Chemical Research in Toxicology

ARTICLE

pubs.acs.org/crt

Chemical and Enzymatic Reductive Activation of Acylfulvene to Isomeric Cytotoxic Reactive Intermediates

Kathryn E. Pietsch,[†,‡] James F. Neels,[†,∥] Xiang Yu,[§] Jiachang Gong,[†,∥] and Shana J. Sturla*[,‡]

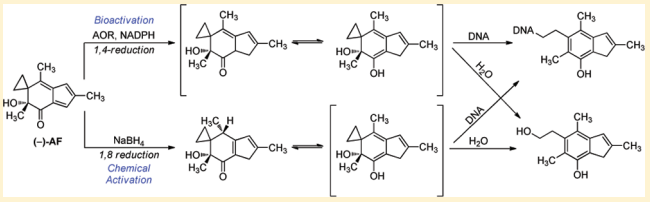
[†]Department of Medicinal Chemistry, College of Pharmacy, University of Minnesota, Minneapolis, Minnesota 55455, United States

[‡]Institute of Food, Nutrition and Health, ETH Zurich, 8092 Zurich, Switzerland

[§]Cancer Cell and Biology Branch, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20814, United States

Supporting Information

ABSTRACT: Acylfulvenes (AFs), a class of semisynthetic analogues of the sesquiterpene natural product illudin S, are cytotoxic toward cancer cells. The minor structural changes between illudin S and AFs translate to an improved therapeutic window in preclinical cell-based assays and xenograft models. AFs are, therefore, unique tools for addressing the chemical and biochemical basis of cytotoxic selectivity. AFs elicit cytotoxic responses by alkylation of biological targets, including DNA. While AFs are capable of direct alkylation, cytosolic reductive bioactivation to an electrophilic intermediate is correlated with enhanced cytotoxicity. Data obtained in this study illustrate chemical aspects of the process of AF activation. By tracking reaction mechanisms with stable isotope-labeled reagents, enzymatic versus chemical activation pathways for AF were compared for reactions involving the NADPH-dependent enzyme prostaglandin reductase 1 (PTGR1) or sodium borohydride, respectively. These two processes resulted in isomeric products that appear to give rise to similar patterns of DNA modification. The chemically activated isomer has been newly isolated and chemically characterized in this study, including an assessment of its relative stereochemistry and stability at varying pH and under bioassay conditions. In mammalian cancer cells, this chemically activated analogue was shown to not rely on further cellular activation to significantly enhance cytotoxic potency, in contrast to the requirements of AF. On the basis of this study, we anticipate that the chemically activated form of AF will serve as a useful chemical probe for evaluating biomolecular interactions independent of enzyme-mediated activation.



INTRODUCTION

Cellular bioactivation involves the transformation of generally inactive compounds to biologically reactive intermediates capable of interacting with, and often covalently modifying, biological targets such as proteins and DNA. Bioreductive alkylating agents, such as acylfulvenes (AFs), are converted to reactive chemical species by preferential enzymatic activation in target cells.[1,2] AFs are semisynthetic derivatives of the natural products illudin M and S (Chart 1), sesquiterpenes produced by the mushroom *Omphalotus olearius*.[3−8] The illudins are potent tumor cell toxins but exhibit low therapeutic indices, meaning that the therapeutic dose is very close to the toxic dose of the drug,[9,10] while AFs have superior therapeutic indices.[6,7,11,12] For both types of structures, reductive metabolism can be catalyzed by prostaglandin reductase 1 (PTGR1), also known as alkenal/one oxidoreductase (AOR), an NADPH-dependent cytosolic medium chain reductase. However, reductive bioactivation of AFs, but not illudin S, is implicated in differentiating sensitive from resistant cells, and AFs offer, therefore, unique chemical tools for probing the role of bioactivation in cytotoxic selectivity.[13,14]

The cytotoxicity of AFs can be attributed to reductive biotransformation-coupled alkylation of critical biomolecules, including DNA.[11,15−18] Bioactivation is mediated by an enzyme or enzymes primarily located in the cytosol of drug-sensitive cells and requires NADPH as a cofactor.[6,19,20] AFs are substrates for the inducible cytosolic NADPH-dependent enzyme PTGR1.[13,14,21] PTGR1 is invoked in the detoxification of electrophiles, such as lipid peroxidation products, and prevents biological adduct formation.[21] For AF, however, a positive correlation exists between cellular PTGR1 levels, cell sensitivities, and DNA adduct levels,[13,14,22,23] suggesting that cells with high levels of PTGR1 are more capable of activating AFs to a reactive species that alkylates DNA and induces toxicity.

Despite the recent nature of data connecting PTGR1 and AFs, on the basis of the chemical structure of the major cytosolic metabolite **2** (Scheme 1), a pathway for AF bioactivation was put forth by McMorris and co-workers over 20 years ago. It involved conjugate addition to the α,β-unsaturated ketone to form putative intermediate **1** (Scheme 1), keto−enol tautomerism, and nucleophilic opening of the cyclopropyl ring. It was hypothesized that hydride delivery from NADPH to the α,β-unsaturated ketone gives rise to an electrophilic intermediate

Received: September 17, 2011
Published: September 22, 2011

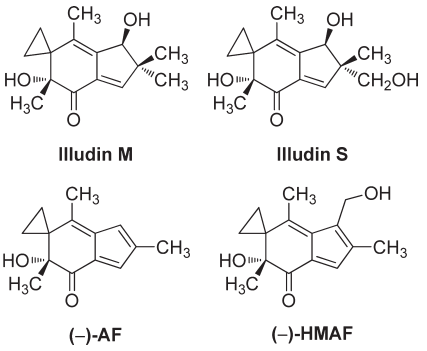
capable of alkylating cellular nucleophiles, thus yielding metabolites or adducts (Scheme 1).[5] The ability to isolate the intermediate that reacts with biomolecules, or a chemical surrogate, would enable mechanistic studies of chemical toxicity. The reduced species presumably is extremely unstable and reactive and has, therefore, not been isolated. Considering the important contributions of PTGR1-mediated activation and the reactivity of the reduced form of AF in dictating the activity and selective toxicity of the drug, it is of interest to characterize the chemical and biochemical aspects of the mechanism of AF reduction and nature of its activated intermediate.

Herein, we report the generation and isolation of a chemically activated AF analogue and studies of its reactivity toward calf thymus DNA (ctDNA), providing new insights into AF cytotoxicity. In addition, we have elucidated chemical pathways that contribute to AF toxicity and are independent of enzymatic activity. This study provides access to a new AF-derived mechanistic probe of toxicity and detailed chemical and biochemical mechanistic information regarding reductive processes that activate AF to an electrophilic species.

EXPERIMENTAL PROCEDURES

Chemicals and Enzymes. Illudin S was provided by MGI Pharma. Rat PTGR1 (rPTGR1) was purified as previously described.[21] Sodium deuteroxide (NaOD) [99.5% D, 40% in deuterium oxide (D_2O)], D_2O (99.9% D), and sodium borodeuteride ($NaBD_4$) (90−95% purity) were purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA). D_2O (100.0% D) was purchased from Acros Organics (Waltham, MA). NADPH was purchased from Calbiochem (San Diego, CA). Dimethyl sulfone was dried under reduced pressure while heating (60 °C) for 24 h.

Chart 1. Natural Product Illudins and Semisynthetic Analogues AF and HMAF

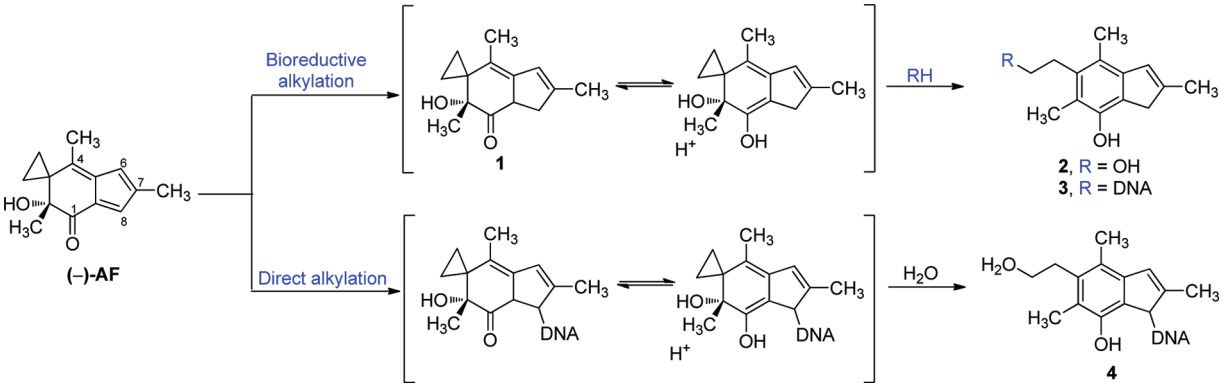


Pyridine was dried with a MBraun (Stratham, NH) solvent purification system. SiliaFlash P60 silica gel (40−63 μm, 230−400 mesh) was purchased from Silicycle (Quebec City, Quebec, Canada). All solvents were HPLC grade. Deoxyadenosine (dAdo) and deoxyguanosine (dGuo) hydrate were purchased from MP Biomedicals (Solon, OH) and Tokyo Chemical Industry (Tokyo, Japan), respectively. ctDNA, Dowex 50-X8, and DEAE cellulose were purchased from Sigma (St. Louis, MO). ctDNA concentration was determined by measuring the absorbance at 260 nm (16.7 A_{260}/mg solid). Reported yields represent an average of two or more experiments of material that is greater than 95% pure. 4-*d*-$NADP^+$ was prepared by a published procedure[24] on a 250 mg scale with a 52% yield. 4,4′-d_2-NADPH (NADPD) was prepared by a published procedure[24] on a 125 mg scale with a 16% yield. ^{1}H NMR spectra for both compounds matched the published spectra. On the basis of ^{1}H NMR, the isotopic purity of NADPD was >90%. The reactivity of the labeled cofactor was confirmed by reaction with benzylidene acetone.[21]

Instrumentation and Apparatus. One-dimensional (1D) ^{1}H and ^{13}C NMR analysis was performed on a 400, 500, or 600 MHz Varian NMR spectrometer, and chemical shifts were assigned on the basis of residual solvent signals. Two-dimensional (2D) nuclear Overhauser effect spectroscopy (NOESY) spectra were acquired on a 600 MHz Varian NMR spectrometer. Qualitative HPLC-ESI-MS/MS analyses were carried out on an Agilent 1100 capillary flow HPLC interfaced with a Thermo Finnigan LCQ Deca ion trap. High-resolution mass spectroscopy (HRMS) spectra were recorded on a Bruker BioTOF II mass spectrometer with an ESI source using polypropethylene glycol (PPG) as a matrix. UV absorbances were determined with a Varian Cary UV 100 Bio UV−visible spectrophotometer. Xcalibur software was used for the acquisition and processing of MS data.

Qualitative HPLC-ESI-MS/MS analyses were carried out on an Agilent 1100 capillary flow HPLC with a Phenomenex MAX-RP Synergi column (150 mm × 0.5 mm, 4 μm particle size), interfaced with a Thermo Finnigan LCQ Deca ion trap. The HPLC flow rate was 10 μL/min, and the mobile phase was methanol/0.1% formic acid in H_2O (v/v). The mobile phase gradient elution is as follows: 5% methanol in 0.1% formic acid in H_2O (v/v) to 95% methanol in 0.1% formic acid in H_2O (v/v) over 19 min, then 95% methanol in 0.1% formic acid in H_2O (v/v) for 11 min, returning to initial conditions over 2 min, and re-equilibration for 15 min. The ESI source was set in positive ion mode with the following parameters: capillary temperature, 250 °C; voltage, 3 kV; current, 80 μA; isolation width, 1.5 *m*/*z*; normalization collision energy, 40%; activation Q, 0.250; and activation time, 30 ms. Mass transitions monitored for 3-AF-Ade and 7-AF-Gua were *m*/*z* 336 to 201 and *m*/*z* 352 to 201, respectively. The relative abundance of these compounds was estimated by integrating *m*/*z* 201 peaks with Xcalibur Qual Browser software (boxcar smoothing, seven points). Samples were analyzed in duplicate, and data reported are the average of duplicate measurements.

Scheme 1. Proposed Pathways for the Formation of AF Adducts via Direct Alkylation and Bioreductive Alkylation



(−)-AF. The title compound was synthesized from illudin S by minor modification to the reported procedure.[5] Illudin S (205 mg, 0.78 mmol) was dissolved in 70 mL of H_2O, followed by the addition of 4 N aqueous sulfuric acid (H_2SO_4) (24 mL, 96 mmol). The reaction was stirred at 25 °C for 21 h. The solution changed from colorless to yellow, and an orange precipitate formed. Ethyl acetate (40 mL) was added to dissolve the precipitate. The aqueous layer was separated and extracted with ethyl acetate (2 × 20 mL). The combined organic layers were washed successively with saturated aqueous sodium bicarbonate (2 × 30 mL) and then brine, and dried with magnesium sulfate (anhydrous), filtered, and concentrated by rotary evaporation. The crude product was purified by flash chromatography [90% hexanes/ethyl acetate (v/v)]. The title compound was isolated as a yellow-orange solid (40% yield). ^{1}H NMR matched published data.[5]

(4′*S*,6′*R*)-6′-Hydroxy-2′,4′,6′-trimethyl-4′,6′-dihydrospiro-[cyclopropate-1,5′-inden]-7′(1′*H*)-one (5). A suspension of sodium borohydride ($NaBH_4$) (16 mg, 0.4 mmol) in 10 mL of pyridine was stirred at 0 °C for 10 min under an atmosphere of nitrogen. A solution of (−)-AF (20 mg, 0.09 mmol) in 2 mL of pyridine was added dropwise to the $NaBH_4$ suspension over the course of 2 min. The mixture turned from yellow to brown while stirring at 25 °C for up to 20 min. [The efficiency of $NaBH_4$-mediated reduction of AF is sensitive to the activity of the reducing reagent, and the reported procedure involves results from studies with a freshly opened bottle of $NaBH_4$ or $NaBD_4$. The mixture is allowed to stir at room temperature until a complete color change from yellow to pale brown is observed (usually less than 20 min). If a second color change to dark brown occurs, the desired product has degraded, and the resulting ^{1}H NMR signal is that of an intractable mixture.] The reaction was quenched by adding 3 mL of 10% (w/v) aqueous potassium iodate. The resulting mixture was partitioned between water and ethyl acetate, followed by extraction with ethyl acetate (2 × 30 mL). The combined organic layers were washed with brine, dried with magnesium sulfate (anhydrous), and concentrated by rotary evaporation. The crude product was adsorbed onto SiliaFlash P60 silica gel (Silicycle) and purified by flash chromatography with elution by a solvent gradient of 100% hexanes → 90% hexanes/ethyl acetate (v/v). For purification, pentanes were sometimes substituted for hexanes, with no change in results. Phosphomolybdic acid stain was used to visualize **5** by TLC. The title compound was isolated as a pale brown oil (4.7 mg, 22 μmol, 24% yield). Caution: Compound **5** sublimes under high vacuum. The reported yield is obtained if the sample is under vacuum (2×10^{-1} Torr) for 3−5 h. ^{1}H NMR (600 MHz, $CDCl_3$): δ 0.33−0.36 (m, 1H), 0.42−0.45 (m, 1H), 0.67−0.71 (m, 2H), 0.95 (d, 3H, J = 4.5), 1.40 (s, 3H), 2.17 (s, 3H), 3.13−3.36 (m, 3H), 3.67 (br, 1H), 6.28 (s, 1H). ^{13}C NMR (600 MHz, $CDCl_3$): δ 197.0, 170.0, 157.2, 132.5, 128.5, 74.4, 42.3, 32.9, 30.9, 24.6, 17.3, 12.8, 3.3, 2.1. HRMS m/z 241.1185 ($[M + Na]^+$); calcd for $C_{14}H_{18}O_2Na$, 241.1199.

(4′*S*,6′*R*)-4′-Deutero-6′-hydroxy-2′,4′,6′-trimethyl-4′,6′-dihydrospiro[cyclopropane-1,5′-inden]-7′(1′*H*)-one (6). The title compound was prepared and purified by the same procedure as **5** detailed above but with $NaBD_4$. The product was obtained as a pale brown oil (4.5 mg, 21 μmol, 23% yield). ^{1}H NMR (600 MHz, $CDCl_3$): δ 0.33−0.35 (m, 1H), 0.42−0.45 (m, 1H), 0.68−0.71 (m, 2H), 0.94 (s, 3H), 1.39 (s, 3H), 2.17 (s, 3H), 3.13−3.36 (m, 2H), 3.66 (br, 1H), 6.28 (s, 1H). ^{13}C NMR (600 MHz, $CDCl_3$): δ 197.0, 170.0, 157.2, 132.7, 128.5, 74.4, 42.4, 30.9, 29.9, 24.6, 17.3, 12.7, 3.3, 2.1. HRMS m/z 242.1282 ($[M + Na]^+$); calcd for $C_{14}H_{17}DO_2Na$, 242.1262.

Conversion of 5 to 2. H_2SO_4 (1.5 mL, 4 N aqueous) was added to a solution of **5** (8 mg, 0.04 mmol) in 0.8 mL of THF. The reaction was allowed to stir for 24 h. Compound **2** precipitated as a pale yellow solid and was isolated by filtration using a Büchner funnel and filter paper (1.0 mg, 5 μmol, 13% yield). The ^{1}H NMR matched the published spectrum for **2**.[20]

Conversion of 6 to 2. H_2SO_4 (1 mL, 0.09 M) was added to a solution of **6** (5 mg, 0.02 mmol) in 0.5 mL of THF. The mixture was allowed to stir for 19 h. Compound **2** precipitated as a pale yellow solid and was isolated by filtration using a Büchner funnel and filter paper (0.5 mg, 2 μmol, 10% yield). The ^{1}H NMR matched the published spectrum for **2**.[20]

Conversion of AF to 2. By a procedure adapted from a previous report,[20] AF (15 mg, 0.069 mmol) was dissolved in 6 mL of acetone−H_2O (1:1 v/v). Zinc dust (160 mg, 2.5 mmol) and 0.75 mL of 10% aqueous H_2SO_4 (v/v) were added at 25 °C. The reaction mixture turned from yellow to colorless over 1 h. The solution pH was adjusted to pH 8−9 by adding 10% potassium carbonate (w/v) (∼2 mL, monitored with pH paper), followed by extraction with ethyl acetate (2 × 10 mL). The combined organic layers were washed with brine, dried with magnesium sulfate (anhydrous), and concentrated by rotary evaporation. The crude product was purified by flash chromatography, with gradient elution [10 → 30% ethyl acetate/hexanes (v/v)] (5.9 mg, 0.027 mmol, 39% yield). The ^{1}H NMR matched the published spectrum for **2**.[20]

Reactions of Nucleic Acids with 5. In a microcentrifuge tube (1.5 mL), a mixture of **5** (20 μL, 100 mM) and nucleoside (dAdo or dGuo, 2.0 μmol) or ctDNA (0.25 mg) was combined and diluted to a final volume of 620 μL by adding H_2O. In the presence or absence of test compound, samples were allowed to react at 37 °C in a water bath for 24 h, vortex mixed, heated at 90 °C for 1 h, and then concentrated to dryness by rotary evaporation. The resulting solids were extracted with methanol (MeOH) (3 × 300 μL per sample). Combined extracts were filtered through a 0.45 μm syringe filter, and the filter was rinsed with 150 μL of MeOH. Filtered extracts were concentrated to dryness by rotary evaporation. The resulting residue was transferred in MeOH to HPLC vials (2 dram vials containing 250 μL inserts) and again concentrated to dryness by rotary evaporation. Samples were reconstituted in 16 μL of MeOH and analyzed by HPLC-ESI-MS/MS as described above.

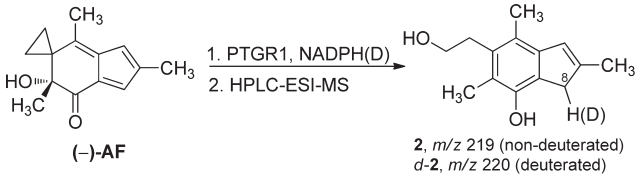
Reactions of Nucleic Acids with AF. In a microcentrifuge tube (1.5 mL), a mixture of AF (20 μL, 100 mM) and nucleoside (dAdo or dGuo, 2.0 μmol) or ctDNA (0.25 mg) was combined and diluted to a final volume of 620 μL by adding H_2O. In the presence or absence of test compound, samples were allowed to react at 37 °C in a water bath for 24 h, vortex mixed, heated at 90 °C for 1 h, and then concentrated to dryness by rotary evaporation.

For bioactivated AF reactions, a mixture of AF (20 μL, 100 mM), nucleoside (dAdo or dGuo, 2.0 μmol) or ctDNA (0.25 mg), rPTGR1 (4.2 μL, 1.7 mg/mL), and NADPH (100 μL, 1.5 mM) was combined and diluted to a final volume of 624 μL with H_2O. The reactions were incubated in a 37 °C water bath, and additional aliquots of NADPH (100 μL, 1.5 mM) were added after 4 and 24 h. After 24 h, an additional aliquot of PTGR1 (4.2 μL, 1.7 mg/mL) was added to the samples. These reactions were incubated for a total of 26.5 h, vortex mixed, heated at 90 °C for 1 h, and then concentrated to dryness by rotary evaporation.

The resulting solids were extracted with MeOH (3 × 300 μL per sample). Combined extracts were filtered through a 0.45 μm syringe filter, and the filter was rinsed with 150 μL of MeOH. Filtered extracts were concentrated to dryness by rotary evaporation. The resulting residue was transferred in MeOH to HPLC vials (2 dram vials containing 250 μL inserts) and again concentrated to dryness by rotary evaporation. Samples were reconstituted in 16 μL of MeOH and analyzed by HPLC-ESI-MS/MS as described above.

Plasmid Construction. Human PTGR1 (hPTGR1) was polymerase chain reaction (PCR)-cloned from a human liver cDNA library (Clontech, Mountain View, CA) according to the NCBI GenBank sequence using the following primer set: forward, 5′-GTCGCGGAATTCAGCTTCAGGATGGTTCGTACTAAGACATGG; reverse, 5′-GTCGCGCTCGAGTTACTATCATGCTTTCACTATTGTCTTCCCC. The PCR product was cleaned and ligated into pBlueScript between *Eco*RI and *Xho*I sites. The DNA insert was confirmed by sequencing and subcloned into episomal vector pCEP4.

Scheme 2. Strategy for Probing the AF Bioactivation Mechanism Using HPLC-ESI-MS



Cell Culture and Transfection. 293T cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and maintained in Dulbecco's modified Eagle's medium (DMEM) (high glucose, Invitrogen, Inc., Carlsbad, CA) with 10% heat-inactivated fetal bovine serum (v/v). Cells were incubated at 37 °C in a humidified atmosphere containing 5% CO_2. Cells were transiently transfected with Lipofectamine 2000 reagents (Invitrogen, Inc.).

Cell Viability Assay. pCEP4 and pCEP4-hAOR transfected 293T cells were maintained in DMEM supplemented with fetal bovine serum and seeded 24 h prior in 96-well plates at a density of 4000 cells/well. Treatments were initiated by replacing the media with those containing AF or **5** at indicated concentrations (cisplatin used as a positive control). Cell viability was measured 24 h later via CellTiter-Blue assay, which measures the metabolic capacity of viable cells to reduce the dye resazurin, to a fluorescent compound (Promega Corp., Madison, WI). Linear regression analysis and IC_{50} calculations were performed using SigmaPlot (version 11.0).

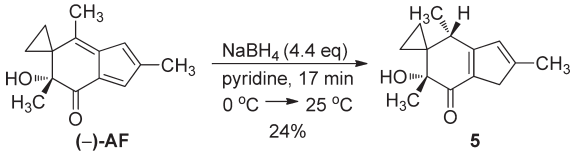
Determination of Rate of Conversion of 5 to 2. H_2SO_4 (1 mL, 4 N) was added to a solution of **5** (6.8 mg) and dimethyl sulfone (6.4 mg) in THF-d_8 (0.5 mL) in an NMR tube. The ensuing reaction was monitored by NMR (600 MHz, Varian) using a preacquisition delay experiment, taking four scans every 5 min for 12 h. Integrals were measured manually and normalized to the integral of the internal standard. Plots were generated using KaleidaGraph (version 4.0). As the reaction proceeds, peaks attributable to **5** decrease and peaks for **2** appear and increase. Initially, only peaks associated with the starting material, **5**, are observed, but spectral changes are observed after the first time point. Using KaleidaGraph (version 4.0), the data were fit to a simple monoexponential equation. The first-order rate constant at 25 °C, based on the decay of methyls A–C (Figure S6 in the Supporting Information), was $1.7 \pm 0.2 \times 10^{-5}$ s^{-1}, corresponding to a $t_{1/2}$ of 11.6 h.

Stability of 5 in Cell Media with Serum. To study the reactivity of **5** cell media, DMEM with 10% fetal bovine serum (v/v) (0.4 mL) was added to an NMR tube containing a solution of **5** (3 mg) and maleic acid (2.84 mg) in DMSO-d_6 (0.4 mL). The solution was monitored by ^{1}H NMR for 24 h at 37 °C as described above for the acid-catalyzed conversion. No spectral changes were observed.

RESULTS

Enzymatic Activation of AF. The regiochemistry of PTGR1-mediated AF reduction was investigated by comparing products formed from reactions with NADPH or NADPD as the cofactor (Scheme 2). The rationale for selection of this enzyme and cofactor is the established relationship between PTGR1, AF bioactivation, and AF toxicity and that the enzyme requires NADPH.[13,14,21] AF reduction can proceed by a number of possible mechanisms including a 1,4-pathway, that is, conjugate addition to the α,β-unsaturated ketone at C8 to form **1** (Scheme 1). Other possibilities include a 1,6-pathway, with addition occurring to the extended conjugated system at C6, or a 1,8-pathway, with addition occurring at C4. When NADPH is used as a reducing agent, the major metabolite isolated is **2**.[20] This species was characterized by mass spectrometry (m/z 219) and NMR. The formation of **2** is consistent with reduction, by one of the above-mentioned pathways, followed by hydrolytic cyclopropane ring opening and subsequent aromatization of the six-membered ring (Scheme 1, top). The same experiment[21] was carried out with NADPD as the cofactor (Scheme 2). In this case, the major product observed, *d*-**2**, had m/z 220 (Figure S1 in the Supporting Information), consistent with it having arisen from either a 1,4- or 1,6-reduction. The 1,8-reduction mechanism can be ruled out because this pathway would generate a product with m/z 219 arising from deuterium addition at the 4-position followed by deuterium loss during the conversion to **2**. In addition, the only change observed in the NMR spectra of *d*-**2** relative to **2** is that the singlet at δ 3.2, which corresponds to the C8 protons, broadens and integrates to one proton.

Scheme 3. Chemical Reduction of AF with $NaBH_4$



Chemical Activation of AF. In an effort to obtain an isolable reductively activated form of AF and to further probe the activated AF structure and reactivity, we screened a number of chemical reducing agents. Among these, catecholborane[25] and Stryker's reagent[26] produced complex reaction mixtures that did not appear to contain a reduced fulvene product. However, we found that the $NaBH_4$-mediated reduction of AF in pyridine[27] yielded a stable product, **5** (Scheme 3, ^{1}H NMR in Figure S2 in the Supporting Information and ^{13}C NMR in Figure S3 in the Supporting Information), assigned on the basis of diagnostic ^{1}H NMR and MS signals. In particular, the C4 proton gives rise to a doublet at 0.95 ppm, consistent with coupling with the protons from the C4 methyl group, which is consistent with the compound formulated as **5** (Figure 1C). NOESY data further support this structural assignment (**5**, Figure 2, top). In addition, the molecular weight m/z 218 corresponding to **5** is consistent with the observed m/z 241 $[M+Na]^+$. Collectively, these data suggest that **5** results from chemically mediated hydride addition to C4, the exocyclic fulvene double bond of AF.

The regiochemistry of borohydride-mediated AF reduction was confirmed by carrying out the reaction with $NaBD_4$. The resulting product (**6**) was characterized by ^{1}H and ^{13}C NMR (Figures S4 and S5 in the Supporting Information). The ^{1}H NMR doublet at δ 0.95 ppm, associated with the C4 proton of **5** (Figure 1C), collapses to a singlet in the case of the deuterated analogue **6** (Figure 1D). The signal corresponding to protons at C4 and C8 in **5** is a $q + q_{AB}$ signal at δ 3.13–3.36 ppm (Figure 1A). In **6**, the q_{AB} signal corresponding to the same protons is also at δ 3.13–3.36 ppm (Figure 1B). These data are consistent with deuteration at C4 and more generally support the assertion that the hydride (deuteride) is delivered to C4.

The relative and absolute stereochemistry at C4 was assigned on the basis of correlations in the 2D NOESY spectra of **5** and **6** (Figure 2). Relevant NOESY cross-peaks are labeled (I–IV) in Figure 2. Nuclear Overhauser effect (NOE) interactions I and II

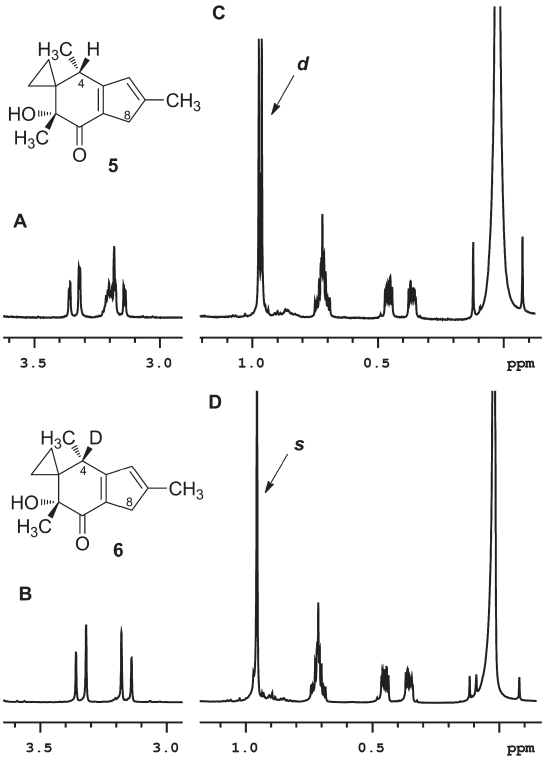


Figure 1. NMR spectra for **5** (A and C) and **6** (B and D). (A) $q + q_{AB}$ signal associated with C4 and C8 protons in **5**. (B) q_{AB} signal associated with C8 protons in **6**. (C) *d* resulting from C4-CH_3 coupling to C4 proton of **5**. (D) *s* associated with C4-CH_3 in **6**.

arise from interactions between protons at C8, which are on either side of the plane of the cyclopentadiene, and are present in both **5** and **6** spectra. NOE cross-peaks V and IV correspond to the interaction of H4 with C13-CH_3 and C10-CH_3, respectively. These data, together with the lack of NOE interaction between C13-CH_3 and C10-CH_3, suggests that the new proton H-4 is on the same face of the molecule as C10-CH_3. Analogous cross-peaks are not present in the NOESY spectra obtained for **6**. The lack of NOE IV and V further suggests that the chemical reduction produces the *S*-stereochemistry pictured in Figure 2.

Reactivity of Reduced AF Analogue 5. To evaluate whether reduced AF analogue **5** is a viable chemical precursor to AF metabolite **2**, we tested its chemical reactivity at varying pH and in the presence of nucleosides and DNA. Because **1**-enol is a common tautomer of both **1** and **5**, it was anticipated that **5** would give rise to the same major metabolite as bioactivated AF, that is, **2**. Such a conversion would involve hydrolytic cyclopropyl ring opening, leading to the formation of **2**. Indeed, we found that in 20 h at 25 °C in a solution of THF/H_2SO_4, both **5** and **6** converted to **2**. Thus, to quantitatively gauge how facile this process is, we assessed the stability of **5** under acidic, neutral, and basic conditions.

Because these conditions successfully produced metabolite **2** and to gain a more precise assessment of the facility of this process, the rate of the acid-mediated conversion of **5** to **2** was measured by time-course NMR. Thus, NMR experiments were carried out in THF-d_8, and dimethyl sulfone was used as an internal standard.[28] H_2SO_4 was added to a solution of **5** in THF-d_8, and the transformation was monitored by acquiring an NMR spectrum every 5 min for 12 h. Chemical shifts were referenced

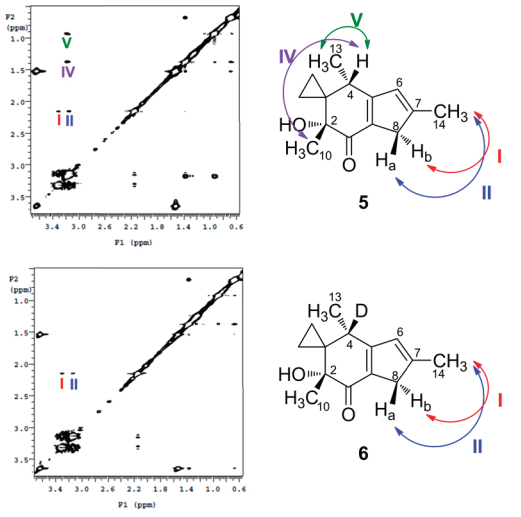


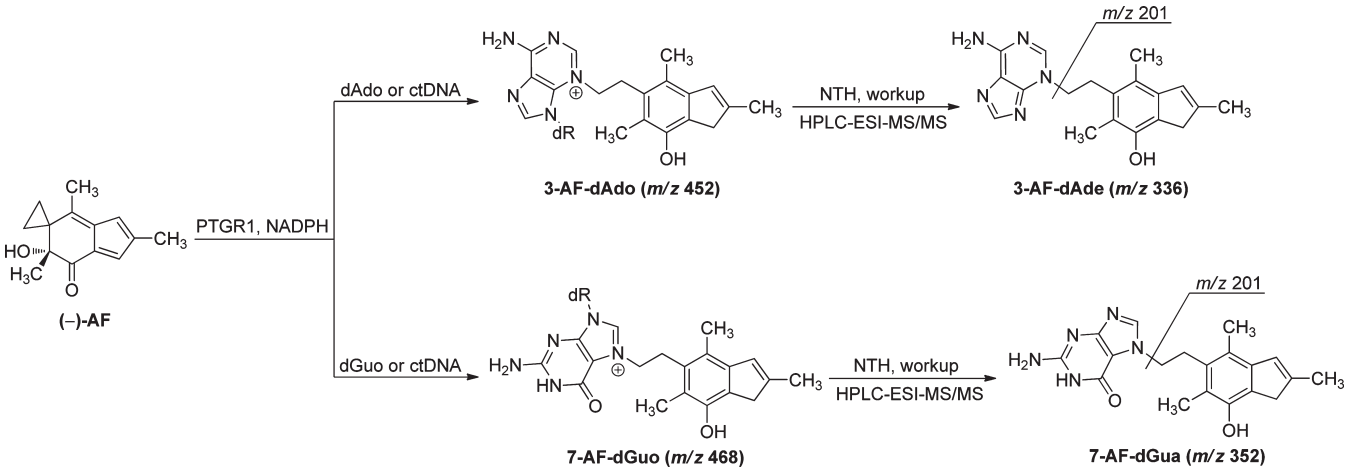
Figure 2. NOE interactions in **5** (top) and **6** (bottom).

to THF-d_8 at δ 1.72 and 3.58 ppm, and peak area integrals were standardized to that of the internal standard, dimethyl sulfone (δ 3.00, s, 6H). Figure S6A in the Supporting Information displays representative data. The observed rate constant k_{obs} was calculated on the basis of the well-distinguished methyl groups highlighted in Figure S6B in the Supporting Information. Figure S6C in the Supporting Information shows the time dependence of decay and growth of peaks associated with **5** and **2**, respectively. The average first-order rate constant at 25 °C was $1.7 \pm 0.2 \times 10^{-5}$ s^{-1}, corresponding to a $t_{1/2}$ of 11.6 h. These data suggest that **5** may react with nucleophiles, converting it through a common AF bioactivation pathway on a time scale similar to AF toxicity and DNA reactivity. Furthermore, this chemical information is of practical importance for the general chemical handling in further studies regarding the biological behavior of this novel compound. Upon addition of NaOD, **5** rapidly decomposed to an intractable mixture that did not contain **2**. A standardized solution of **5** in D_2O appeared stable for over 30 days, at which time no changes could be observed in the spectrum.

Reactions of 5 with Nucleosides and ctDNA. For **5** to be a viable mechanistic probe for studying AF-mediated alkylation of cellular targets, its reactivity should mirror that of enzymatically reduced AF. Thus, in the present study, we aimed to determine whether **5** alkylates DNA in a similar manner as bioactivated AF. In the presence of PTGR1 and NADPH or rat liver cytosol, AF has been shown to preferentially alkylate purine bases as single nucleosides and in ctDNA, and the corresponding adducts have been detected in cells. The major DNA adducts, 3-AF-dAdo and 7-AF-dGuo, formed by AF in the presence of PTGR1 and NADPH, are thermally unstable and depurinate during neutral thermal hydrolysis (NTH)[29] (Scheme 4). These adducts have been previously identified and characterized.[23]

The reaction of **5** with purine nucleosides or ctDNA was compared with the corresponding PTGR1-mediated reactions of AF. Compound **5** was reacted with dAdo, dGuo, or ctDNA in aqueous solution (pH 7) at 37 °C for 24 h. Samples were heated to 90 °C to induce hydrolysis[29] of modified bases. After removal of water by rotary evaporation, the dried mixture was reconstituted in MeOH and assayed by LC-MS to determine whether 3-AF-Ade and 7-AF-Gua were formed.[23] Reactions with dAdo or dGuo resulted in the appearance of peaks with *m*/*z* 336 or 352,

Scheme 4. Reaction of Bioactivated AF with Purine Bases or ctDNA[a]



[a] The DNA adducts and resulting fragments are identified on the basis of known m/z values.

respectively, both of which have fragment ions with m/z 201, representative of AF's characteristic indene moiety (Scheme 4). Similarly shaped extracted ion chromatograms corresponding to additional fragments of the base portion of the adduct [m/z 136 for adenine (Ade), m/z 152 for guanine (Gua)] provide further evidence for the AF-DNA adducts. The identity of resulting adducts was confirmed by coinjection with authentic standards of 3-AF-Ade and 7-AF-Gua and matching retention times.

Mass spectra for reactions of enzyme bioactivated AF with purine bases gave rise to comparable signals to **5** with the same substrates (Figure 3). The reaction of AF with dGuo in the presence of PTGR1 and NADPH gave rise to peaks that fragment to m/z 201 (Figure 3Ai) and m/z 152 (Figure 3Aii), characteristic of AF adduct formation. The larger peaks (for both fragments) elute at a retention time similar to that of the of the 7-AF-Gua standard. It is possible that the earlier eluting peaks may arise from the fragmentation of the 3-AF-Gua adducts, as it is known that the 3-AF-Gua adduct has a shorter retention time than that of 7-AF-Gua.[23] A similar peak, arising from the reaction of **5** with dGuo, albeit smaller, also elutes at the same time as the m/z 201 fragment of the 3-AF-Ade standard (~17 min) but is not the major product peak of the reaction. The major peak of this reaction mixture does not correlate to any peaks resulting from the reaction of bioactivated AF with dGuo, which suggests that chemically activated AF may be capable of yielding another depurinating adduct with m/z 352.

Reactions of bioactivated AF or **5** with dAdo were analyzed by LC-MS. The retention time of the major peak for both conditions (Figure 3C,D) matches that of the Ade standard, and the corresponding mass spectrum shows that fragmentation of 3-AF-Ade results in m/z 201 and 136 (m/z 136 not labeled in Figure 3D).

The reactivity of bioactivated AF or chemically activated AF (**5**) with ctDNA was examined in a manner similar to studies with individual nucleosides described above. It is evident that the reaction of ctDNA with bioactivated AF yields two products with m/z 352, with the latter and more prevalent peak corresponding to 7-AF-Gua fragments (Figure 4Ai,ii). In the case of the Gua adducts, the larger peak resulting from the reaction of ctDNA with **5** has a fragment m/z 201, but the chromatogram associated with fragment m/z 152, as compared to m/z 201, is extremely minor. Therefore, it is possible that **5** forms the 7-AF-Gua adduct in the presence of ctDNA but at low levels. The formation of 3-AF-Ade in the reaction of **5** with ctDNA is confirmed, as the resulting m/z 336 peak that fragments to m/z 201 and 136 and has a retention time of 17 min (Figure 4D), which matches the retention times of both authentic 3-AF-Ade standard and the adduct resulting from the reaction of bioactivated AF with ctDNA (Figure 4C).

Cytotoxicity of 5. To test the intermediacy of **5** in PTGR1-mediated AF bioactivation, the cytotoxicity of **5** was assayed in HEK-293T cells transiently transfected with a control or PTGR1-overexpressing vector.[21] These engineered cells were utilized in previous studies[13,14] aiming to examine the role of PTGR1 in AF cytotoxicity. Briefly, cells engineered with a PTGR1-overexpressing vector are sensitive to AF, as they are more proficient in activating the drug. Cells transiently transfected with a blank vector are resistant to AF. If **5** is a preactivated species, it should not require further PTGR1-mediated activation to be cytotoxic. Thus, we hypothesized that its IC_{50} values should be similar between PTGR1-transfected and control cells, despite differences in PTGR1 expression. To examine the chemical stability of **5** under the cell assay conditions, a solution of **5** in media supplemented with 10% fetal bovine serum was monitored by ^{1}H NMR at 37 °C for 24 h. The ^{1}H NMR spectra for **5** did not change over the course of the experiment, suggesting that the compound is stable to these conditions.

The IC_{50} values measured (Table 1 and Figure S7 in the Supporting Information) suggest that **5** reacts like a bioactivated form in AF in cells. The cytotoxicity of **5** in cells transiently transfected with hPTGR1-overexpressing vector was approximately 3 μM as compared to 10 μM in control cells transfected with a blank vector. Consistent with previous studies,[13,14] cells transfected to overexpress PTGR1 are more sensitive to AF, which is used as a positive control in this case. Specifically, there is a 40-fold difference in potency between the control and the test cells. Thus, the 3-fold difference is relatively small as compared to the difference seen in the positive control between the two types of cells. These data suggest, therefore, that **5** does not require further PTGR1-mediated activation by enzymatic reduction; that is, cells are similarly sensitive to the compound even with large differences in enzyme levels (Table 1). These data, together with

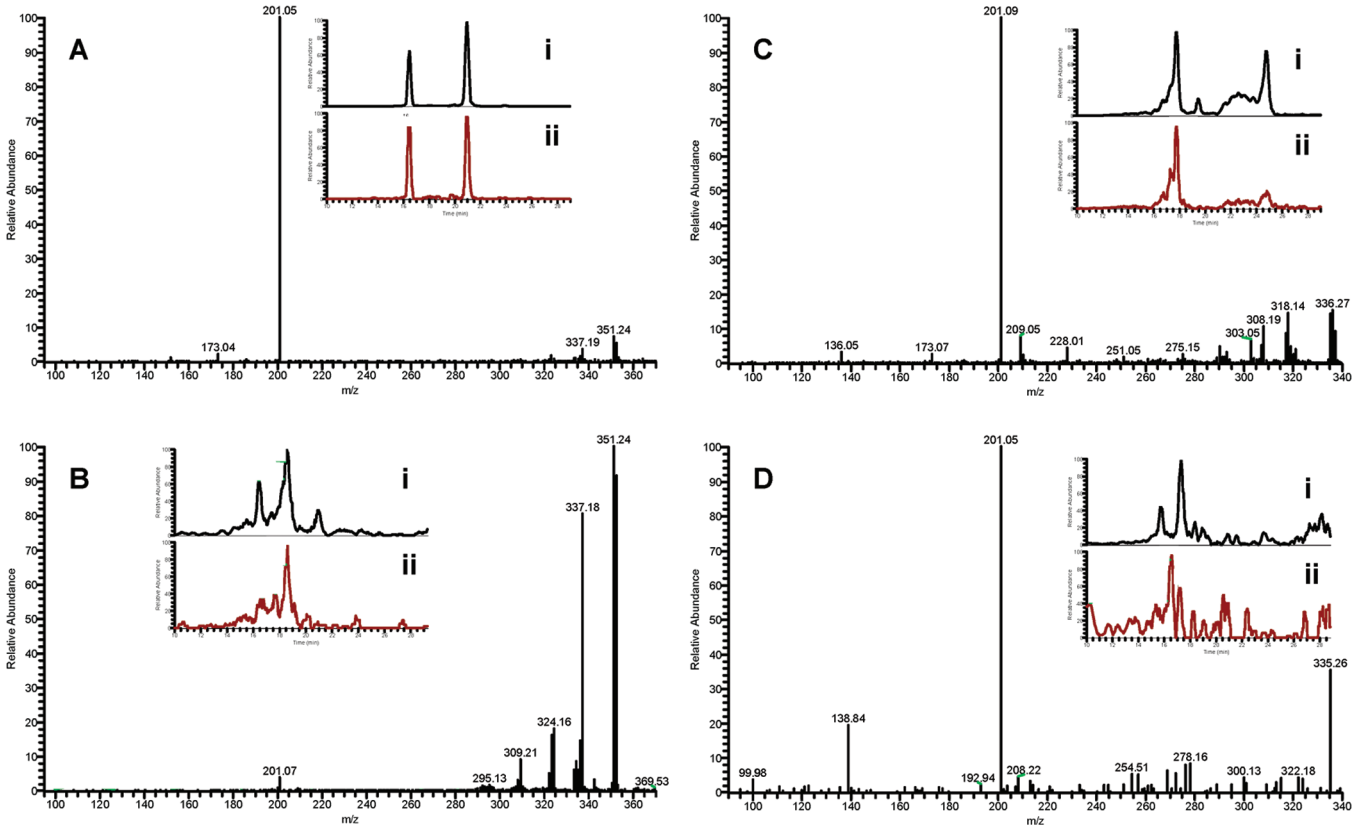


Figure 3. HPLC-MS^2 analysis of reactions of monomeric nucleosides with **5** or bioactivated AF. (A) MS^2 m/z 352 → 201 mass spectra for the reaction of bioactivated AF with dGuo. Extracted ion chromatographs for MS^2 m/z 352 → 201 (i) and MS^2 m/z 352 → 152 (ii). (B) MS^2 m/z 352 → 201 mass spectra for the reaction of chemically activated AF (**5**) with dGuo. Extracted ion chromatographs for MS^2 m/z 352 → 201 (i) and MS^2 m/z 352 → 152 (ii). (C) MS^2 m/z 336 → 201 mass spectra for the reaction of bioactivated AF with dAdo. Extracted ion chromatographs for MS^2 m/z 336 → 201 (i) and MS^2 m/z 336 → 136 (ii). (D) MS^2 m/z 336 → 201 mass spectra for the reaction of chemically activated AF (**5**) with dAdo. Extracted ion chromatographs for MS^2 m/z 336 → 201 (i) and MS^2 m/z 336 → 136 (ii).

the adduct formation data, chemical reactivity, and data regarding conversion to the AF metabolite suggest that **5** does not require activation. However, it is strictly possible that another enzyme, present at equal levels in the two cells, may bioactivate **5**.

DISCUSSION

The importance of reductase-mediated activation of AFs in chemotherapeutic applications motivates interest in this reaction mechanism. Independent generation of a chemically activated AF analogue fosters a better understanding of biological adduct formation and cytotoxicity independent of enzymatic bioactivation. For bioreductive alkylating agents, it is often informative to test chemical activation when trying to isolate biologically relevant reactive intermediates and/or explain the formation of observed products.[30] In the case of mitomycin C, for example, a number of reagents were employed throughout the elucidation of its reduction mechanism.[31−34] The present study details the chemoselectivity of AF bioreduction, the synthesis and purification of a chemically reduced form of AF (**5**), as well as the characterization of its stability and reactivity profile with DNA.

The regiochemistry of AF reduction catalyzed by PTGR1 was evaluated by carrying out enzyme-mediated reduction reactions with isolated enzyme and deuterium-labeled cofactor NADPD. The resulting product **2** had m/z 220, consistent with a 1,4- or 1,6-reduction. The 1,4-reduction mechanism has precedent in previous data[21] indicating that PTGR1 catalyzes the reduction of α,β-unsaturated ketones or aldehydes via hydride addition to the β-carbon. However, on the basis of data available in this and previous studies, a potential 1,6-pathway cannot be strictly excluded.

The enzymatic (PTGR1-mediated) and chemical ($NaBH_4$-mediated) activation processes give rise to different isomeric intermediates. PTGR1 catalyzes hydride addition to C8 yielding **1** in the case of the 1,4-pathway, while $NaBH_4$ delivers the hydride to C4 yielding **5**. Compound **5** is an isomer of the proposed bioactivation product. Furthermore, the regiochemistry of the $NaBH_4$-mediated reduction is different than previous reactions with simple α,β-unsaturated ketone substrates, which undergo $NaBH_4$-mediated 1,4-addition in pyridine.[27] Literature examples demonstrate that strong hydride reducing agents, like alkyllithium reagents[35] and lithium triethylborohydride,[36] are generally required to reduce fulvenes, but here, we demonstrate fulvene reduction with the mild reducing agent $NaBH_4$. The regioselectivity of the chemical reduction can be rationalized on the basis of a dipolar resonance structure with an aromatic cyclopentadiene ring, suggested earlier by McMorris et al.[5,7] (Scheme 5), where positive charge is localized at C4 and negative charge is delocalized in the cyclopentadiene ring. In addition, it is possible that the adjacent carbonyl may tune the reduction potential of the fulvene, suggesting a possible means for modifying the reactivity of the molecule, thus enabling the reduction with $NaBH_4$.

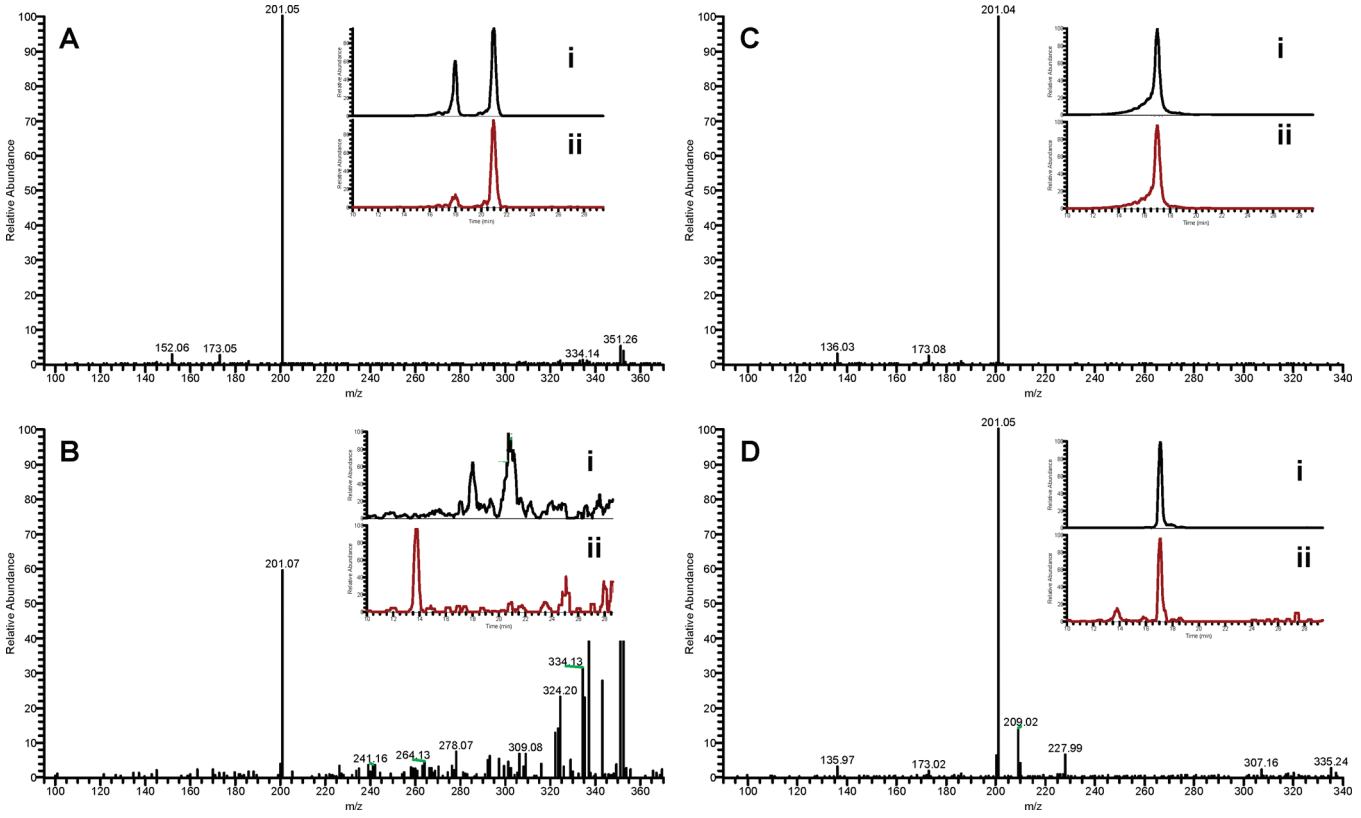


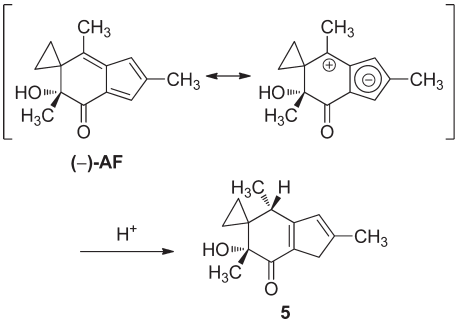
Figure 4. HPLC-MS^2 analysis of ctDNA reactions with **5** and bioactivated AF. (A) MS^2 m/z 352 → 201 mass spectra for the reaction of bioactivated AF with ctDNA. Extracted ion chromatographs for MS^2 m/z 352 → 201 (i) and MS^2 m/z 352 → 152 (ii). (B) MS^2 m/z 352 → 201 mass spectra for the reaction of chemically activated AF (**5**) with ctDNA. Extracted ion chromatographs for MS^2 m/z 352 → 201 (i) and MS^2 m/z 352 → 152 (ii). (C) MS^2 m/z 336 → 201 mass spectra for the reaction of bioactivated AF with ctDNA. Extracted ion chromatographs for MS^2 m/z 336 → 201 (i) and MS^2 m/z 336 → 136 (ii). (D) MS^2 m/z 336 → 201 mass spectra for the reaction of chemically activated AF (**5**) with ctDNA. Extracted ion chromatographs for MS^2 m/z 336 → 201 (i) and MS^2 m/z 336 → 136 (ii).

Table 1. IC_{50} Values for AF and 5 in HEK-293T Cells Transfected with a Blank (Control) or hPTGR1 Overexpressing Vector

compd	control[a] (μM)	hPTGR1[a] (μM)
(−)-AF[b]	3.3 ± 1.3	0.077 ± 0.048
5[c]	9.7 ± 1.9	3.2 ± 1.1

[a] Values are expressed as mean ± SE. [b] $n = 3$, $P < 0.05$. [c] $n = 3$, $P < 0.01$.

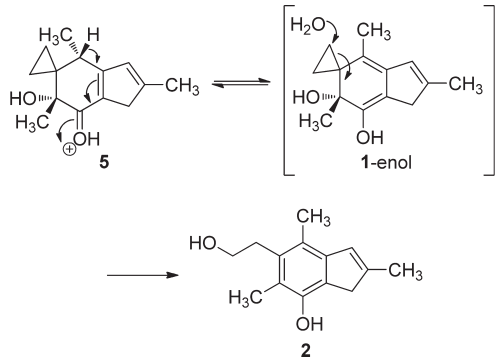
Scheme 5. Reduction of AF to Yield 5 and Instructive Dipolar Resonance Precursor Structure



Results described here regarding the reaction of $NaBH_4$ with AF suggest that this chemical reduction process may be diastereoselective, leading to the exclusive formation of *S*-**5** resulting from hydride addition to the *si* face of the molecule. The observed facial stereoselection of the only observed and isolated product is opposite of what might be expected for a structure in which the tertiary alcohol participates in chelation-controlled direction of hydride addition to the *re* face of the AF scaffold, corresponding to the conformation illustrated in Scheme 3. A potential explanation for the inverse diastereoselectivity is that unfavorable steric interactions in the product are minimized. Thus, if hydride delivery to C4 were directed by the hydroxyl group to the *re* face, the methyl groups at C2 and C4 would be *syn* in the product, that is, 1,3-diaxial to one another (Figure S8A in the Supporting Information). Instead, delivery of the hydride to the *si* face (*syn* to C2-CH_3) occurs, leading to a product with *anti* C2 and C4 methyl groups, thereby minimizing steric interactions (Figure S8B in the Supporting Information). However, as a final caveat, on the basis of the <25% yield, we cannot strictly discount the possibility that we may be isolating a single diastereomeric product from a mixture resulting from a nondiastereoselective reaction.

Because reduced AF analogue **5** is an isomer of the proposed bioactivation intermediate, it was anticipated that this chemically activated species would be capable of being converted to metabolite **2**. A consistent chemical mechanism therefore involves

Scheme 6. Proposed Mechanism for the Conversion of 5 to 2 in Aqueous Acid



tautomerization to **1**-enol followed by hydrolytic cyclopropane cleavage (Scheme 6). We demonstrated that this reaction takes place in the presence of acid, and this reactivity profile suggests that **5** is a competent chemical model for enzymatically reduced AF. Under the same conditions, **6** is also converted to **2**, which further supports the putative reaction mechanism illustrated in Scheme 6.

In a cell-free system, **5** alkylates nucleic acids less efficiently than bioactivated AF. For reactions with monomeric nucleosides, 7-AF-Gua and 3-AF-Ade adducts arising from **5** were on average 400- and 50-fold less abundant, respectively, than adducts resulting from covalent modification by bioactivated AF. Adducts formed in ctDNA from **5** were less abundant than those resulting from bioactivated AF but were similar in scale (both were on average 300-fold less abundant). Compound **5** is stable despite being chemically activated, as demonstrated by testing its stability in cell media. While this observation fails to explain the difference in adduct abundance resulting from treatments with **5** vs bioactivated AF, such reduced reactivity of chemically vs biologically activated intermediates has been observed previously. Examples include leinamycin[37] and a small molecule leinamycin analogue.[38] Leinamycin primarily relies on thiol-dependent activation to alkylate DNA; however, it was discovered that thiol-independent activation results in the same DNA damage but at a slower rate and lower abundance,[37] which is also the case for its analogue.[38] The observation that these two activation pathways yield the same products suggest that they share a common intermediate. Thus, by analogy, the conversion of AF and **5** under acidic conditions to metabolite **2** also suggests a common intermediate such that the compound is an effective model on a pharmacodynamics basis but not a pharmacokinetic basis. On the basis of MS data, reactions of **5** with ctDNA appeared to proceed with fewer additional/unknown products than those with individual nucleosides, especially when comparing the chromatograms corresponding to 3-AF-Ade adduct fragments (Figure 3D vs Figure 4D). AF is planar, similar to nucleobases, and it is possible that AF and **5** are capable of noncovalently associating with DNA, possibly intercalating within the duplex, prior to alkylation. Analogous noncovalent preassociations have been suggested for a number of alkylating agents, such as aflatoxin-B_1,[39] CC-1065,[40] and benzo[*a*]pyrene's metabolically activated *anti*-BP-diol epoxide (BPDE).[1,41,42] It is also interesting to note that **5** has an extra stereocenter relative to bioactivated AF. Knowing that the (+)-enantiomers of AFs are less potent than their (−)-counterparts,[13] it is interesting to consider how the additional stereocenter in **5** may influence its reactivity with chiral biomolecules and ultimately its activity in cells.

Transiently transfected cells are a convenient and generally informative model; however, there is typically wide variability in enzyme overexpression levels and cells stop overexpressing the enzyme—PTGR1 in this case—over time. Consequently, the data obtained with this model should be interpreted with these caveats in mind, and only relative comparisons within a given experiment seem informative. The data obtained here suggest that **5** is toxic to cells with little dependence on bioactivation capacity. Yet, there is a small but statistically significant difference between the cells with high and low levels of PTGR1 expression. However, as compared to the 20−100-fold differences of drug potencies for AF or hydroxymethylacylfulvene (HMAF) that have been observed in the same model in the current and previous studies,[13,14] the approximate 3-fold difference for compound **5** is relatively minor and cannot be interpreted as contributing to toxicity to a similar extent as AF or HMAF. Further studies in stable cell lines may suggest possible factors that contribute to susceptibility differences toward **5**, such as transport or metabolism.

CONCLUSION

The biochemical and chemical activation pathways of AF with PTGR1 and $NaBH_4$, respectively, result in isomeric reactive intermediates that yield the same metabolite **2** and similar profiles of DNA adducts. A new AF derivative that is chemically activated has been obtained and chemically characterized. The cytotoxicity of **5**, as a function of high or low PTGR1 expression, suggests that it does not require further activation to be cytotoxic in cells. Thus, **5** appears to be a new and chemically competent model for bioactivated AF, and is expected to open new possibilities for evaluating covalent interactions with biomolecules, such as DNA and protein, independent of its dependence on enzyme-mediated activation.

ASSOCIATED CONTENT

Supporting Information. Mass spectra of *d*-**2**, NMR spectra of **5** and **6**, NMR arrays of acid-mediated conversion of **5** to **2**, cytotoxicity curves of **5**, and models of AF hydride reduction (Figures S1−S8). This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Author

*E-mail: sturlas@ethz.ch.

Present Addresses

‖Bristol-Myers Squibb Pharmaceutical Research Institute, Princeton, New Jersey 08543.

Funding Sources

We acknowledge the National Cancer Institute (R01 CA123007) for support of this research. The Masonic Cancer Center Analytical Biochemistry Facility, University of Minnesota, is supported in part by the Cancer Center Support Grant (CA 77598). The MN Structural Biology NMR Facility, University of Minnesota, is supported by the University of Minnesota Medical School, the Minnesota Medical Foundation, and funding from the National Science Foundation (BIR 961477).

■ ACKNOWLEDGMENT

We thank Dr. Beverly Ostrowski for assistance with NMR experiments and Dr. Sarah Kliegman for assistance with data analysis and helpful discussion. We also thank Professor Courtney Aldrich for helpful comments regarding the manuscript.

■ ABBREVIATIONS

AFs, acylfulvenes; PTGR1, prostaglandin reductase 1; AOR, alkenal/one oxidoreductase; HMAF, hydroxymethylacylfulvene; ctDNA, calf thymus DNA; rPTGR1, rat PTGR1; NaOD, sodium deuteroxide; D_2O, deuterium oxide; $NaBD_4$, sodium borodeuteride; dAdo, deoxyadenosine; dGuo, deoxyguanosine; NOESY, nuclear Overhauser effect spectroscopy; HRMS, high-resolution mass spectroscopy; PPG, polypropethylene glycol; H_2SO_4, sulfuric acid; $NaBH_4$, sodium borohydride; MeOH, methanol; hPTGR1, human PTGR1; DMEM, Dulbecco's modified Eagle's medium; NADPD, 4,4′-d_2-NADPH; NTH, neutral thermal hydrolysis.

■ REFERENCES

(1) Boger, D. L., and Garbaccio, R. M. (1999) Shape-dependent catalysis: Insights into the source of catalysis for the CC-1065 and duocarmycin DNA alkylation reaction. *Acc. Chem. Res. 32*, 1043–1052.

(2) Lin, A. J., Cosby, L. A., Shansky, C. W., and Sartorelli, A. C. (1972) Potential bioreductive alkylating agents. 1. Benzoquinone derivatives. *J. Med. Chem. 15*, 1247–1252.

(3) Anchel, M., Hervey, A., and Robbins, W. J. (1952) Production of illudin M and of a fourth crystalline compound by Clitocybe Illudens. *Proc. Natl. Acad. Sci. U.S.A. 38*, 927–928.

(4) McMorris, T. C., and Anchel, M. (1965) Fungal metabolites. The structures of the novel sesquiterpenoids illudin-S and -M. *J. Am. Chem. Soc. 87*, 1594–1600.

(5) McMorris, T. C., Kelner, M. J., Wang, W., Diaz, M. A., Estes, L. A., and Taetle, R. (1996) Acylfulvenes, a new class of potent antitumor agents. *Experientia 52*, 75–80.

(6) McMorris, T. C., Kelner, M. J., Wang, W., Yu, J., Estes, L. A., and Taetle, R. (1996) (Hydroxymethyl)acylfulvene: An illudin derivative with superior antitumor properties. *J. Nat. Prod. 59*, 896–899.

(7) McMorris, T. C. (1999) Discovery and development of sesquiterpenoid derived hydroxymethylacylfulvene: A new anticancer drug. *Bioorg. Med. Chem. 7*, 881–886.

(8) Schobert, R., Knauer, S., Seibt, S., and Biersack, B. (2011) Anticancer active illudins: Recent developments of a potent alkylating compound class. *Curr. Med. Chem. 18*, 790–807.

(9) Kelner, M. J., McMorris, T. C., and Taetle, R. (1990) Preclinical evaluation of illudins as anticancer agents: Basis for selective cytotoxicity. *J. Natl. Cancer Inst. 82*, 1562–1565.

(10) Kelner, M. J., McMorris, T. C., Beck, W. T., Zamora, J. M., and Taetle, R. (1987) Preclinical evaluation of illudins as anticancer agents. *Cancer Res. 47*, 3186–3189.

(11) Woynarowska, B. A., Woynarowski, J. M., Herzig, M. C., Roberts, K., Higdon, A. L., and MacDonald, J. R. (2000) Differential cytotoxicity and induction of apoptosis in tumor and normal cells by hydroxymethylacylfulvene (HMAF). *Biochem. Pharmacol. 59*, 1217–1226.

(12) MacDonald, J. R., Muscoplat, C. C., Dexter, D. L., Mangold, G. L., Chen, S. F., Kelner, M. J., McMorris, T. C., and Von Hoff, D. D. (1997) Preclinical antitumor activity of 6-hydroxymethylacylfulvene, a semisynthetic derivative of the mushroom toxin illudin S. *Cancer Res. 57*, 279–283.

(13) Gong, J., Neels, J. F., Yu, X., Kensler, T. W., Peterson, L. A., and Sturla, S. J. (2006) Investigating the role of stereochemistry in the activity of anticancer acylfulvenes: Synthesis, reductase-mediated bioactivation, and cellular toxicity. *J. Med. Chem. 49*, 2593–2599.

(14) Dick, R. A., Yu, X., and Kensler, T. W. (2004) NADPH alkenal/one oxidoreductase activity determines sensitivity of cancer cells to the chemotherapeutic alkylating agent irofulven. *Clin. Cancer Res. 10*, 1492–1499.

(15) Kelner, M. J., McMorris, T. C., Montoya, M. A., Estes, L., Uglik, S. F., Rutherford, M., Samson, K. M., Bagnell, R. D., and Taetle, R. (1998) Characterization of acylfulvene histiospecific toxicity in human tumor cell lines. *Cancer Chemother. Pharmacol. 41*, 237–242.

(16) Kelner, M. J., McMorris, T. C., Montoya, M. A., Estes, L., Uglik, S. F., Rutherford, M., Samson, K. M., Bagnell, R. D., and Taetle, R. (1999) Characterization of MGI 114 (HMAF) histiospecific toxicity in human tumor cell lines. *Cancer Chemother. Pharmacol. 44*, 235–240.

(17) Herzig, M. C. S., Arnett, B., MacDonald, J. R., and Woynarowski, J. M. (1999) Drug uptake and cellular targets of hydroxymethylacylfulvene (HMAF). *Biochem. Pharmacol. 58*, 217–225.

(18) Woynarowski, J. M., Napier, C., Koester, S. K., Chen, S.-F., Troyer, D., Chapman, W., and MacDonald, J. R. (1997) Effects on DNA integrity and apoptosis induction by a novel antitumor sesquiterpene drug, 6-hydroxymethylacylfulvene (HMAF, MGI 114). *Biochem. Pharmacol. 54*, 1181–1193.

(19) McMorris, T. C., Elayadi, A. N., Yu, J., Hu, Y., and Kelner, M. J. (1999) Metabolism of antitumor hydroxymethylacylfulvene by rat liver cytosol. *Drug Metab. Dispos. 27*, 983–985.

(20) McMorris, T. C., Elayadi, A. N., Yu, J., and Kelner, M. J. (1999) Metabolism of antitumor acylfulvene by rat liver cytosol. *Biochem. Pharmacol. 57*, 83–88.

(21) Dick, R. A., Kwak, M. K., Sutter, T. R., and Kensler, T. W. (2001) Antioxidative function and substrate specificity of NAD(P)H-dependent alkenal/one oxidoreductase. A new role for leukotriene B4 12-hydroxydehydrogenase/15-oxoprostaglandin 13-reductase. *J. Biol. Chem. 276*, 40803–40810.

(22) Neels, J. F., Gong, J., Yu, X., and Sturla, S. J. (2007) Quantitative correlation of drug bioactivation and deoxyadenosine alkylation by acylfulvene. *Chem. Res. Toxicol. 20*, 1513–1519.

(23) Gong, J., Vaidyanathan, V. G., Yu, X., Kensler, T. W., Peterson, L. A., and Sturla, S. J. (2007) Depurinating acylfulvene-DNA adducts: Characterizing cellular chemical reactions of a selective antitumor agent. *J. Am. Chem. Soc. 129*, 2101–2111.

(24) Ichinose, K., Kodera, M., Leeper, F. J., and Battersby, A. R. (1999) Biosynthesis of porphyrins and related macrocycles. Part 51. Proof that a reductive step occurs during the biosynthesis of vitamin B_{12} by the microaerophilic organism, *Propionibacterium shermanii*. *J. Chem. Soc. Perkin Trans. 1 8*, 879–887.

(25) Evans, D. A., and Fu, G. C. (1990) Conjugate reduction of α,β-unsaturated carbonyl compounds by catecholborane. *J. Org. Chem. 55*, 5678–5680.

(26) Mahoney, W. S., Brestensky, D. M., and Stryker, J. M. (1988) Selective hydride-mediated conjugate reduction of α,β-unsaturated carbonyl compounds using $[(Ph_3P)CuH]_6$. *J. Am. Chem. Soc. 110*, 291–293.

(27) Jackson, W. R., and Zurqiyah, A. (1965) The occurrence of 1,2- or 1,4-addition in the reduction of some α,β-unsaturated ketones with metal hydrides. *J. Chem. Soc.* 5280–5287.

(28) Hays, P. A. (2005) Proton Nuclear Magnetic Resonance Spectroscopy (NMR) Methods for determining the purity of reference drug standards and illicit forensic drug seizures. *J. Forensic Sci. 50*, 1342.

(29) Anthoney, D. A., and Twelves, C. J. (2001) DNA: still a target worth aiming at? A review of new DNA-interactive agents. *Am. J. PharmacoGenomics 1*, 67–81.

(30) Lin, A. J., and Sartorelli, A. C. (1973) 2,3-Dimethyl-5,6-bis-(methylene)-1,4-benzoquinone. The active intermediate of bioreductive alkylating agents. *J. Org. Chem. 38*, 813–815.

(31) Zein, N., and Kohn, H. (1987) Covalent binding of mitomycin C to nucleosides under reductive conditions. *J. Am. Chem. Soc. 109*, 1576–1577.

(32) Tomasz, M., Chowdary, D., Lipman, R., Shimotakahara, S., Veiro, D., Walker, V., and Verdine, G. L. (1986) Reaction of DNA with chemically or enzymatically activated mitomycin C: isolation and structure of the major covalent adduct. *Proc. Natl. Acad. Sci. U.S.A. 83*, 6702–6706.

(33) Iyer, V. N., and Szybalski, W. (1964) Mitomycins and porfiromycin: Chemical mechanism of activation and cross-linking of DNA. *Science 145*, 55–58.

(34) Tomasz, M., and Lipman, R. (1979) Alkylation reactions of mitomycin C at acid pH. *J. Am. Chem. Soc. 101*, 6063–6067.

(35) Erker, G., Nolte, R., Aul, R., Wilker, S., Krueger, C., and Noe, R. (1991) Cp-substituent additivity effects controlling the stereochemistry of the propene polymerization reaction at conformationally unrestricted $(Cp\text{-}CHR^1R^2)_2ZrCl_2$/methylalumoxane catalysts. *J. Am. Chem. Soc. 113*, 7594–7602.

(36) Sweeney, N. J., Mendoza, O., Müller-Bunz, H., Pampillón, C., Rehmann, F.-J. K., Strohfeldt, K., and Tacke, M. (2005) Novel benzyl substituted titanocene anti-cancer drugs. *J. Organomet. Chem. 690*, 4537–4544.

(37) Breydo, L., Zang, H., Mitra, K., and Gates, K. S. (2001) Thiol-independent DNA alkylation by leinamycin. *J. Am. Chem. Soc. 123*, 2060–2061.

(38) Chatterji, T., Kizil, M., Keerthi, K., Chowdhury, G., Pospísil, T., and Gates, K. S. (2003) Small molecules that mimic the thiol-triggered alkylating properties seen in the natural product leinamycin. *J. Am. Chem. Soc. 125*, 4996–4997.

(39) Gopalakrishnan, S., Byrd, S., Stone, M. P., and Harris, T. M. (1989) Carcinogen-nucleic acid interactions: equilibrium binding studies of aflatoxin B1 with the oligodeoxynucleotide $d(ATGCAT)_2$ and with plasmid pBR322 support intercalative association with the B-DNA helix. *Biochemistry 28*, 726–734.

(40) Boger, D. L., Invergo, B. J., Coleman, R. S., Zarrinmayeh, H., Kitos, P. A., Thompson, S. C., Leong, T., and McLaughlin, L. W. (1990) A demonstration of the intrinsic importance of stabilizing hydrophobic binding and non-convalent van der waals contacts dominant in the non-covalent CC-1065/B-DNA binding. *Chem.-Biol. Interact. 73*, 29–52.

(41) Geacintov, N. E., Yoshida, H., Ibanez, V., and Harvey, R. G. (1981) Non-covalent intercalative binding of 7,8-dihydroxy-9,10-epoxybenzo(a)pyrene to DNA. *Biochem. Biophys. Res. Commun. 100*, 1569–1577.

(42) Guza, R., Kotandeniya, D., Murphy, K., Dissanayake, T., Lin, C., Giambasu, G. M., Lad, R. R., Wojciechowski, F., Amin, S., Sturla, S. J., Hudson, R. H. E., York, D. M., Jankowiak, R., Jones, R., and Tretyakova, N. Y. (2011) Influence of C-5 substituted cytosine and related nucleoside analogs on the formation of benzo[a]pyrene diol epoxide-dG adducts at CG base pairs of DNA. *Nucleic Acids Res. 39*, 3988–4006.