*z* 387) molecular ion, as expected from an adduct of dG with 2,6-diMeA. In addition, the detection of an intense ion at *m*/*z* 271 is consistent with loss of the deoxyribosyl fragment from the parent ion. The UV spectrum of adduct **2** (Figure 2) had a major absorption band at 284 nm, which represents a bathochromic shift of ca. 30 nm compared to dG, presumably due to significant conjugation between the arylamine and dG chromophores. This observation was the first suggestion that substitution did not occur through the arylamine nitrogen, since the steric hindrance caused by the presence of the two ortho methyl groups should decrease the ability of the arylamine nitrogen to act as an electron donor. For instance, while the UV absorption

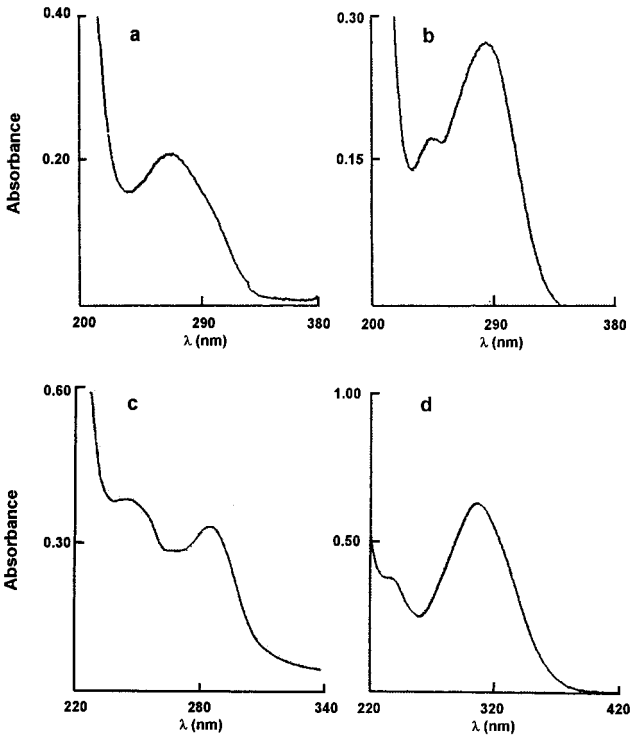

Figure 2. UV spectra of the isolated 2,6-diMeA adducts, recorded in methanol: (a) dG-C8-2,6-diMeA (**1**), (b) dG-N^2-2,6-diMeA (**2**), (c) dG-O^6-2,6-diMeA (**3**), and (d) dA-N^6-2,6-diMeA (**4**).

of dG-C8-2,6-diMeA had a bathochromic shift of only 15 nm compared to dG, shifts of up to 30 nm have been observed for other dG-C8-Ar adducts without ortho substituents in the arylamine moiety (*19*, *22*). The ^{1}H NMR spectrum of adduct **2** provided conclusive structural proof. Thus (Table 1), a six-proton singlet at 2.08 ppm and a two-proton nonexchangeable singlet at 7.00 ppm were fully consistent with substitution in the aromatic ring, para to the arylamine nitrogen. The observation of a one-proton singlet at 7.95 ppm, assigned to guanine H8, further indicated that substitution must have occurred through one of the dG heteroatoms. With the exception of the arylamine NH_2, presumably obscured by the water resonance, all the expected exchangeable signals were detected. Of these, two downfield one-proton signals, at 8.26 and 10.34 ppm, were essential for structure elucidation (Figure 3). The latter was assigned to the guanine imino proton. Both the location (ca. 2 ppm downfield from the N^2-H_2 protons in dG) and the intensity of the signal at 8.26 ppm were consistent with substitution through the exocyclic nitrogen of dG. The ^{1}H NMR spectrum further indicated the presence of all the expected deoxyribose resonances, and the ^{13}C NMR spectrum confirmed the presence of all the expected carbon resonances. Taken together, the spectroscopic data for adduct **2** are fully compatible with the formation of a covalent bond between the N^2 atom of dG and the C4 atom of 2,6-diMeA.

(2) dG-O^6-2,6-diMeA (3). The ESI mass spectrum of adduct **3** in the positive ion mode indicated the presence of the protonated (*m*/*z* 387) and sodiated (*m*/*z* 409) molecular ions expected from an adduct of dG with 2,6-diMeA. Like adduct **2**, an intense ion at *m*/*z* 271, consistent with loss of the deoxyribosyl fragment from the parent ion, was detected. In contrast with a single major absorption band observed for dG-C8-2,6-diMeA and dG-N^2-2,6-diMeA, the UV spectrum of this adduct (Figure 2) had two absorption bands with similar intensities, centered at 237 and 286 nm, which suggested that two relatively independent chromophores were present in the molecule. A similar UV spectral pattern has been reported recently for an O^6-substituted adduct formed by reaction of dG with the arylnitrene generated from 2-methylnitrobenzene (*25*). The ^{1}H NMR spectrum of **3** showed all the expected nonexchangeable sugar resonances, as well as a singlet at 8.14 ppm, assigned to the guanine H8 (Table 1). A six-proton singlet at 2.08 ppm and an aromatic pattern of two equivalent protons at 6.65 ppm were consistent with substitution in the 2,6-diMeA ring, para to the amino group. Four sets of labile protons were observed. Two of these, at 4.98 and 5.27 ppm, accounted for one proton each and were assigned to the two sugar hydroxyls, 5′-OH and 3′-OH, respectively, on the basis of their coupling patterns. The two additional sets of exchangeable signals, at 4.44 and 6.35 ppm, accounted for two protons each. This suggests the presence of two exocyclic NH_2 groups, one in the arylamine fragment and the other in the dG moiety. Furthermore, no downfield resonance consistent with the presence of a dG imino proton was observed. Taken together, these data suggest substitution through the O^6 atom of dG. Further support for the identification of adduct **3** as dG-O^6-2,6-diMeA stemmed from the observation of downfield shifts (3−5 ppm) of the C2 and C6 resonances, compared to the same resonances in dG (*31*), suggesting full aromatization of the purine ring system. Similar downfield shifts of the C2 and C6 resonances have been observed in other O^6-substituted derivatives of dG (*25*, *35*).

Adducts from the Reaction with dG3′p. The HPLC profile from the reaction of *N*-AcO-2,6-diMeA with dG3′p was virtually identical to that obtained with dG. The major adducts, dG3′p-C8-2,6-diMeA and dG3′p-N^2-2,6-diMeA, were isolated by reversed-phase HPLC. Both had UV spectra identical to those of the corresponding dG adducts, into which they were converted upon treatment with alkaline phosphatase. On the basis of the UV pattern of the reaction products, an O^6-substituted adduct, dG3′p-O^6-2,6-diMeA, was also present in the reaction mixture. However, this adduct was formed in low yield and was not isolated.

Adduct from the Reaction with dA (dA-N^6-2,6-diMeA, 4). The HPLC profile from the reaction of *N*-AcO-2,6-diMeA with dA indicated the presence of one putative major adduct, obtained in 2% yield, which eluted in the 30−40% methanol fraction from Sephadex LH-20

The ESI mass spectrum of this species in the positive ion mode was consistent with formation of a dA adduct from 2,6-diMeA. Thus, the parent peak (*m*/*z* 371) corresponded to the protonated molecular ion, and a prominent ion at *m*/*z* 255 resulted from loss of the deoxyribosyl fragment. The UV spectrum of adduct **4** had one major absorption band at 306 nm (Figure 2), which represents a bathochromic shift of ca. 50 nm compared to dA, suggesting the occurrence of extensive conjugation between the arylamine and dA chromophores. The ^{1}H and ^{13}C NMR spectra of this adduct are shown in Figure 4 (panels a and b, respectively). As indicated in Figure 4b, all the expected carbon resonances were present. The detection of one singlet due to two equivalent methyl groups (2.08 ppm) and one singlet due to two equivalent aromatic protons (7.20 ppm) in the ^{1}H NMR spectrum of

Table 1. Proton Chemical Shifts Relevant for Structural Elucidation of the 2,6-diMeA Adducts

	δ (ppm)[a]			
	dG-C8-2,6-diMeA (**1**)	dG-N^2-2,6-diMeA (**2**)	dG-O^6-2,6-diMeA (**3**)	dA-N^6-2,6-diMeA (**4**)
CH_3	2.13 (s, 6H)	2.08 (s, 6H)	2.08 (s, 6H)	2.08 (s, 6H)
ArH	7.04 (m, 3H)	7.00 (s, 2H)	6.65 (s, 2H)	7.20 (s, 2H)
$ArNH/ArNH_2$[b]	7.84 (s, 1H)	ND[c]	4.44 (s, 2H)	4.38 (s, 2H)
N^2-H/N^2-H_2[b]	6.33 (s, 2H)	8.26 (s, 1H)	6.35 (s, 2H)	–
N^6-H[b]	–	–	–	9.33 (s, 1H)
H8	–	7.95 (s, 1H)	8.14 (s, 1H)	8.24 (s, 1H)
H2	–	–	–	8.40 (s, 1H)
N1H[b]	10.56 (s, 1H)	10.34 (s, 1H)	–	–

[a] Recorded in DMSO-d_6. [b] Exchanged with D_2O. [c] ND, not detected.

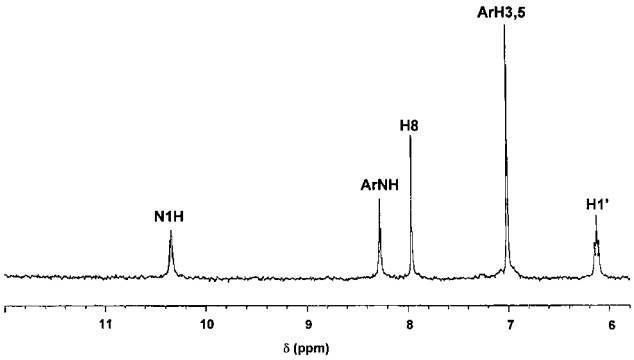

Figure 3. Downfield region (6−12 ppm) of the ^{1}H NMR spectrum of dG-N^2-2,6-diMeA (**2**), recorded in DMSO-d_6. Full chemical shift assignments are given in Materials and Methods.

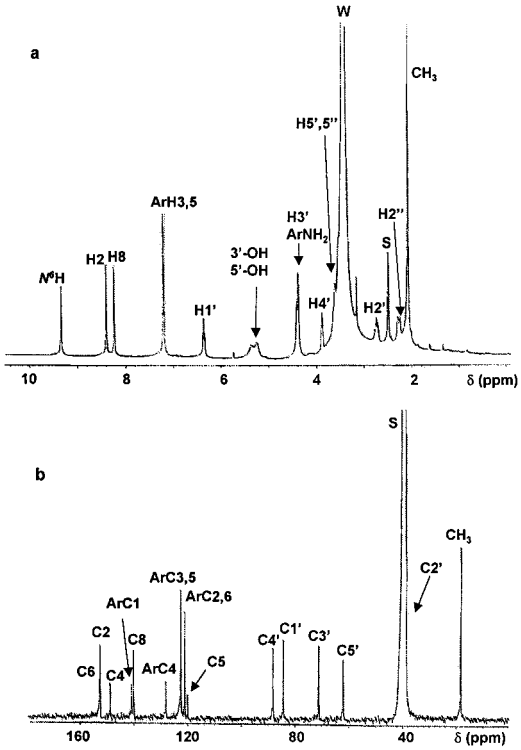

Figure 4. ^{1}H NMR spectrum (a) and proton broadband-decoupled ^{13}C NMR spectrum (b) of dA-N^6-2,6-diMeA (**4**), recorded in DMSO-d_6. Full chemical shift assignments are given in Materials and Methods. S represents the DMSO resonance and W the water resonance.

adduct **4** (Figure 4a and Table 1) indicated that, like dG-N^2-2,6-diMeA and dG-O^6-2,6-diMeA, substitution occurred through the C4 atom of 2,6-diMeA. This was further confirmed by the presence of an exchangeable two-proton singlet at 4.38 ppm, assigned to the arylamine NH_2. All the sugar resonances were observed, as were two singlets at 8.24 and 8.40 ppm, assigned to adenine H8 and H2, respectively. Finally, the presence of a one-proton exchangeable signal at 9.33 ppm (Figure 4a), ca. 2 ppm downfield from the N^6-H_2 resonance in dA (not shown), strongly indicated the existence of a bond between the exocyclic nitrogen of dA and the arylamine C4. Thus, the identification of adduct **4** as 4-(deoxyadenosin-N^6-yl)-2,6-dimethylaniline (dA-N^6-2,6-diMeA) is fully consistent with the spectroscopic data.

Adducts from the Reaction with DNA. Like other *N*-acetoxyarylamines, *N*-AcO-2,6-diMeA has been shown to yield the C8-substituted dG derivative, dG-C8-2,6-diMeA, as a major adduct when reacted with DNA in vitro (*19*). Following solvent extractions and enzymatic hydrolysis of the DNA to nucleosides, we compared the UV profiles and HPLC retention times of the reaction products with those of the new adduct standards obtained from the reaction of *N*-AcO-2,6-diMeA with dG and dA (not shown). When the extinction coefficients determined for each of the four 2,6-diMeA adduct standards were taken into consideration (cf. Materials and Methods), we concluded that the relative yields were approximately 46% for dA-N^6-2,6-diMeA, 22% for dG-N^2-2,6-diMeA, 20% for dG-O^6-2,6-diMeA, and 12% for dG-C8-2,6-diMeA. The fact that the dA derivative is the adduct formed in greatest proportion upon reaction with DNA is consistent with the relative yields obtained in the reactions with the nucleosides. In addition, it should be noted that although dG-C8-2,6-diMeA was the major adduct obtained from the reaction of *N*-AcO-2,6-diMeA with dG, the lower yields of the dG-N^2-2,6-diMeA and dG-O^6-2,6-diMeA adducts isolated in that reaction are likely to have resulted from the laborious purification procedure. This product profile contrasts markedly with the usual predominance of dG-C8-Ar adducts when other alkylated aniline derivatives are reacted with DNA (*19*, *22*).

^{32}P-Postlabeling Studies. With the aim of evaluating the contribution of structural factors to the efficiency of ^{32}P-postlabeling of specific DNA adducts, we conducted a comparative kinetic analysis of the PNK-catalyzed phosphorylation reaction, using 2′-deoxyguanosine 3′-phosphate (dG3′p) and the dG3′p adducts from 2,6-diMeA (dG3′p-C8-2,6-diMeA and dG3′p-N^2-2,6-diMeA). For comparative purposes, dG3′p-N^2-BPDE, the dG3′p adduct from BPDE, was included in the study.

The experimental conditions for this type of study have been described (*29*). Briefly, an incubation time of 5 min was chosen on the basis of our previous results (*29*) with dG3′p and *N*-(deoxyguanosin-8-yl)-4-aminobiphenyl 3′-phosphate (dG3′p-C8-4-ABP), which indicated that the linear region of the time course curve spanned approximately 10 min regardless of the substrate. A PNK concentration of 150 milliunits/μL (12 nM) was selected

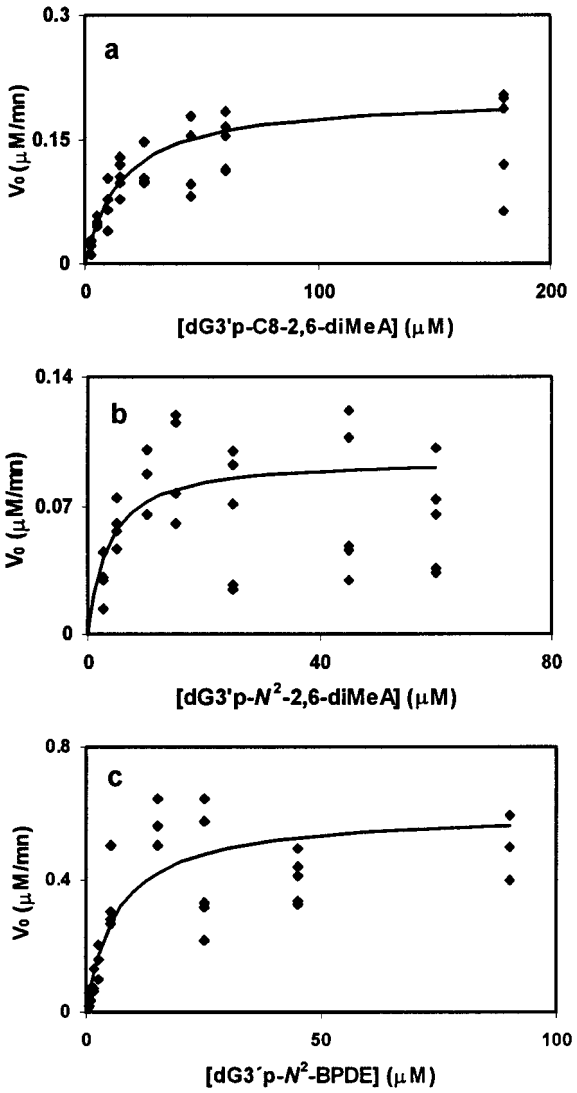

Figure 5. Variation of the initial velocity of phosphorylation as a function of substrate concentration, in the presence of 240 μM ATP and 150 milliunits/μL PNK, using (a) dG3′p-C8-2,6-diMeA, (b) dG3′p-N^2-2,6-diMeA, and (c) dG3′p-N^2-BPDE as substrates. The incubation conditions are outlined in Materials and Methods.

because this enzyme concentration falls within the range of PNK concentrations (100−1000 milliunits/μL) typically used in ^{32}P-postlabeling protocols (*26*, *27*) and is sufficiently low to prevent the accumulation of bisphosphate products, thus ensuring stationary state conditions (*29*). The ATP concentration was set at 240 μM because our previous study (*29*) indicated that this was a near-saturating concentration, which allowed the determination of satisfactory kinetic data while preventing excessive exposure to ^{32}P. Since our previously reported kinetic parameters for the parent nucleotide, dG3′p, were obtained with a lower ATP concentration (101 μM), we also analyzed the labeling of this substrate at 240 μM ATP.

The experimental curves displaying the initial velocity of phosphorylation as a function of substrate concentration are shown in Figure 5 for each of the three dG3′p adducts. The calculated kinetic parameters are listed in Table 2. In all instances, a greater number of data points was collected in the region of low substrate concentration for better accuracy in defining the experimental curve (*34*). The substrate concentrations ranged from 1.0 to 200 μM in the incubation mixtures, with the higher concentrations selected to ensure that the V_{max} plateau was reached. The results indicated that Michaelis−Menten kinetics was followed for all the substrates. As shown in Table 2, the apparent PNK turnover number (k_{cat} = 160 min^{-1}) and specificity constant (k_{cat}/K_m = 19 μM^{-1} min^{-1}) obtained with dG3′p were substantially higher than those determined with any of the dG3′p-adducted substrates. This is fully consistent with our previous results using a series of dG3′p-C8-Ar adducts and strongly suggests that ^{32}P-postlabeling of adducted nucleotides is less efficient than that of dG3′p. In addition, it should be noted that the PNK specificity constant determined with dG3′p in this study is approximately 3-fold higher than the one we reported previously (*29*), which demonstrates the importance of conducting the kinetic measurements at a higher ATP concentration.

Despite a consistently lower efficiency of labeling of all the adducts compared to that of the parent nucleotide, significant differences were apparent depending on the adduct structures. Thus, while the enzyme specificity constant obtained with dG3′p-C8-2,6-diMeA (k_{cat}/K_m = 1.1 μM^{-1} min^{-1}) was comparable to that previously determined with dG3′p-C8-4-ABP [k_{cat}/K_m = 1.4 μM^{-1} min^{-1} (*29*)], the labeling efficiency of dG3′p-N^2-2,6-diMeA [k_{cat}/K_m = 2.4 μM^{-1} min^{-1} (Table 2)] was approximately 2-fold higher. This result suggests that N^2 adducts from dG3′p are intrinsically better substrates than their C8 analogues for PNK. The enzyme specificity constant (k_{cat}/K_m = 7.9 μM^{-1} min^{-1}) determined with the bulkier N^2 adduct, dG3′p-N^2-BPDE, is consistent with this interpretation. In addition, since the BPDE derivative was the adduct labeled with greatest efficiency, the results further imply that the bulky aromatic moiety may play a role in the binding of the substrate to the active site of the enzyme.

Discussion

Multiplicity of 2,6-diMeA Adducts. In this study, we have shown that *N*-AcO-2,6-diMeA, a metabolically plausible electrophilic derivative of 2,6-diMeA, forms an unusual pattern of DNA adducts. Unlike *N*-acyloxy derivatives of other alkylanilines, which give dG-C8-Ar adducts as the predominant products of reaction with DNA in vitro (*19*, *22*), *N*-AcO-2,6-diMeA yielded three major dG adducts, dG-C8-2,6-diMeA, dG-N^2-2,6-diMeA, and dG-O^6-2,6-diMeA, and one major dA adduct, dA-N^6-2,6-diMeA. Furthermore, the dA adduct was the product formed in the greatest proportion upon reaction with DNA, while dG-C8-2,6-diMeA was the minor adduct of the four. This unique behavior also contrasts markedly with the characteristic pattern of adduct formation from known aromatic amine carcinogens, which tend to yield dG-C8-Ar adducts as the major lesions in vivo (*6*−*8*). Nonetheless, exceptions to this typical behavior have been reported; for instance, *N*-hydroxy-1-naphthylamine predominantly forms dG adducts through the exocyclic O^6 atom (*36*), while N^2-dG and N^6-dA species have been characterized as minor adducts from 2-naphthylamine (*37*).

The peculiar multiplicity of adduct structures obtained from *N*-AcO-2,6-diMeA reveals a low selectivity of the reactive intermediate toward specific nucleophilic sites in the purine bases. Extensive work on the mechanisms of hydrolysis of *N*-acyloxyarylamines and *N*-acyl-*N*-acyloxyarylamines (*38*−*41*, and references therein) has

Table 2. Kinetic Parameters Obtained for the Phosphorylation of dG3′p and dG3′p Adducts

substrate[a]	K_m (μM)[b]	V_{max} (μM min^{-1})[b]	k_{cat} (min^{-1})[c]	k_{cat}/K_m (μM^{-1} min^{-1})[d]
dG3′p	8.4 ± 0.93	1.9 ± 0.12	160	19
dG3′p-C8-2,6-diMeA	15.9 ± 2.5	0.20 ± 0.016	17	1.1
dG3′p-N^2-2,6-diMeA	3.4 ± 1.6	0.096 ± 0.013	8.2	2.4
dG3′p-N^2-BPDE	6.5 ± 1.4	0.60 ± 0.069	51	7.9

[a] Substrate concentrations ranged from 1.0 to 200 μM in the incubation mixtures. The ATP concentration was 240 μM. [b] Calculated for the Michaelis−Menten equation using the weighted least-squares (relative weights) fitting program Leonora (*34*). Both K_m and V_{max} are apparent values and are presented ± the standard error. [c] Apparent turnover number, defined as $V_{max}/[E]_0$. The PNK concentration in the incubation mixture was 150 milliunits/μL (12 nM). [d] Apparent specificity constant.

indicated that these reactions proceed by N−O bond heterolysis, leading to a nitrenium/carbenium ion intermediate as the cationic component of a tight ion pair, which may undergo diffusion to a solvent-separated ion pair. In general, trapping of the cationic species by external nucleophiles or internal return of the carboxylate anion, followed by nucleophilic attack on a resulting cyclohexadiene derivative, has been invoked to rationalize the structures of solvolysis products, including those obtained from carboxylate esters of *N*-OH-2,6-diMeA (*40*, *41*). The observed substitution in the arylamine ring for three of the adducts (dG-N^2-2,6-diMeA, dG-O^6-2,6-diMeA, and dA-N^6-2,6-diMeA), along with the unusual formation of a predominant dA adduct upon reaction with DNA, is fully consistent with the involvement of a nitrenium/carbenium ion intermediate in the rate-limiting step. The occurrence of aryl ring substitution para to the arylamine nitrogen instead of the more common ortho position (*6*−*8*), which was prevented by the presence of the two ortho methyl substituents, further supports this interpretation. It seems plausible that the steric hindrance imposed on the arylamine nitrogen by the two ortho methyl substituents may have been the factor determining a decreased extent of aryl *N*-substitution (i.e., formation of dG-C8-2,6-diMeA), while favoring nucleophilic attack at the less hindered para position. Thus, the ability of 2,6-diMeA to yield multiple adducts may stem, for the most part, from the steric constraints in the vicinity of the nitrogen atom.

The possible implications of the formation of multiple 2,6-diMeA−DNA adducts to the biological response elicited by this arylamine have yet to be established. As indicated above, there is substantial evidence (*12*, *13*) that 2,6-diMeA undergoes activation in vivo through *N*-hydroxylation. *N*-Hydroxyalkylanilines substituted ortho to the nitrogen tend to be more mutagenic than their analogues not bearing ortho substituents (*18*, *19*), which has been interpreted as implying that ortho-substituted dG-C8-Ar adducts may have higher intrinsic mutagenicities (*19*). However, while *N*-OH-2,6-diMeA is the most mutagenic of the entire series of *N*-(hydroxymethyl)-, *N*-(hydroxydimethyl)-, and *N*-(hydroxyethyl)anilines (*18*, *19*), this property does not seem to result from a higher efficiency of formation of the C8-substituted dG adduct (*19*, *23*). Instead, it appears that one or more of the additional 2,6-diMeA adducts may contribute significantly to the increased mutagenicity of *N*-OH-2,6-diMeA compared to other *N*-hydroxyalkylanilines. While specific adducts are expected to have characteristic mutagenicities and mutational spectra, the relative contributions of each adduct to the toxicological effects induced by 2,6-diMeA are presently unknown.

^{32}P-Postlabeling Studies. Due to its high sensitivity and versatility, ^{32}P-postlabeling is one of the most widely used methodologies for the detection and quantitation of carcinogen−DNA adducts (*26*−*28*). However, the precision of measured adduct levels is highly dependent on a number of assumptions, including the complete digestion of the DNA, the total recovery of the adducts during the enrichment and chromatography steps, and an equivalent efficiency of conversion of normal and adducted nucleotide 3′-phosphates to their corresponding 3′,5′-bisphosphates. Despite potential problems with these assumptions, these premises underlie the widespread use of the relative adduct labeling (RAL) approach for quantitative purposes (*42*). Although some reports have suggested that certain aromatic and bulky adducts may be better substrates than normal nucleotides for PNK (*43*, *44*), a substantial body of evidence has indicated that a variety of structurally different adducts are labeled with low efficiencies (reviewed in ref *29*). Arylamine−DNA adduct levels, in particular, seem to be consistently underestimated by RAL, as compared to other DNA adduct detection methodologies (*45*, *46*). When the results of our previous comparative kinetic study of the PNK-catalyzed ^{32}P-postlabeling of dG3′p and a series of dG3′p-C8-Ar adducts (*29*) are taken into account, this underestimation appears to be due, in part, to the lower efficiency of labeling of these adducts compared to that of the normal nucleotide. In particular, we were able to demonstrate that optimal labeling of dG3′p-C8-Ar adducts required ATP concentrations higher than those used in current ^{32}P-postlabeling protocols (*29*). In the study presented here, we have used the same methodology to investigate whether the site of attachment of the same arylamine fragment to the nucleotide (dG3′p) affects the labeling efficiency. Toward this purpose, we conducted a comparative kinetic analysis of the PNK-catalyzed phosphorylation of dG3′p-C8-2,6-diMeA and dG3′p-N^2-2,6-diMeA. Under the same experimental conditions, both adducts were labeled with much lower efficiencies (18- and 8-fold lower, respectively) than dG3′p, as indicated by the corresponding apparent specificity constants [k_{cat}/K_m (Table 2)]. This observation suggests that, under kinetically controlled conditions, normal nucleotides will be labeled preferentially compared to dG3′p-N^2-Ar adducts, which is similar to our previous observation with dG3′p-C8-Ar adducts (*29*). Nonetheless, the fact that the labeling efficiency of dG3′p-N^2-2,6-diMeA was approximately 2-fold higher than that of dG3′p-C8-2,6-diMeA confirms that the site of attachment of the aromatic fragment to the nucleotide affects the specificity of PNK toward the adducted substrate. In addition, it should be noted that all the dG3′p-C8-Ar adducts analyzed in our previous work (*29*) were labeled with efficiencies lower than that of dG3′p-N^2-2,6-diMeA, which suggests that dG3′p-N^2 adducts may be intrinsically better substrates for PNK than dG3′p-C8 adducts.

This is further substantiated by the results obtained with the bulky dG3′p-N^2-BPDE adduct. Despite being labeled with lower efficiency than the parent nucleotide, this adduct showed the best labeling efficiency of all the adducts that were tested, both in this study and in our previous report (*29*), as measured by the corresponding apparent specificity constant (Table 2). The reasons underlying this increased affinity of the bulkier substrate, and of N^2 adducts in general, toward the enzyme are not clear. Nonetheless, it is conceivable that the location of the aromatic fragment in the adduct may favor noncovalent stacking interactions with amino acid residues in the active site of the enzyme. We should also note that the labeling efficiency obtained for dG3′p-C8-2,6-diMeA was on the same order of magnitude previously determined for dG3′p-C8-4-ABP, but 5−25-fold higher than that obtained for other dG3′p-C8-Ar adducts from alkylanilines (*29*). This result was somewhat surprising, since we have observed consistently lower labeling efficiencies with adducts derived from ortho-substituted anilines. Although the reasons for the better labeling of dG3′p-C8-2,6-diMeA are obscure, it is possible that the steric hindrance in the vicinity of the arylamine nitrogen may enhance the stability of the adduct under the harsh alkaline conditions used for the labeling reaction.

In conclusion, this ^{32}P-postlabeling study confirms our previous observation that under kinetically controlled conditions, normal nucleotides are labeled preferentially to adducted substrates, which may lead to the underestimation of adduct levels in typical ^{32}P-postlabeling experiments. Nonetheless, the results demonstrate that compared to their C8-substituted analogues, aromatic adducts through the N^2 atom of dG are labeled with higher efficiencies.

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