Pyridyloxobutyl Adduct O^6-[4-Oxo-4-(3-pyridyl)butyl]guanine Is Present in 4-(Acetoxymethylnitrosamino)-1-(3-pyridyl)-1-butanone-Treated DNA and Is a Substrate for O^6-Alkylguanine-DNA Alkyltransferase

Lijuan Wang,[†,▽] Thomas E. Spratt,[‡] Xiao-Keng Liu,[†,∥] Stephen S. Hecht,[†,⊥] Anthony E. Pegg,[§] and Lisa A. Peterson*[,†,▽]

Divisions of Chemical Carcinogenesis and of Pathology and Toxicology, American Health Foundation, 1 Dana Road, Valhalla, New York 10595, and Department of Cellular and Molecular Physiology, Milton S. Hershey Medical Center, Pennsylvania State University College of Medicine, Hershey, Pennsylvania 17033

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The lung carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) is activated to reactive metabolites that methylate or pyridyloxobutylate DNA. Previous studies demonstrated that pyridyloxobutylated DNA interferes with the repair of O^6-methylguanine (O^6-mG) by O^6-alkylguanine-DNA alkyltransferase (AGT). The AGT reactivity of pyridyloxobutylated DNA was attributed to (pyridyloxobutyl)guanine adducts. One potential AGT substrate adduct, 2′-deoxy-O^6-[4-oxo-4-(3-pyridyl)butyl]guanosine (O^6-pobdG), was prepared. This adduct was stable at pH 7.0 for greater than 13 days and to neutral thermal hydrolysis conditions (pH 7.0, 100 °C, 30 min). Under mild acid hydrolysis conditions (0.1 N HCl, 80 °C), O^6-pobdG was depurinated to yield O^6-[4-oxo-4-(3-pyridyl)butyl]guanine (O^6-pobG). O^6-pobdG was hydrolyzed to 4-hydroxy-1-(3-pyridyl)-1-butanone and guanine under strong acid hydrolysis conditions (0.8 N HCl, 80 °C). O^6-pobG was detected in 0.1 N HCl hydrolysates of DNA alkylated with the model pyridyloxobutylating agent 4-(acetoxymethylnitrosamino)-1-(3-[5-^{3}H]pyridyl)-1-butanone ([5-^{3}H]NNKOAc). When [5-^{3}H]NNKOAc-treated DNA was incubated with either rat liver or recombinant human AGT, O^6-pobG was removed, presumably a result of transfer of the pyridyloxobutyl group from the O^6-position of guanine to AGT's active site.

Introduction

The tobacco-specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK[1]) selectively causes lung tumors in laboratory animals (*1*−*3*). α-Hydroxylation of this asymmetric nitrosamine forms two DNA reactive species. Methyl hydroxylation generates pyridyloxobutyl DNA adducts, whereas methylene hydroxylation leads to methyl DNA adducts (*4*−*6*) (Scheme 1). Formation and persistence of O^6-methylguanine (O^6-mG) is critical for lung tumor induction in NNK-treated A/J mice (*6*). The repair protein O^6-alkylguanine-DNA alkyltransferase (AGT) repairs O^6-mG in a reaction where the methyl group is transferred from the O^6-position of guanine to an active site cysteinyl residue (*7*). Studies in A/J mice indicated that pyridyloxobutylation enhanced the tumorigenic activity of DNA methylation apparently by increasing the levels and persistence of O^6-mG in lung DNA (*6*). Therefore, we have postulated that pyridyloxobutyl adducts interfere with O^6-mG repair, resulting in increased persistence of this promutagenic adduct.

Pyridyloxobutylated DNA is capable of interfering with O^6-mG repair *in vitro* using rat liver AGT (*8*, *9*). The instability of pyridyloxobutyl adducts to chemical or enzymatic hydrolysis of DNA complicated the precise chemical characterization of the AGT substrate adducts. The majority of these adducts decompose to 4-hydroxy-1-(3-pyridyl)-1-butanone (HPB, **4**; Scheme 1) upon enzymatic or strong acid hydrolysis of DNA (*5*, *10*). Previous studies indicated that a (pyridyloxobutyl)guanine adduct was responsible for the AGT reactivity of this DNA (*9*). Since mammalian AGT primarily reacts with O^6-alkylguanine residues in DNA, an O^6-(pyridyloxobutyl)guanine adduct is a possible AGT substrate adduct (Scheme 2). The model pyridyloxobutylating agent 4-(acetoxymethylnitrosamino)-1-(3-pyridyl)-1-butanone (NNKOAc, **1**) hydrolyzes to the corresponding α-hydroxynitrosamine metabolite in the presence of esterase. This compound decomposes to 4-oxo-4-(3-pyridyl)-1-butane diazohydroxide (**3**) (*11*) (Scheme 1). The diazonium ion derived from this species can react directly with guanine at the O^6-position to form an open-chain adduct or indirectly via a cyclic oxonium ion (**2**) to form a cyclic adduct. Therefore, there are two possible O^6-(pyridyloxobutyl)guanine structures: an open-chain adduct, O^6-[4-oxo-4-(3-pyridyl)butyl]-

* To whom correspondence should be addressed. Phone: (914) 789-7229. Fax: (914) 592-6317.
† Division of Chemical Carcinogenesis.
‡ Division of Pathology and Toxicology.
§ Milton S. Hershey Medical Center.
▽ Current address: Division of Carcinogenesis and Molecular Epidemiology, American Health Foundation.
∥ Current address: AMBI Inc., Tarrytown, NY 10591.
⊥ Current address: University of Minnesota Cancer Center, Minneapolis, MN 55455.
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[1] Abbreviations: AGT, O^6-alkylguanine-DNA alkyltransferase; DTT, dithiothreitol; HEPES, *N*-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HPB, 4-hydroxy-1-(3-pyridyl)-1-butanone; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; NNKOAc, 4-(acetoxymethylnitrosamino)-1-(3-pyridyl)-1-butanone; O^6-bzdG, 2′-deoxy-O^6-benzylguanosine; O^6-pobdG, 2′-deoxy-O^6-[4-oxo-4-(3-pyridyl)butyl]guanosine; O^6-pobG, O^6-[4-oxo-4-(3-pyridyl)butyl]guanine; O^6-mG, O^6-methylguanine; TCA, trichloroacetic acid.

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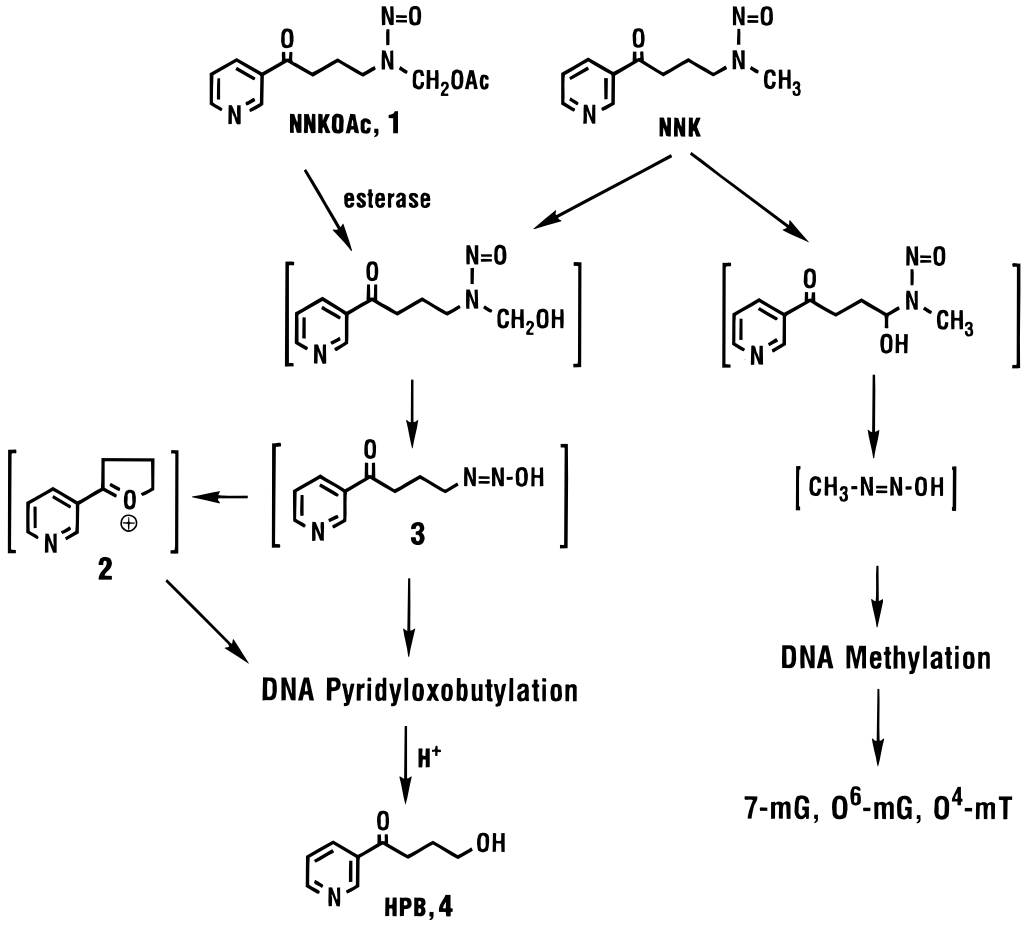
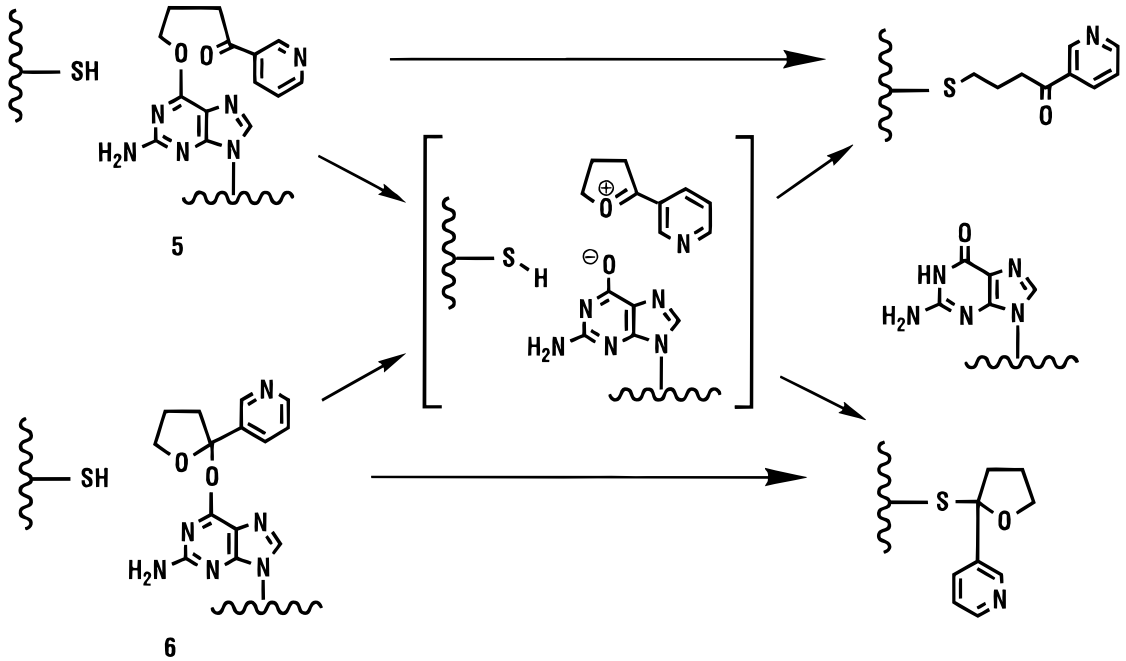
Scheme 1. Proposed Activation Pathways for NNK and NNKOAc



Scheme 2. Proposed Mechanism of Reaction of Two Potential O^6-(Pyridyloxobutyl)guanine Adducts with AGT



guanine (**5**, O^6-pobG), and a cyclic adduct, O^6-[2-(3-pyridyl)-2,3,4,5-tetrahydrofuran-2-yl]guanine (**6**; Scheme 2). Either one of these adducts will decompose under hydrolysis conditions to generate HPB.

In this paper, the preparation and characterization of 2′-deoxy-O^6-[4-oxo-4-(3-pyridyl)butyl]guanosine (O^6-pobdG, **9**) are described. The stability of this compound was investigated. O^6-[4-Oxo-4-(3-pyridyl)butyl]guanine (O^6-pobG) was detected in NNKOAc-treated DNA and was a substrate for rat liver and human AGT.

Experimental Procedures

Caution: *NNKOAc is mutagenic as well as carcinogenic in laboratory animals.*

Materials. NNKOAc, [5-^{3}H]NNKOAc (specific activity, 850 mCi/mmol), 2′-deoxy-O^6-benzylguanosine, 2′-deoxy-N^2-isobutyrylguanosine 3′,5′-diisobutyrate, and [^{3}H]methylated DNA were prepared as previously described (*8*, *11*−*14*). 3-[2-(3-Pyridyl)-1,3-dithian-2-yl]propan-1-al was reduced with $NaBH_4$ in MeOH to yield 3-[2-(3-pyridyl)-1,3-dithian-2-yl]propan-1-ol (*15*). [5-^{3}H]NNKOAc-treated DNA was prepared as previously reported (*8*) and dialyzed against water overnight prior to use. Rat liver AGT was obtained according to published procedures (*9*). Human AGT with a polyhistidine tag at the amino terminus was expressed and purified as previously reported (*16*). Calf thymus DNA and porcine liver esterase were purchased from Sigma Chemical Co. (St. Louis, MO).

Instrumental Analyses. NMR spectra were acquired with a Bruker Model AM360 WB spectrometer and are reported in ppm relative to an external standard. UV spectra were collected on a Hewlett Packard 8425A diode array spectrophotometer, which was computer controlled by HP 89530 MS-DOS UV−vis operating software. HPLC analyses were carried out with a Waters 510 system (Millipore, Waters Division, Milford, MA) with a Shimadzu SPD-UV−vis detector or β-Ram radioflow detector (IN/US Systems, Inc., Tampa, FL). Electrospray MS analyses were obtained on a Finnigan Model TSQ 700 tandem quadruple mass spectrometer with a LC interface.

2′-Deoxy-N^2-isobutyryl-O^6-[3-[2-(3-pyridyl)-1,3-dithian-2-yl]propyl]guanosine 3′,5′-Diisobutyrate (7). 2′-Deoxy-N^2-isobutyrylguanosine 3′,5′-diisobutyrate (0.239 g, 0.5 mmol), triphenylphosphine (0.197 g, 0.75 mmol), and 4-(1,3-dithian-2-yl)-4-(3-pyridyl)butan-1-ol (0.191 g, 0.75 mmol) were combined, dried over P_2O_5 under vacuum for 1−2 days, and dissolved in 10 mL of anhydrous 1,4-dioxane. Diethyl azodicarboxylate (0.131 g, 0.75 mmol) in anhydrous 1,4-dioxane (2 mL) was added dropwise at room temperature (*17*, *18*). After 18 h, the reaction mixture was concentrated under reduced pressure. CH_2Cl_2 (250 mL) was added, and the solution was washed twice with H_2O, dried over $MgSO_4$, and filtered. The product was purified by preparative TLC (PZF 254S, 20 × 20 cm, 1 mm thick; EM Separations Technology, Gibbstown, NJ) with elution by CH_2Cl_2/ethyl acetate (1:1). The lowest band contained **7** (268 mg, 0.38 mmol, 75% yield, >95% pure): ^{1}H NMR ($CDCl_3$) δ 9.22 (d, J = 1.9 Hz, 1H, 2-pyridyl), 8.55 (dd, J = 4.9, 1.1 Hz, 1H, 6-pyridyl), 8.46 (d, J = 8.1 Hz, 1H, 4-pyridyl), 8.23 (s, 1H, N^2-H), 7.95 (s, 1H, C8-H), 7.49 (dd, J = 8.1, 5.0 Hz, 1H, 5-pyridyl), 6.37 (dd, J = 8.0, 6.1 Hz, 1H, 1′-H), 5.42−5.41 (m, 1H, 3′-H), 4.49−4.30 (m, 5H, O^6-C**H**$_2$, 5′-H, 4′-H), 3.00−2.55 [m, 9H, 2′-CH$_2$, SC**H**$_2$CH$_2$C**H**$_2$S, (CH$_3$)$_2$C**H**], 2.25−2.20 (m, 2H, O^6-CH$_2$-CH$_2$C**H**$_2$), 1.99−1.86 (m, 4H, SCH$_2$C**H**$_2$CH$_2$S, O^6-CH$_2$C**H**$_2$), 1.29−1.13 [m, 18H, (C**H**$_3$)$_2$CH]; positive ESI-MS/MS m/z (rel intensity) 715 (M + 1, 100), 238 (M − 2′-deoxy-N^2-isobutyrylguanosine 3′,5′-diisobutyrate, 90). See Supporting Information for ^{1}H NMR spectrum.

2′-Deoxy-O^6-[3-[2-(3-pyridyl)-1,3-dithian-2-yl]propyl]guanosine (8). NaOH (2 N, 10 mL) was added to **7** (30 mg, 0.042 mmol) in MeOH (10 mL) at room temperature. After 3 h, the pH was adjusted to 7 with 80% aqueous acetic acid, and the reaction mixture was extracted with ethyl acetate. The organic layers were combined, washed with H_2O, dried over $MgSO_4$, and filtered. Compound **8** was purified by preparative TLC, eluting with toluene/ethyl acetate/MeOH (10:8:3) (18 mg, 0.036 mmol, 85% yield, >95% pure): ^{1}H NMR ($CDCl_3$) δ 9.14 (d, J = 2.2 Hz, 1H, 2-pyridyl), 8.49 (dd, J = 4.7, 1.4 Hz, 1H, 6-pyridyl), 8.21 (dt, J = 8.1, 2.1 Hz, 1H, 4-pyridyl), 7.61 (s, 1H, C8-H), 7.29 (dd, J = 8.1, 4.7 Hz, 1H, 5-pyridyl), 6.69 (d, J = 11.1 Hz, 1H, 5′-OH), 6.22 (dd, J = 9.4, 5.6 Hz, 1H, 1′-H), 4.96 (s, 2H, N^2-H$_2$), 4.75 (bd, J = 4.6 Hz, 1H, 3′-H), 4.36 (t, J = 6.6 Hz, 2H, O^6-CH$_2$), 4.20 (s, 1H, 5′-H$_a$), 3.96 (d, J = 12.1 Hz, 1H, 4′-H), 3.75 (t, J = 11.4 Hz, 1H, 5′-H$_b$), 3.03−2.96 (m, 2H, 3′-OH, 2′-H$_a$), 2.73−2.59 (m, 4H, SC**H**$_2$CH$_2$C**H**$_2$S), 2.26 (dd, J = 13.3, 5.6 Hz, 1H, 2′-H$_b$), 2.21−2.16 (m, 2H, O^6-CH$_2$CH$_2$C**H**$_2$), 2.00−1.79 (m, 4H, O^6-CH$_2$C**H**$_2$, SCH$_2$C**H**$_2$CH$_2$S); positive ESI-MS/MS m/z (rel intensity) 505 (M + 1, 100), 389 (M − 2′-deoxyribose, 51), 238 (M − 2′-deoxyguanosine, 49); λ_{max} (MeOH) 250 nm (ϵ = 8700 M^{-1} cm^{-1}), 281 (ϵ = 7650). See Supporting Information for ^{1}H NMR spectrum.

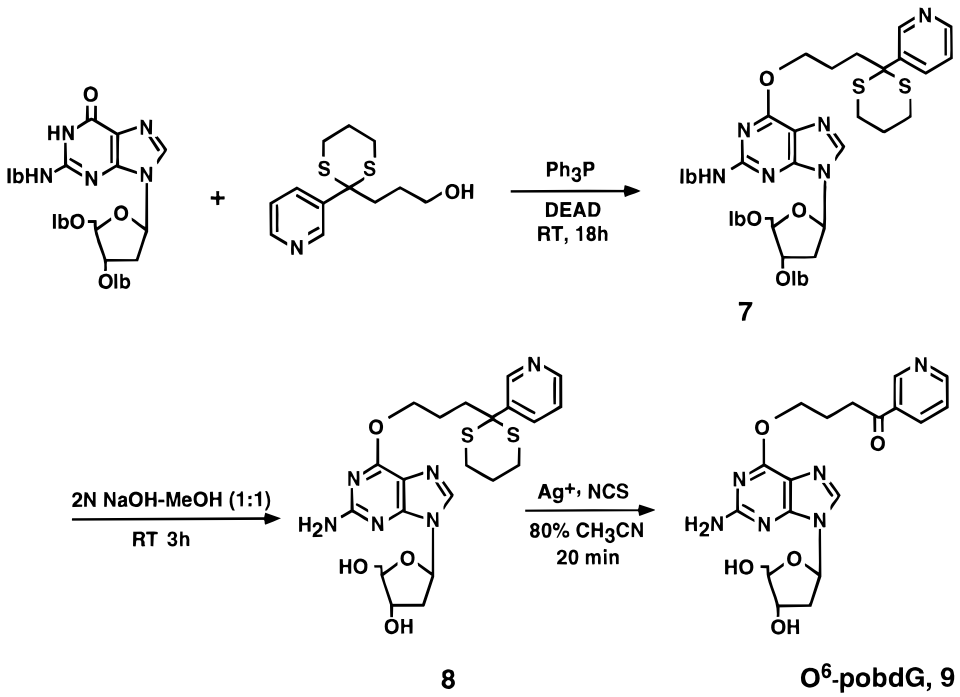
2′-Deoxy-O^6-[4-oxo-4-(3-pyridyl)butyl]guanosine (O^6-pobdG, 9). A solution of **8** (10 mg, 0.02 mmol) in acetonitrile (0.2 mL) was added in one portion to a well-stirred solution of *N*-chlorosuccinimide (10.4 mg, 0.08 mmol) and silver nitrate (15 mg, 0.088 mmol) in 80% aqueous acetonitrile (1 mL) at 25 °C (*19*). After 20 min, saturated aqueous solutions of Na_2SO_3, Na_2CO_3, and NaCl were successively added (0.2 mL each). Then CH_2Cl_2 (5 mL) was added, and the mixture was filtered through Celite. After the filter cake was washed thoroughly with CH_2Cl_2, the organic filtrate was dried with anhydrous $MgSO_4$ and filtered. Compound **9** was purified by preparative TLC, eluting with toluene/ethyl acetate/MeOH (10:8:3) (7.4 mg, 18 μmol, 90% yield, >95% pure): ^{1}H NMR ($CDCl_3$) δ 9.18 (d, J = 1.9 Hz, 1H, 2-pyridyl), 8.77 (dd, J = 4.8, 1.7 Hz, 1H, 6-pyridyl), 8.24 (dt, J = 8.0, 1.9 Hz, 1H, 4-pyridyl), 7.62 (s, 1H, C8-H), 7.41 (dd, J = 7.9, 4.8 Hz, 1H, 5-pyridyl), 6.70 (bs, 1H, 5′-OH), 6.22 (dd, J = 9.6, 5.5 Hz, 1H, 1′-H), 4.86 (s, 2H, N^2-H$_2$), 4.77 (d, J = 4.9 Hz, 1H, 3′-H), 4.62 (t, J = 6.0 Hz, 2H, O^6-C**H**$_2$), 4.20 (s, 1H, 5′-H$_a$), 3.98 (dd, J = 12.8, 1.5 Hz, 1H, 4′-H), 3.77 (d, J = 12.7 Hz, 1H, 5′-H$_b$), 3.47 (s, 1H, 3′-OH), 3.24 (t, J = 7.0 Hz, 2H, O^6-CH$_2$CH$_2$C**H**$_2$CO), 3.08−3.00 (m, 1H, 2′-H$_a$), 2.36−2.29 (m, 2H, O^6-CH$_2$C**H**$_2$), 2.25 (dd, J = 13.5, 5.5 Hz, 1H, 2′-H$_b$); UV λ_{max} (MeOH) 234 nm (ϵ = 14 100 M^{-1} cm^{-1}), 275 (ϵ = 10 400); positive ESI-MS/MS m/z (rel intensity) 415 (M + 1, 60), 299 (M − 2′-deoxyribose, 100), 268 (M − pyridyloxobutyl, 38), 152 [M − (pyridyloxobutyl + 2′-deoxyribose), 50], 148 (M − 2′-deoxyguanosine, 55); high-resolution FAB MS calcd for $C_{19}H_{22}N_6O_5$ 415.1727, found 415.1734. See Supporting Information for ^{1}H NMR spectrum.

***O*^{6}-[4-Oxo-4-(3-pyridyl)butyl]guanine (O^6-pobG).** O^6-pobdG (2 mg, 4.8 μmol) was added to 0.1 N HCl (5 mL) and heated at 80 °C for 30 min. After cooling to room temperature, the mixture was neutralized with 0.1 N NaOH (5 mL) and applied to a C18 Sep-pak cartridge (Waters Corp., Milford, MA). The product was eluted with H_2O:MeOH (1:1) and identified as O^6-pobG: ^{1}H NMR (DMSO-d_6) δ 12.55 (s, 1H, N^{9}H), 9.14 (d, J = 1.5 Hz, 2-pyridyl), 8.78 (dd, J = 4.8, 1.5 Hz, 1H, 4-pyridyl), 8.33−8.31 (m, 1H, 6-pyridyl), 7.78 (s, 1H, C8-H), 7.56 (dd, J = 7.9, 4.9 Hz, 1H, 5-pyridyl), 6.20 (s, 2H, N^{2}H$_2$), 4.45 (t, J = 6.6 Hz, 2H, O^6-CH$_2$), 3.26 (t, J = 6.8 Hz, 2H, O^6-CH$_2$CH$_2$C**H**$_2$), 2.15−2.09 (m, 2H, O^6-CH$_2$C**H**$_2$); positive ESI-MS/MS m/z (rel intensity) 299 (M + 1, 90), 152 (M − pyridyloxobutyl, 50), 148 (M − guanine, 100); UV λ_{max} (1:1 MeOH:H_2O) 276 nm.

Stability Studies. (a) O^6-pobdG (1.1 mM) was incubated in 50 mM HEPES (pH 7.8) in the presence or absence of 1.2 mM DTT for 30 min at 37 °C. (b) O^6-pobdG (1 mM) was heated in 10 mM sodium cacodylate (pH 7.0) at 100 °C for 60 min prior to HPLC analysis. (c) O^6-pobdG (1 mM) in 10 mM sodium cacodylate (pH 7.0) and 10 mM sodium azide were incubated at 37 °C for up to 13 days prior to HPLC analysis. Following the addition of 0.1 vol of 1 N HCl, O^6-pobdG (1 mM) in 10 mM sodium cacodylate (pH 7.0) was heated at 80 °C for 30 min prior to HPLC analysis. (d) Finally, the stability of O^6-pobdG was also determined under strong acid hydrolysis conditions (0.8 N HCl, 80 °C, 1 h). All mixtures were analyzed by HPLC using a Phenomenex Bondaclone C18 column (300 × 3.9 mm; Torrance, CA) eluted with solvent A (20 mM sodium phosphate, pH 7.0) and solvent B (95% methanol) with a linear gradient from 100% solvent A to 50% solvent A/50% solvent B over 20 min followed by a 10 min hold at 50% solvent A/50% solvent B (flow rate, 1 mL/min). The retention times (min) of the compounds are as follows: guanine (11.0), HPB (20.0), O^6-pobG (26.6), and O^6-pobdG (27.2).

DNA Hydrolysis. [5-^{3}H]NNKOAc-treated DNA was heated in 0.1 N HCl at 80 °C for 0.5 h and frozen at −20 °C until analysis. The sample was neutralized with 1 M potassium phosphate (pH 7.4, 0.2 mL) prior to analysis by HPLC with radioflow detection. The mixture was separated on a Phenomenex Bondaclone C18 column (300 × 3.9 mm; Torrance, CA) with a linear gradient from 100% solvent A to 50% solvent A/50% solvent B over 60 min (flow rate, 1 mL/min). The retention time

Scheme 3. Synthetic Route to *O*⁶-pobdG[a]



[a] Ib = isobutyryl, Ph_3P = triphenylphosphine, DEAD = diethyl azodicarboxylate, NCS = *N*-chlorosuccinimide.

of *O*⁶-pobG in this system was 57 min. Total levels of HPB-releasing adducts were determined as previously described (*6*). *O*⁶-pobG and HPB levels were expressed relative to the guanine concentrations of the same samples (*4*).

The radioactive peak that coeluted with *O*⁶-pobG standard was collected using a linear gradient from 100% A to 50% A/50% B over 50 min. A portion of this fraction was reacted with $NaBH_4$ and reanalyzed by HPLC with radioflow detection. HCl was added to another portion of this fraction (final concentration, 0.8 N HCl). This solution was heated at 80 °C for 4 h and reanalyzed following neutralization by HPLC with radioflow detection. The retention times (min) of the compounds were HPB (34.0), *O*⁶-pobG (50.0), and *O*⁶-pobdG (53.5).

Incubation of *O*⁶-pobdG with AGT. *O*⁶-pobdG (0.9 mM) or *O*⁶-benzyl-2′-deoxyguanosine (*O*⁶-bzdG; 0.7 mM) was incubated with rat liver AGT (0.67 pmol) in the presence or absence of calf thymus DNA (20 μg) in 50 mM HEPES (pH 7.8), 1 mM DTT, and 1 mM EDTA (total volume, 1 mL). The nucleosides were previously dissolved in the buffer at their maximum solubility (*O*⁶-pobdG, 1.1 mM; and *O*⁶-bzdG, 0.9 mM). After 30 min at 37 °C, [^{3}H]methylated DNA was added, and the incubations were continued for an additional 30 min at 37 °C. AGT activity was determined as previously described (*9*).

Incubation of [5-^{3}H]NNKOAc-Treated DNA with AGT. [5-^{3}H]NNKOAc-treated DNA was incubated with either semipurified rat liver AGT (9 pmol) or purified human AGT with a polyhistidine tag (6 pmol) in 35 mM HEPES, 1 mM DTT, 1 mM EDTA, and 5% glycerol (pH 7.8) for 30 min at 37 °C (total volume, 1 mL). Controls were performed either in the presence of boiled rat liver protein or in the absence of AGT. The reaction was stopped upon addition of bovine serum albumin (0.8 mg) and 1.0 N HCl (0.1 mL). The resulting mixture was heated at 80 °C for 30 min. Following centrifugation, the supernatant was removed, filtered, and analyzed by HPLC linked with radioflow detection (see above). *O*⁶-pobG and HPB levels are expressed relative to guanine concentrations of the same samples.

Results

The postulated *O*⁶-(pyridyloxobutyl)guanine DNA adduct *O*⁶-pobdG (**9**) was prepared as outlined in Scheme 3. ^{1}H NMR, UV, and MS analyses were consistent with the assigned structure. The stability of this compound was examined under a variety of conditions. Incubation of *O*⁶-pobdG in the AGT assay buffer demonstrated that the compound was stable to the AGT reaction conditions (pH 7.8, 37 °C for 30 min). Subsequent studies demonstrated that this derivative was stable in pH 7 buffer at 37 °C for at least 13 days. It was also stable at 100 °C for 30 min. When *O*⁶-pobdG was heated in 0.1 N HCl for 30 min, a new peak appeared in the HPLC trace that eluted immediately before *O*⁶-pobdG. Preparative isolation and characterization by NMR, MS, and UV analyses demonstrated that this new compound was *O*⁶-pobG. When *O*⁶-pobdG was heated at 80 °C in 0.8 N HCl, it decomposed to HPB and guanine with the intermediate formation of *O*⁶-pobG. *O*⁶-pobdG was not stable under basic conditions. A new compound was formed that did not coelute with any of the standards.

With the knowledge that *O*⁶-pobdG depurinates to *O*⁶-pobG under 0.1 N HCl hydrolysis conditions, we performed similar hydrolyses of [5-^{3}H]NNKOAc-treated DNA to determine if this adduct was present. HPLC analysis of the hydrolysates with radioflow detection indicated the presence of a radioactive peak that coeluted with the *O*⁶-pobG standard (Figure 1A). Following reaction with $NaBH_4$, the standard and the radioactive peak eluted 4 min earlier, consistent with reduction. In addition, the adduct released radioactivity that coeluted with HPB when it was heated in 0.8 N HCl at 80 °C for 4 h. These results are consistent with the presence of *O*⁶-pobG in the NNKOAc-treated DNA. Adduct levels in the 0.1 N HCl hydrolysates were 5 ± 1 pmol of *O*⁶-pobG/μmol of guanine and 42 ± 8 pmol of HPB-releasing adducts/μmol of guanine (n = 6). This compares to 73 pmol of HPB-releasing adducts/μmol of guanine measured in 0.8 N HCl hydrolysates (n = 2).

*O*⁶-pobG was removed from [5-^{3}H]NNKOAc-treated DNA by AGT. Weak acid hydrolysates of [5-^{3}H]-NNKOAc-treated DNA that had been incubated with partially purified rat liver AGT lacked the radioactive

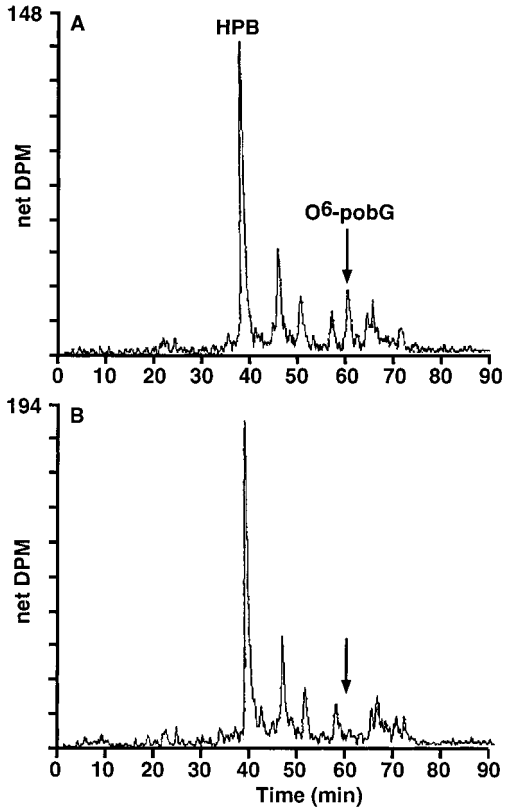


Figure 1. Representative HPLC radiograms of 0.1 N HCl hydrolysates of [5-^{3}H]NNKOAc-treated DNA previously incubated with (A) boiled rat liver AGT or (B) active rat liver AGT.

peak that coeluted with O^6-pobG (Figure 1B). O^6-pobG levels were reduced to the limits of detection (less than 1.5 pmol of O^6-pobG/μmol of guanine), demonstrating that the adduct is a substrate for the repair protein. Controls were performed in the presence of heat-treated protein or bovine serum albumin. Similar results were obtained with purified human AGT with a polyhistidine tag, confirming that O^6-pobG is a substrate for AGT.

High background complicated attempts to measure radioactivity associated with the protein. The nucleoside O^6-pobdG was not active toward rat liver AGT under conditions that yielded complete inhibition of the protein by O^6-bzdG.

Discussion

In this paper we report the first identification of a pyridyloxobutyl DNA adduct, O^6-pobG (**9**), in NNKOAc-treated calf thymus DNA. The presence of other radioactive peaks in the 0.1 N HCl hydrolysates suggests that there are other adducts that are also stable to these hydrolysis conditions. They are possibly depurinated guanine or adenine adducts.

The stability of O^6-pobdG under neutral thermal hydrolysis conditions was unexpected. AGT substrate adducts in NNKOAc-treated calf thymus DNA were removed from DNA during neutral thermal hydrolysis (*8*). In contrast, the ability of NNKOAc-treated 19-mers to react with AGT was stable to neutral thermal hydrolysis conditions (*9*). The reason for these differences is unclear. However, it is possible that the adduct in NNKOAc-treated DNA (generated as double-stranded DNA) has an alternative conformation than that in the oligomers (which were generated as single-stranded DNA) and the nucleoside. These conformations may have different stabilities. It is also possible that the cyclic adduct **6** represents a greater percentage of the AGT substrate adducts in NNKOAc-treated DNA. This adduct is expected to have lower stability under hydrolysis conditions (*20*).

O^6-pobG in DNA was repaired by rat and human AGT, demonstrating that it is a substrate for this repair protein. Therefore, it can potentially compete with O^6-mG for reaction with this protein. These studies indicate that the ability of NNKOAc-treated DNA to interfere with repair of O^6-mG is through a competition between these two adduct types for reaction with AGT. Our previous studies indicate that approximately 3−30% of the original HPB-releasing adduct level are AGT substrate adducts, depending on the DNA preparation (*8*, *9*). O^6-pobG represented 7% of the total HPB-releasing adducts in the present study. At this time, we cannot exclude the possibility that there are other potential AGT substrate adducts in NNKOAc-treated DNA.

AGT only reacts with O^6-pobG when it is incorporated into DNA; O^6-pobdG was unable to interfere with AGT's ability to repair O^6-mG. This observation is consistent with previous reports on the reactivity of other O^6-alkylguanine derivatives. AGT reacts more quickly with O^6-alkylguanine adducts in double-stranded DNA than as free bases or nucleosides (*21*−*27*). The binding of AGT to DNA is an important step in the initiation of adduct repair and likely places the adduct in a better position for reaction with the active site cysteine (*23*, *27*).

The removal of O^6-pobG from DNA by AGT indicates that the pyridyloxobutyl group is transferred from the O^6-position of guanine to the cysteine residue at AGT's active site. This transfer can be effected either via direct S_N2 displacement or via the involvement of an oxonium ion intermediate generating an alkylated protein (Scheme 2). The cyclic adduct **6** could also undergo similar reactions. We have proposed that the transfer reaction may be facilitated by neighboring group participation of the carbonyl oxygen. Future studies will examine the mechanism of pyridyloxobutyl group transfer.

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Supporting Information Available: ^{1}H NMR spectra of compounds **7**−**9** (3 pages). Ordering information is given on any current masthead page.

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