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Regioselective Formation of Acrolein-Derived Cyclic 1,N^2-Propanodeoxyguanosine Adducts Mediated by Amino Acids, Proteins, and Cell Lysates

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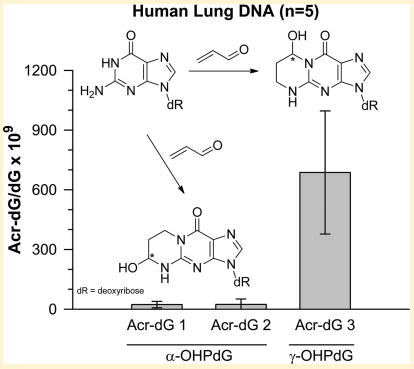
ABSTRACT: Acrolein (Acr) is a major component in cigarette smoke and a ubiquitous environmental pollutant. It is also formed as a product of lipid peroxidation. Following ring closure via the Michael addition, Acr modifies deoxyguanosine (dG) in DNA by forming cyclic 1,N^2-propanodeoxyguanosine adducts (OHPdG). The reactions of Acr with dG yield, depending on the direction of ring closure, two regioisomers, α- and γ-OHPdG, in approximately equal amounts. However, previous ^{32}P-postlabeling studies showed that the γ isomers were detected predominantly in the DNA of rodent and human tissues. Because of the potential differential biological activity of the isomeric OHPdG adducts, it is important to confirm and study the chemical basis of the regioselective formation of γ isomers *in vivo*. In this study, it is confirmed that γ-OHPdG adducts are indeed the major isomers formed *in vivo* as evidenced by a LC-MS/MS method specifically developed for Acr-derived dG adducts. Furthermore, we have shown that the formation of γ-isomers is increased in the presence of amino-containing compounds, including amino acids, proteins, and cell lysates. A product of Acr and arginine that appears to mediate the regioselective formation of γ isomers was identified, but its structure was not fully characterized due to its instability. This study demonstrates that intracellular amino-containing compounds may influence the regiochemistry of the formation of OHPdG adducts and reveals a mechanism for the preferential formation of γ-OHPdG by Acr *in vivo*.

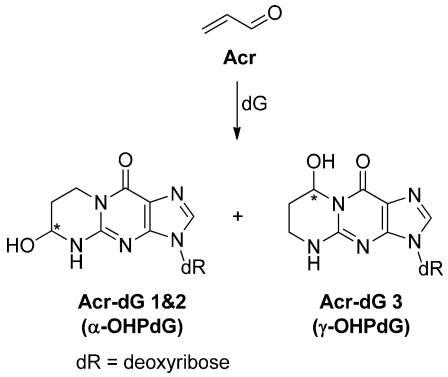


INTRODUCTION

Acr is a common environmental pollutant found in cigarette smoke and automobile exhaust, and evolves from cooking oil at high temperatures. In addition, it can be formed endogenously by lipid peroxidation.[1] It is mutagenic, potentially carcinogenic, and may play a role in human cancers.[2] Acr can readily modify DNA bases, such as guanine, by forming cyclic adducts.[3] The earlier detection of high levels of cyclic 1,N^2-propanodeoxyguanosine adducts of Acr (OHPdG) in the tissue DNA of rodents and humans as background lesions[4] has spurred extensive research efforts aiming at understanding the biological roles of these adducts in cancer. The high frequency of endogenous damage in the genome compared to that from environmental chemicals implicates the endogenous lesions, such as the Acr-derived cyclic adducts, in tumor development, if they are not efficiently repaired. Therefore, it is important to understand the chemical mechanism for the formation of OHPdG *in vivo*.

Reactions of Acr with dG yield two regioisomers, namely, 3*H*-6-hydroxy-3-(β-D-2′-deoxyribofuranosyl)-5,6,7,8-tetrahydropyrido[3,2-*a*]purine-9-one (designated as Acr-dG 1 and 2 or α-OHPdGs) and 3*H*-8-hydroxy-3-(β-D-2′-deoxyribofuranosyl)-5,6,7,8-tetrahydropyrido[3,2-*a*]purine-9-one (Acr-dG 3 or γ-OHPdG) (Scheme 1), due to the opposite direction of ring closure via the Michael addition.[3] As diastereomers, α-OHPdGs are detected as two peaks that are formed in equilibrium, but the γ-OHPdGs appear as a single peak in the HPLC system (Figure 1).

Scheme 1. α-OHPdG (Acr-dG 1 & 2) and γ-OHPdG (Acr-dG 3) Formation from the Reaction of Acr with dG[a]



[a]α and γ isomers are formed as a result of the opposite direction of ring closure via the Michael addition reaction.

Studies showed OHPdG in DNA can be repaired by the nucleotide excision repair pathway.[5] The mutagenicity of OHPdG has been extensively investigated. Acr itself induces mutagenicity in *Salmonella typhimurium* tester strains TA 100 and 104,[6] and

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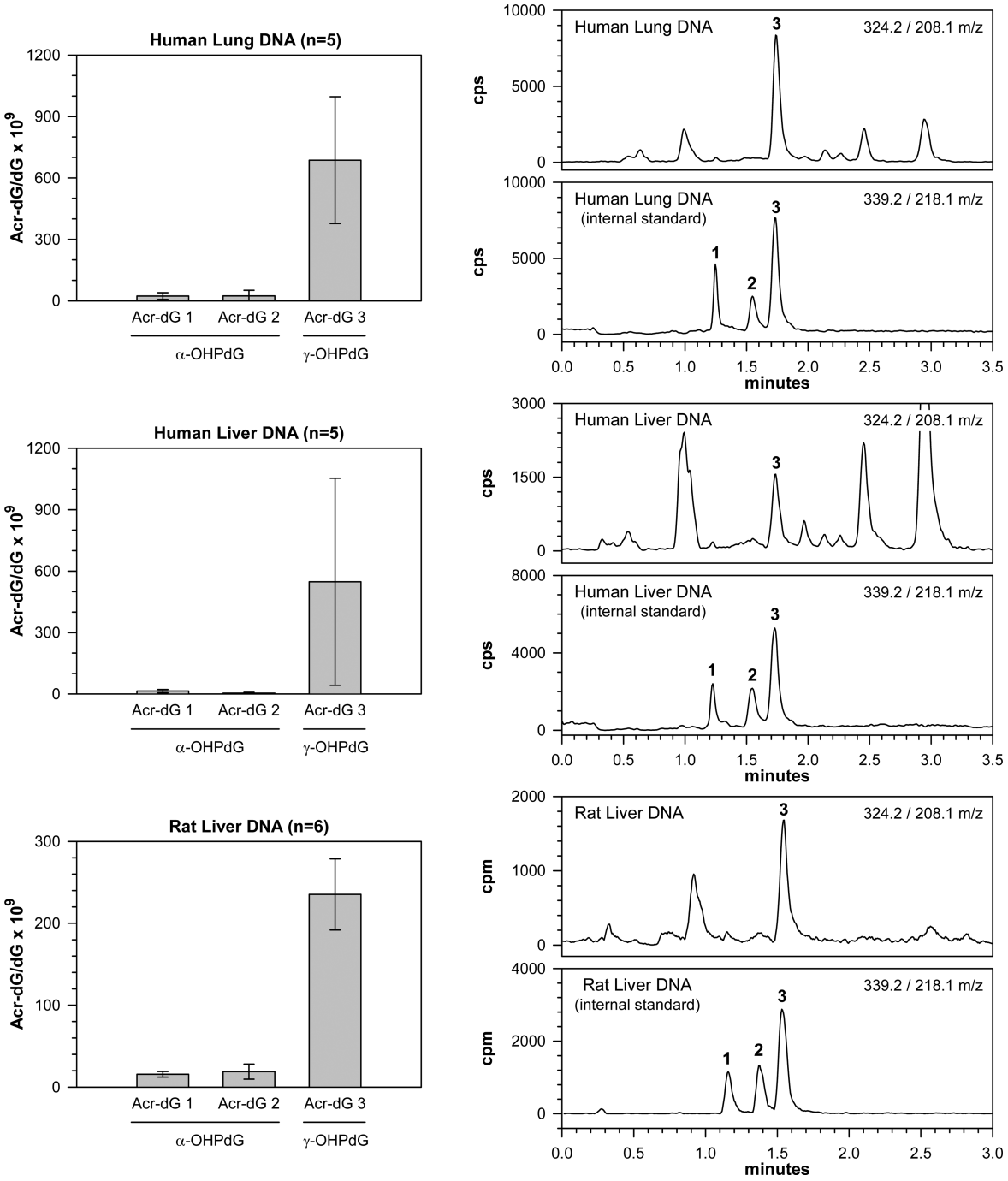

Figure 1. γ-OHPdG (Acr-dG 3), but little or no α-OHPdG (Acr-dG 1 and 2), was detected in lung and liver tissues of humans and rodents by the LC-MS/MS method. Multiple samples of each tissue were analyzed (5 human lungs, 5 human livers, and 6 rat livers). [$^{13}C_{10}$,$^{15}N_5$]-OHPdG were used as internal standards. The mass chromatograms show that γ-OHPdG (Acr-dG 3) is the predominant peak that comigrates with the corresponding internal standard in all samples.

importantly, OHPdGs are found in DNA isolated from these strains under the conditions in which mutation occurs.[7] The site-specific mutagenesis studies also showed that OHPdGs are pro-mutagenic, causing base-substitution, mainly G to T transversion, and frame-shift mutations.[8−11] However, their mutagenic potencies may depend on the regiochemistry and assay conditions. For example, α-OHPdGs are mutagenic in double-stranded DNA in the mammalian cells, but γ-OHPdGs when present in single-stranded DNA are mutagenic in assays using mammalian cells but not in double-stranded DNA, presumably due to ring-opening when paired with deoxycytosine.[12] Regardless of the differences in mutagenicity between the α and γ isomers, Acr-derived dG adducts have been shown to be implicated in the cancer of human lung cells of smokers by observations that the binding pattern by Acr in the p53 gene, presumably via OHPdG formation, is similar to that of the p53 mutational hotspots, notably at codons 248 and 249 where G to T transversions occur.[13]

Earlier *in vitro* studies showed that reactions of Acr with dG yield α- and γ-OHPdGs in almost equal amounts.[3] Interestingly, using an HPLC-based ^{32}P-postlabeling method we consistently detected γ-isomers as the predominant adducts *in vivo*.[4] A recent study, however, reported that an LC-MS/MS method detected

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both α- and γ-isomers in smoker and nonsmoker lung DNA, with a majority of the lung DNA samples having a greater level of α- than γ-isomers.[14] Because of the potential differential roles that γ- and α-isomers may play in tumorigenesis, it is important to confirm the regiochemistry of OHPdG *in vivo* and understand the chemical basis of its formation. In this study, using an LC-MS/MS method specifically developed for Acr-derived dG adducts, we confirmed the earlier studies by detecting γ-OHPdGs as the predominant isomeric adducts in liver and lung tissues of rodents and humans. Furthermore, we demonstrated a preferential formation of γ-OHPdG by Acr in the presence of amino-containing compounds, including amino acids, proteins, and cell lysates. These results shed light on a possible mechanism that explains the regioselective formation of γ-OHPdG *in vivo*.

MATERIALS AND METHODS

Chemicals. Acetonitrile (ACN) was purchased from EMD (EMD Chemicals, Gibbstown, NJ). HPLC grade water was generated in-house by a reverse osmosis−ion exchange water purification station equipped with a water polisher (Millipore, Billerica, MA). Acr stabilized with hydroquinone and the borane *tert*-butylamine complex were purchased from Alfa Aesar (Alfa Aesar, Ward Hill, MA). L-Arginine was obtained from Acros Organics (Thermo Fisher Scientific Inc., Waltham, MA). Spermidine and sodium phosphate monobasic certified dehydrate were bought from Fisher Chemicals (Thermo Fisher Scientific Inc., Waltham, MA). Sodium hydroxide solution was purchased from Fluka (Sigma-Aldrich Corp., St. Louis, MO). Sodium borohydride, 2-amino-2-hydroxymethyl-propane-1,3-diol (Tris), 2′-deoxyguanosine monohydrate (dG), L-lysine, L-histidine, L-threonine, L-serine, L-cysteine, calf thymus DNA, histone from calf thymus, deoxyribonuclease I from bovine pancreas Type II (DNase I), and purified phosphodiesterase I from *Crotalus adamanteus* venom were obtained from Sigma (Sigma-Aldrich Corp., St. Louis, MO). [$^{13}C_{10}$,$^{15}N_5$]-2′-deoxyguanosine ([$^{13}C_{10}$,$^{15}N_5$]-dG) was obtained from Spectra Stable Isotopes (Columbia, MD now Cambridge Isotope Laboratories, Andover, MA).

Alkaline phosphatase grade I from calf intestine (AP) was purchased from Roche (Roche Applied Science, Indianapolis, IN). BEAS-2B cells were bought from American Type Culture Collection (Manassas, VA), and cell cultures were supplied by Mediatech (Manassas, VA). BondElut C18, 200 mg/1 mL SP columns came from Agilent (Agilent Technologies, Santa Clara, CA). All other reagents used were of analytical or HPLC grade obtained from Sigma (Sigma-Aldrich Corp., St. Louis, MO) or Fisher Chemical (Fair Lawn, NJ).

OHPdG standards were prepared as described before[3] using arginine to improve the reaction yield. Briefly, dG monohydrate (28.5 mg, 0.1 mmol) was allowed to react with Acr (73.5 μL, 1.1 mmol) and arginine (34.84 mg, 0.2 mmol) in 100 mM phosphate buffer at pH 7.4 at 37 °C overnight. The products were purified using HPLC System 4. α-OHPdGs (two peaks) were collected together, whereas the γ-OHPdGs were eluted and collected as a single peak. Their structures were confirmed by ESI-MS/MS as previously published.[14] α-OHPdG: $[M + H]^+$ = 324.1 m/z, $[B + H]^+$ = 208.1 m/z, $[B + H - H_2O]^+$ = 190.1 m/z, [guanine + H]$^+$ = 152.1 m/z. γ-OHPdG: $[M + H]^+$ = 324.1 m/z, $[B + H]^+$ = 208.1 m/z, $[B + H - H_2O]^+$ = 190.1 m/z, $[B + H - CH_3CHO]^+$ = 164.1 m/z, [guanine + H]$^+$ = 152.1 m/z, [guanine − NH_2]$^+$ = 135.0 m/z.

[$^{13}C_{10}$,$^{15}N_5$]-OHPdGs as internal standards were synthesized and purified as described for the normal isotopic standards, except that [$^{13}C_{10}$,$^{15}N_5$]-dG was used instead of dG. [$^{13}C_{10}$,$^{15}N_5$]-α-OHPdG: $[M + H]^+$ = 339.1 m/z, $[B + H]^+$ = 218.1 m/z, $[B + H - H_2O]^+$ = 200.1 m/z, [guanine + H]$^+$ = 162.1 m/z; [$^{13}C_{10}$,$^{15}N_5$]-γ-OHPdG: $[M + H]^+$ = 339.1 m/z, $[B + H]^+$ = 218.1 m/z, $[B + H - H_2O]^+$ = 200.1 m/z, $[B + H - CH_3CHO]^+$ = 174.1 m/z, [guanine + H]$^+$ = 162.1 m/z, [guanine − NH_2]$^+$ = 144.0 m/z. The UV spectra were identical to that of OHPdG.

Human and Rat Tissues. Human tissues were obtained from the Histopathology & Tissue Shared Resource of the Lombardi comprehensive Cancer Center of Georgetown University. After surgery, the tissues were covered in OTC frozen tissue matrix, frozen in an isopentane bath, and then stored at −80 °C. The tissues were histologically confirmed as normal. Rat livers were isolated from Long−Evans Cinnamon rats purchased from Imamichi Institute for Animal Reproduction (Ibaraki 300-0134, Japan).

HPLC Systems. *System 1.* The Agilent 1100 HPLC system was equipped with a G1315B photodiode array detector using a C18 reverse-phase Agilent Eclipse XDB-C18, 5 μm, 150 × 4.6 mm column (Agilent Technologies, Inc., Santa Clara, CA). The solvent used was 1 mM ammonium formate and 3% ACN run on a 2 mL/min isocratic flow for 15 min. Between experiments, the column was washed for 10 min using 100% ACN. Peak retention times were α-OHPdGs (Acr-dG 1 and 2) at 7.5 and 9.5 min, respectively, and γ-OHPdGs (Acr-dG 3) at 10.5 min.

System 2. An Agilent 1200 HPLC system consisting of a G1322A degasser, a G1311A quaternary pump, and a G1315D photodiode array detector (Agilent Technologies, Inc., Santa Clara, CA) were used. The system was equipped with Phenomenex Prodigy ODS-3, 250 × 4.6 mm, 100 Å, 5 μm column protected by a Phenomenex guard cartridge (Phenomenex, Inc., Torrance, CA). The mobile phase (solvent A) was 10 mM phosphate buffer, pH 7; solvent B was 40% ACN and 10 mM phosphate buffer, pH 7. A flow rate of 1 mL/min was established. The gradient program was 0−5 min 100% A; 5−25 min from 100% A to 50% A and 50% B, followed by a washing sequence: 25−28 min 100% water; 28−29 min from 100% water to 100% ACN; 29−39 min 100% ACN; and 39−42 min 100% water. Before each run, the column was stabilized with 100% A for 12 min. Detection wavelengths were at 200, 210, 227, 254, and 280 nm. Spectra for all time-points were recorded from 190 to 400 nm with a resolution of 1 nm.

System 3. The same as system 1; A, 3% ACN and 1 mM ammonium formate; B, ACN, flow rate of 1.5 mL/min. Gradient program: 0 to 5 min 100% A, 5 to 6 min 50% A, then 6 to 12 min 100% B.

System 4. This consisted of a Shimadzu HPLC system comprising a SPD-M10A VP diode array detector, a SCL-10A VP controller, and two LC-10AD VP pumps (Shimadzu Scientific Instruments, Columbia, MD) equipped with Phenomenex Prodigy 250 × 21.2 mm, 5 μm particle size, 100 Å, ODS3 (C18) columns (Phenomenex, Torrance, CA) using an isocratic elution of 5% ACN in water at a flow rate of 10 mL/min and detection at 254 nm.

Detection of OHPdG in Tissue DNA by LC-MS/MS. DNA was isolated from tissues using a modified Marmur's procedure[15] and the purity and concentration determined by a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA). DNA samples (50 to 1000 μg) were dissolved in 5 mM magnesium chloride (800 μL per mg DNA), spiked with 100 fmol of [$^{13}C_{10}$,$^{15}N_5$]-α-OHPdG and 50 fmol of [$^{13}C_{10}$,$^{15}N_5$]-γ-OHPdG as internal standards. The mixture was incubated with DNase I (1300 units/mg DNA) for 30 min at 37 °C. An additional portion of DNase I (1300 units/mg DNA) was added, and the sample was incubated for a further 10 min. Phosphodiesterase I (0.06 units/mg DNA) and alkaline phosphatase (380 units/mg DNA) were added, and the sample was incubated for an additional 60 min at 37 °C. After digestion, a small aliquot of hydrolysate was taken for further dG quantification, and the remaining digest was purified by solid phase extraction (SPE) using C18, 200 mg, BondElut columns(Agilent Technologies, Santa Clara, CA). Prior to use, the columns were conditioned by ACN (3 × 1 mL) and water (3 × 1 mL). After sample loading, the columns were washed with water (2 × 1 mL), then the Acr-dG adducts were collected by eluting with 20% ACN in water (2 × 1 mL). Samples were dried over vacuum using a SpeedVac and kept at −80 °C. Before quantification, assay samples were reconstituted using 50 μL of water and 37 μL of sample, which were then used experimentally. Detection and quantification of adducts was carried out with an ACQUITY UPLC liquid chromatography system (Waters Corporation, Milford, MA) equipped with a 50 × 2.1 mm, 1.7 μm particle size C18 column (Waters Acquity UPLC BEH C18) and coupled to a Applied Biosystems/MDS SCIEX 4000 QTRAP triple quadrupole mass spectrometer (Life Technologies Corporation, Carlsbad, CA). The separation of adducts was performed isocratically by eluting with 3% ACN and 1 mM ammonium formate buffer over 3.5 min using a 0.5 mL/min flow rate at 40 °C, followed by a 100% ACN wash. The ESI source operated in positive mode. The MRM experiment was performed using ion transitions of 324.2→208.1 m/z (OHPdG) and 339.2→218.1 m/z ([$^{13}C_{10}$,$^{15}N_5$]−OHPdG) for quantification, and

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those of 324.2→190.1 m/z (OHPdG) and 339.2→200.1 m/z ([$^{13}C_{10}$,$^{15}N_5$]−OHPdG) for structural confirmation. All other parameters were optimized to achieve maximum signal intensity. Calibration curves were constructed for all three isomers before each analysis using standard solutions of OHPdG and [$^{13}C_{10}$,$^{15}N_5$]-OHPdG. A constant concentration of [$^{13}C_{10}$,$^{15}N_5$]-OHPdG (1 fmol/μL) was used with different concentrations of OHPdG (1.68 amol/μL−220 fmol/μL) and analyzed using 37 μL injections by LC-MS/MS-MRM. To determine the levels of OHPdG, dG was quantified in DNA hydrolysate using HPLC System 3 with detection at 254 nm. A standard curve was constructed using UV quantified dG standard (ε_{254} = 13700M^{-1}·cm^{-1} at 254 nm in water).

Reactions of Acr with dG in the Presence of Amino Acids, Proteins, and Cell Lysates. Arginine, lysine, histidine, threonine, serine, Tris, and spermidine of various concentrations (0.5, 1, 2, 4, and 8 mM) or different amounts (2, 6, and 10 mg/mL) of histones, bovine serum albumin, and the soluble portion of BEAS-2B cell lysate were added to 2 mM dG in pH 7.4 sodium phosphate buffer, followed by adding Acr to achieve an equimolar concentration (2 mM). The reaction mixture was incubated at 37 °C for 24 h and kept at −20 °C until HPLC analysis using System 1. The cell lysates were obtained from BEAS-2B cells grown in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin in a 5% CO_2 humidified incubator at 37 °C. The cells were trypsinized, washed twice in Dulbecco's phosphate-buffered saline (DPBS), suspended in 100 mM pH 7.4 sodium phosphate buffer, and lysed by sonication. The resulting lysates were spun at 14,000 rpm to remove insoluble proteins and cell membranes. The soluble protein concentration was determined using the BioRad assay.

Reactions of Acr with Calf Thymus DNA in the Presence of Arginine and Histone. Calf thymus DNA (500 μg) and 0.5 mM Acr were incubated in either 100 mM, pH 7.4 phosphate buffer or 100 mM, pH 7.4 Tris buffer. Arginine (1 or 4 mM) and histone (0.25 or 1 mg) were added to 500 μg of calf thymus DNA in 100 mM, pH 7.4 sodium phosphate buffer, followed by the addition of Acr at 0.5 mM. These reactions were incubated at 37 °C for 24 h. DNA was precipitated in cold 100% ethanol, washed twice in cold 80% EtOH, dried, and the purity and concentration read on a NanoDrop 2000 spectrophotometer. The DNA was digested, purified, and analyzed by LC-MS/MS as described above.

Reduction of γ- and α-OHPdG by Sodium Borohydride for Confirmation of Identities. To confirm the identity of α- and γ-OHPdGs, the adducts were subjected to sodium borohydride reduction as previously described.[3] Briefly, the HPLC fractions containing the γ adducts were concentrated to 50 μL, then 20 μL of 10 M sodium hydroxide was added, followed by 5 mg of sodium borohydride. The reaction mixture was kept at room temperature for 10 min and then placed on ice, acidified with 20 μL concentrated phosphoric acid, and analyzed immediately using HPLC system 2. Similarly, the HPLC fraction containing the α adducts was concentrated to 50 μL, and 50 μL of 10 M sodium hydroxide was added, followed by 5 mg of sodium borohydride and 5 mg of the borane *tert*-butylamine complex. The reaction mixture was kept at 50 °C for 30 min and then placed on ice, acidified by 50 μL of concentrated phosphoric acid, and analyzed immediately by HPLC system 2.

Reactions of the Intermediate of Acr and Arginine with dG. An aliquot of 650 μL of arginine (100 mM) in 200 mM phosphate buffer at pH 7.4 was mixed with 4 μL of Acr. The reaction was carried out at 37 °C for 5 min. An aliquot of 100 μL of the reaction mixture was analyzed by HPLC system 2. A total of 20 fractions containing products between 2 and 15 min were collected in Eppendorf tubes containing 500 μL of 10 mM dG in 200 mM phosphate buffer at pH 7.4. To identify the active intermediate, the collected fractions were kept at 37 °C overnight then analyzed for Acr-dG using HPLC system 2. To stabilize the intermediate, in a separate experiment the fraction containing the intermediate was collected in an Eppendorf tube with an excess amount of arginine (500 μL of 50 mM) and 5 mM dG in 200 mM phosphate buffer at pH 7.4. The mixture was kept at 37 °C for 46 h, and an aliquot was analyzed by HPLC using system 2.

RESULTS

Detection of γ-OHPdG as the Predominant Lesions by Acr in Rodent and Human Tissue DNA by LC-MS/MS. Our earlier [32] P-postlabeling studies showed that while both OHPdG isomers may be detected *in vivo*, the γ-isomers are by far the predominant lesions present in tissue DNA.[4] To confirm this, we set out to use an LC-MS/MS method specifically developed for γ- and α-OHPdGs. Using this method, γ-OHPdG is invariably detected as the major adduct isomers present in all human lung and liver and rat liver samples studied (Figure 1). The levels of modification in DNA determined by the mass spectrometry assays ranged between one adduct per 10^6 and 10^7 dG residues and are comparable to those reported in our earlier ^{32}P-postlabeling studies.[4] As expected, a substantial variability was seen in human DNA, especially those from liver tissue. Together with the earlier ^{32}P-postlabeling studies, these findings firmly establish the regioselectivity in the formation of γ-OHPdG isomers *in vivo*.

Effects of Tris versus Phosphate Buffer on the Formation of α- and γ-OHPdG. An interesting regiochemical effect of Tris buffer on OHPdG formation was observed, but this effect was not seen with phosphate buffer. Under identical conditions, reactions of Acr with dG in Tris buffer yielded more γ isomers (α/γ = 0.3) than in phosphate buffer (α/γ = 1.1), and the yields of γ isomers are proportional to the Tris concentration from 0.5 to 8 mM (Figure 2). The regiochemical bias induced by Tris

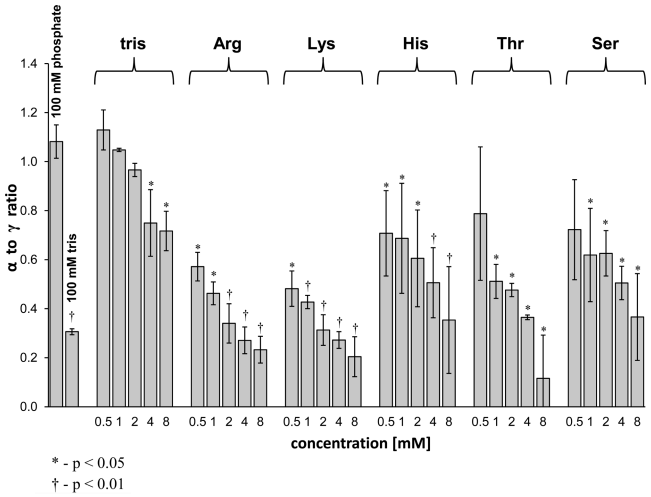

Figure 2. Concentration dependent increase of γ-OHPdG proportional to α-OHPdG in reactions with Acr was observed in the presence of Tris buffer and amino acids. Various concentrations (0.5, 1, 2, 4, and 8 mM) of Tris buffer, arginine, lysine, histidine, threonine, and serine were added to dG in phosphate buffer, followed by Acr, and the reactions were carried out at 37 °C for 24 h. The reaction mixtures were kept at −20 °C until HPLC analysis. *p < 0.05 and $^{\dagger}p$ < 0.01 as compared to reactions with phosphate buffer only.

buffer is also evident in the reactions with calf thymus DNA; a striking increase in the formation of γ-OHPdG with Tris was observed compared to the use of phosphate buffer under the same conditions (Figure 3). These results suggest a possible role of the amino group in Tris in the regioselective bias seen in the formation of γ-OHPdGs.

Increased γ-OHPdG Formation in the Presence of Amino Acids. To determine whether amino acids, like Tris, can also drive the regiochemistry toward γ-isomer formation, Acr was incubated with dG in phosphate buffer containing an amino acid,

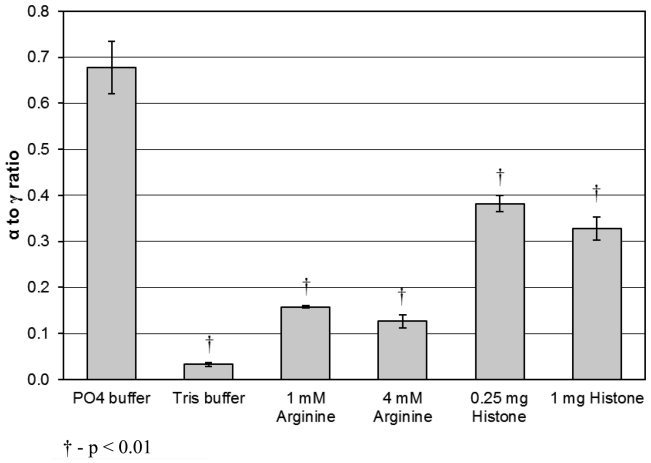

Figure 3. An increased formation of γ-OHPdG proportional to the α isomer in calf thymus DNA modified by Acr in Tris buffer or phosphate buffer containing arginine (1 and 4 mM) and histone (0.25 and 1 mg) as compared to phosphate buffer alone. Calf thymus DNA and Acr were incubated in phosphate buffer or Tris buffer. Arginine or histone was added to a mixture containing calf thymus DNA in phosphate buffer, followed by Acr. The reaction mixture was incubated at 37 °C for 24 h. DNA was then precipitated for enzymatic digestion. Fractions containing adducts were purified and analyzed by LC-MS/MS. $^{\dagger}p < 0.01$ as compared to reactions with phosphate buffer only.

such as arginine, lysine, histidine, threonine, and serine. A concentration dependent increase of γ-OHPdG was observed with the amino acids over a range of 0.5 to 8 mM (Figure 2). Lysine and arginine appeared to have the strongest effects, causing a nearly 2-fold increase of γ-OHPdG at only 0.5 mM. To examine whether the same preferential formation of γ-OHPdG occurs in DNA, calf thymus DNA was incubated with Acr in phosphate buffer containing arginine. Similar to the result with Tris, the yields of γ-OHPdG were dramatically increased in the presence of arginine (Figure 3).

Increased γ-OHPdG Formation with Spermidine, Histone, and Bovine Serum Albumin. The effects of other intracellular amino-containing compounds were examined, specifically spermidine, histone, and bovine serum albumin. The incubations were carried out in phosphate buffer containing various amounts of spermidine from 0 to 8 mM (Figure 4a) or histone and BSA from 0 to 10 mg (Figure 4b). Again, the results clearly showed a dose dependent preferred formation of γ-isomers caused by these compounds. The strongest effect was seen in spermidine with a more than 2-fold increase of γ-isomers at 0.25 mM and an 11-fold increase at 8 mM.

Increased γ-OHPdG Formation with Cell Lysates. To mimic the intracellular matrix, Acr was incubated with dG in the presence of cell lysate proteins to determine whether the latter will shift the reactions toward γ-isomer formation. Various concentrations of cell lysate proteins obtained from BEAS-2B cells from 0 to 2 mg/mL were incubated with Acr and dG in phosphate buffer. Although the overall yields of OHPdG decreased with increasing amounts of cell lysate proteins, likely due to the conjugation of Acr with protein thiols and glutathione, a profound regioselective effect was noted in a concentration dependent manner toward the formation of γ-OHPdG (Figure 5).

Identification of an Intermediate Product of Acr and Arginine for the Formation of γ-OHPdG. To study the chemical basis of regioselectivity, we set out to identify the intermediate product of Acr and arginine to determine whether

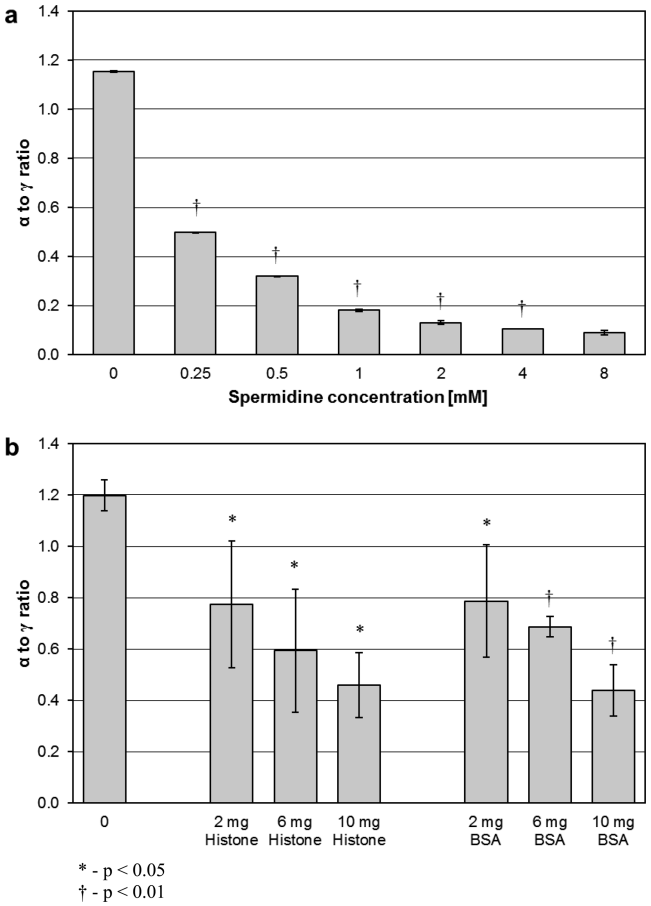

Figure 4. Spermidine (a) and histone and BSA (b) shifted the reactions of dG and Acr in phosphate buffer toward γ-OHPdG formation in a concentration dependent manner. The reactions were carried out in phosphate buffer at 37 °C for 24 h. The reaction mixtures were kept at −20 °C until HPLC analysis. $^{*}p < 0.05$ and $^{\dagger}p < 0.01$ as compared to phosphate buffer.

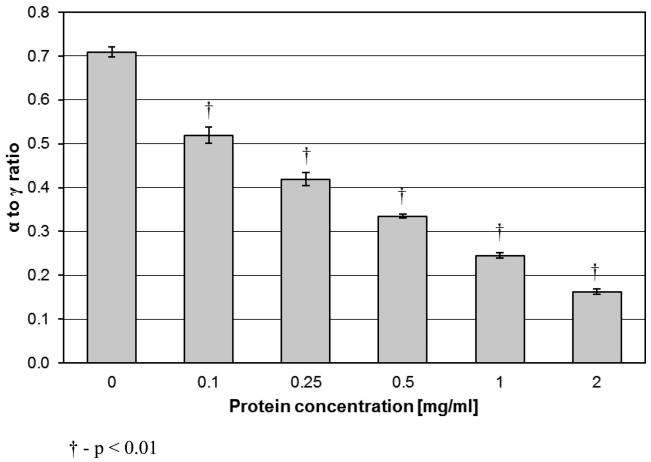

Figure 5. Soluble proteins in cell lysates increased the regioselective formation of γ-OHPdG. The cell lysates were obtained from BEAS-2B cells. The cells were trypsinized, washed in DPBS, suspended in phosphate buffer, and lysed by sonication. The soluble proteins in the lysates were added to dG, followed by Acr. The reactions were carried out at 37 °C for 24 h. The reaction mixture was kept at −20 °C until HPLC analysis. $^{\dagger}p < 0.01$ as compared to phosphate buffer without cell lysates.

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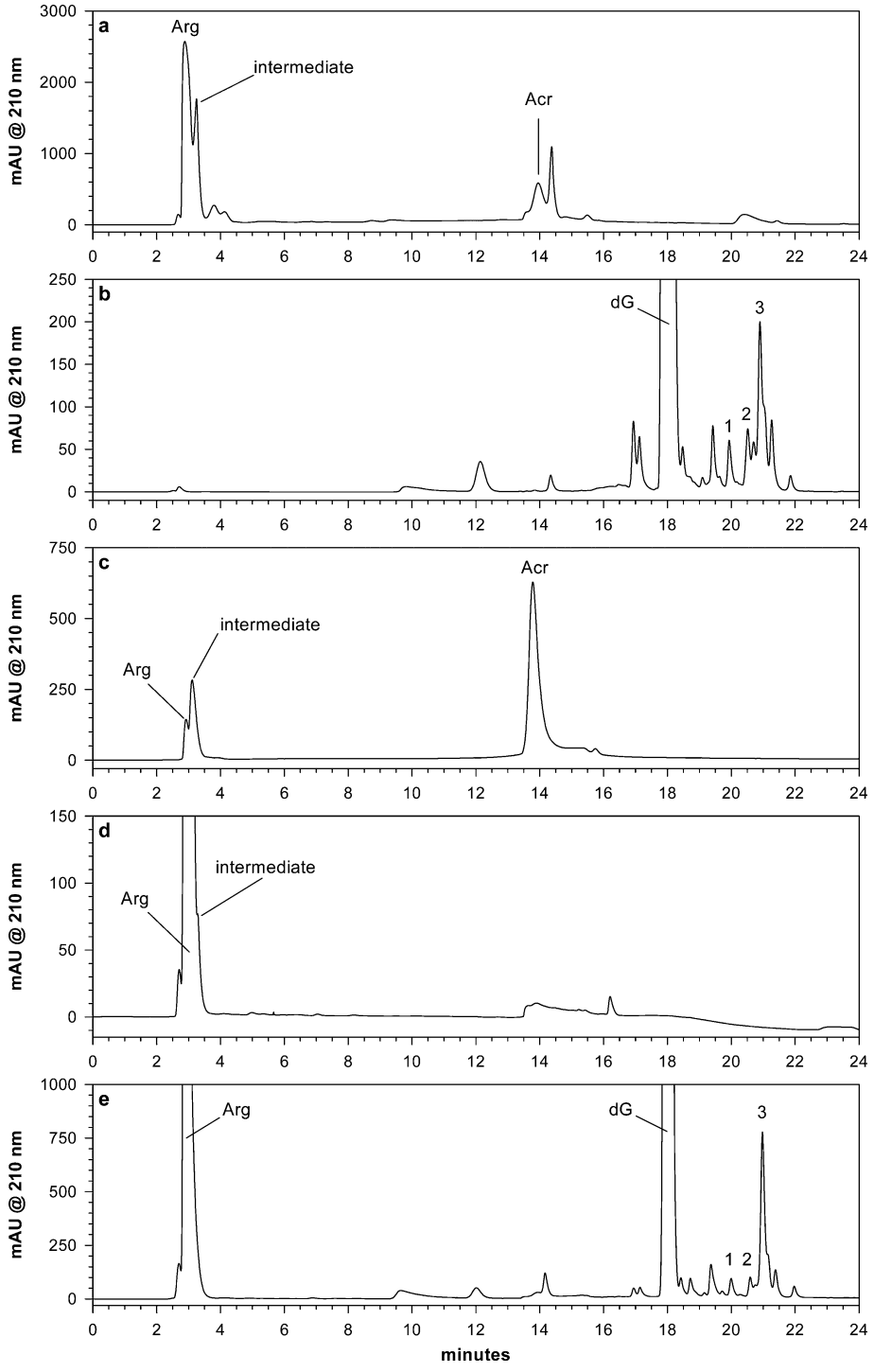

Figure 6. Intermediate product identified from reactions of Acr with arginine was shown to react with dG to preferentially form γ-OHPdG. (a) HPLC chromatogram showed the formation of an intermediate product at 3.3 min; (b) The intermediate upon reaction with dG yielded both α- and γ-OHPdG isomers ($\alpha/\gamma = 0.63$). (c) The intermediate was unstable as it rapidly dissociated to Acr and arginine upon isolation. (d) The intermediate was stabilized (shown as a shoulder adjacent to arginine) in phosphate buffer with excess arginine. (e) The stabilized intermediate yielded γ-OHPdG regioselectively upon reaction with dG ($\alpha/\gamma = 0.17$).

γ-isomers are preferentially formed in its reaction with dG. A total of 20 fractions containing various peaks from HPLC analysis were collected between 2 and 15 min (Figure 6a). Each fraction was subsequently allowed to react with dG in phosphate buffer. Except for the peak at 14 min identified as Acr, only the fraction at 3.3 min containing a product found to rapidly dissociate to Acr and arginine upon isolation yielded OHPdG at an α/γ ratio of 0.63 (Figure 6b and c). The formation of OHPdG was confirmed by coelution with the synthetic standards and by chemical conversions with sodium borohydride/borane *tert*-butylamine complex and sodium borohydride for α and γ isomers, respectively.[3] To stabilize the intermediate product at 3.3 min, it was collected into phosphate buffer containing an excess of arginine (Figure 6d). Subsequent reactions of the fraction containing the stabilized intermediate with dG yielded predominantly γ-isomers ($\alpha/\gamma = 0.17$) at 37 °C (Figure 6e).

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DISCUSSION

Although earlier HPLC-based ^{32}P-postlabeling studies showed that γ-OHPdG is the major isomeric adduct detected *in vivo*,[4] an LC-MS/MS study reported that both isomers are detected in nearly equal amounts, if not a greater quantity of α isomers, in human lung tissues.[14] More recently, however, it was shown that, consistent with the earlier observations by the ^{32}P-postlabeling assay, in human leukocytes γ-isomers are the predominant lesions detected.[16] While the lack of regioselective formation of γ-OHPdG in the human lung DNA is unclear, the discrepancies reported between lung and leukocyte DNA are intriguing and prompted us to carry out further investigations to confirm the regioselectivity of γ-OHPdG formation *in vivo*. In this study, we obtained unequivocal evidence for the regioselective formation of γ-OHPdGs as endogenous adducts of Acr in rodent and human tissues using an LC-MS/MS method specifically developed for these isomers. Furthermore, to investigate the chemical basis of the regioselective formation of γ-OHPdG, we demonstrated that amino-containing compounds, such as amino acids, spermidine, proteins, and cell lysate proteins, can shift the reaction equilibria toward γ isomer formation. These findings implicate a role of intracellular amines in the preferential formation of γ-OHPdGs *in vivo*.

Because α- and γ-OHPdG are repaired equally well *in vitro* by the nucleotide excision repair pathway (Choudhury, S. et al., unpublished results), the fact that more γ isomers are detected *in vivo* is likely due to their preferential formation rather than selective repair. Studies have shown that α- and γ-OHPdG exhibit differential mutagenicity. Whereas most studies support the notion that OHPdG is mutagenic, one study reported a lack of mutagenic activity of OHPdG in mouse and human fibroblasts,[17] but this view has been challenged.[18] The discrepancies in the mutagenicity of OHPdG reported in these studies may be due to a lack of taking into account the isomeric adduct formation in the assays. Site-specific mutagenesis studies have shown that the mutagenicity of OHPdG adducts may vary, depending on the regiochemistry, oligomer sequence context, host system, and single or double stranded vector.[8−11] Because γ-OHPdGs are weak miscoding lesions in double stranded DNA in mammalian cells, it is generally thought that α-OHPdGs may be the more highly mutagenic lesions in double stranded DNA compared to the more abundant γ-isomers. Taken together, these results suggest that although α isomers are the minor DNA lesions *in vivo* they may play an important role in mutagenesis and that the formation of α isomers may be mitigated by intracellular amino compounds.

In this study, we have obtained evidence that may explain the *in vivo* regioselective formation of γ-OHPdG. In this context, it is important to consider the concentrations of amino acids and proteins used. The amino acid concentrations ranged from 0.5 to 8 mM, comparable to that reported in some human tissues.[19] It is known that the protein concentrations in cells are quite high, ranging from several mg/mL to 200 mg/mL.[20] Therefore, both amino acid and protein concentrations used in our experiments are physiologically relevant. The concentration effects of amino acids and proteins on γ-OHPdG formation lend further support to their roles in mediating the regioselective effects. Among the amino acids, lysine and arginine exhibit the strongest effects. Because these particular amino acids contain either a primary amine or a highly basic guanidine, it is possible that that these amines may contribute to the formation of an intermediate via a Schiff's base formation that facilitates γ-isomer formation. In contrast to amino acids, the effects of histone vs BSA on γ isomer formation may be influenced by a variety of factors, such as protein conformation, the number of free amino groups and their accessibility, and the number of thiols. It has been shown that BSA can conjugate with Acr and consequently decrease its reaction with DNA.[21] Therefore, it is not surprising to note that while the total yields of OHPdG decreased as the protein amount increased, the preferential γ isomer formation was still clearly evident and was found to be proportional to protein concentrations.

To provide insights into the chemical mechanism underlying γ-isomer formation, we isolated an intermediate product from reactions of Acr and arginine. However, the rapid dissociation of the intermediate, possibly a Schiff's base, to Acr and arginine upon isolation precluded its full structural characterization. Nevertheless, an intermediate, after being stabilized with an excess of arginine, was identified that can react with dG yielding predominantly γ-isomers. It is plausible that asymmetry or spatial bulkiness of the conjugate may lead to regioselectivity. These results support an *in vivo* mechanism involving an intermediate that is initially formed between Acr and arginine, or possibly other amino compounds, and stabilized by the intracellular amines.

The reaction of enal with Tris buffer was previously described with the cyclic adduct of malondialdehyde via a Schiff's base, suggesting a possible mechanism for cross-linking.[22] Acr was shown to modify lysine by forming N^{ϵ}-(3-formyl-3,4-dehydropiperidino)-lysine, a cyclic lysine derivative via the Michael addition.[23] However, it is unlikely that this stable derivative can react with dG to form OHPdG. Other studies have shown that the formation of cyclic propano dG adducts by acetaldehyde and crotonaldehyde is facilitated by the presence of an amino acid, such as arginine and lysine, and by histone, presumably via a Schiff's base intermediate.[24−26] The present study is the first to demonstrate that the regioisomer ratio in the reaction of dG with Acr can be altered by amino acids or proteins. Because of the high concentrations of these compounds in cells and the differential mutagenicity of the isomeric adducts, these results may have implications for the roles of Acr and its cyclic DNA adducts in mutagenesis and carcinogenesis.

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Notes

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ABBREVIATIONS

ACN, acetonitrile; Acr, acrolein; dG, deoxyguanosine; LC-MS/MS, liquid chromatography−tandem mass spectrometry; OHPdG, Acr-derived 1,N^2-propanodeoxyguanosine

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